

Malgorzata Kloc · Jacek Z. Kubiak  
*Editors*

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# Marine Organisms as Model Systems in Biology and Medicine

# **Results and Problems in Cell Differentiation**

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Volume 65

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Jacek Z. Kubiak, Rennes CX, France

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Editors

# Marine Organisms as Model Systems in Biology and Medicine

 Springer

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# Preface

Recent analyses have estimated that between 700,000 and 1,000,000 species of organisms (excluding viruses and bacteria) live in the marine environment, with around two-third of those still remaining to be identified. The World Register of Marine Species lists about 200,000 identified animal species inhabiting the world's seas and oceans. Considering this marvelous abundance one has to wonder how much scientific and medicine-related information is hidden there and still remains to be reviled.

This volume reviews the current knowledge on cellular, molecular, physiological, developmental, and ecological aspects of various species of marine organisms and their potential or already existing applications in research, medicine, and biotechnology.

The first section of this volume describes oocyte maturation and aging in nemertean worms; the signal transduction pathways in fertilization and their multi-scale modeling; the role of actin in echinoderm fertilization; and the function of nuclear basic proteins in the sperm of marine invertebrates. The last chapter of this section describes modes of sexual differentiation and reproduction and biotechnology methods used in aquaculture to manipulate sexual profile of aquatic organisms.

The second section is devoted to the description of archetypal medusa body plan; current developments such as multi-omics studies in the medusa model research; sea urchin larvae as a model for postembryonic development, and evolution of the nervous system; regulatory role of non-protein-coding RNAs in tunicate development; and genetic regulatory network in notochord formation in ascidians.

The third section focuses on various aspects of cell differentiation, trans-differentiation, and stemness in sponges and modes and mechanisms of extraordinary regeneration abilities of various echinoderms and solitary and colonial tunicates.

The fourth section summarizes biochemical adaptations in marine invertebrates and the biotechnological and biomedical applications of the produced biomolecules and describes modes of coral feeding and importance of secreted mucus not only for the corals but also for an animate and inanimate environment, as well as the feeding strategies and chemosensory mechanisms regulating appetitive behavior in fish. It also describes the biomolecules (glycans) with antiviral activities, the jellyfish

venoms, available treatments and possible applications in medicine, and the functions and regulation of synthesis of echinoderm pigments.

The fifth section concentrates on reef building corals as a genomic tool for climate change research; the “crown-of-thorns starfish” (COTS) genome for testing new genomic technologies and bioinformatics tools; structure and composition of the crab carapace and its usefulness in biomimetic mechanical design; adult brain rejuvenation, visual system, and RNA editing in Octopus; and cubozoan jellyfish camera-type eye as a model for visual information processing.

As an additional benefit to the readers, the chapters are illustrated with the gorgeous images of marine life. We believe that this volume besides being highly informative and scientifically inspiring will ignite an admiration and reverence for the beauty and marvels of marine organisms.

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# Abstract

This book reviews the most recent knowledge on cellular, molecular, physiological, and developmental aspects of various marine species. It also describes formidable regeneration abilities of marine organisms, their usefulness as research model systems and as a source of biological compounds, and bioengineering and robotic ideas for medical applications.

The book covers the following subjects: starfish and sea urchin as models for signal transduction analysis and multi-scale modeling of molecular and cytoskeletal events during fertilization and postembryonic development; marine nemertean worms to study oocyte maturation and aging; nuclear basic proteins in marine organisms' sperm; regeneration and stem cells in sponges, solitary ascidians, and echinoderms; gene regulatory network in tunicate notochord; the role of noncoding RNAs in tunicate development; development and evolution of the nervous system; structures and composition of crab carapace as a model for bioengineering of composite materials; multi-omics studies of medusa and the crown-of-thorns starfish genome assembly as a bioinformatics tools; box jellyfish visual system as a model to study visual perception; brain rejuvenation and gene editing in Octopus, bio-pigments in echinoderms and antiviral activity of marine organism-derived glycans; coral reefs biology; microbiota and modes of feeding; role of secreted mucus; feeding strategies and feeding regulatory mechanisms in fish; and monosex in marine organism aquaculture.

This book is unique in combining such a comprehensive list of diverse subjects on marine organisms in one volume. It should give the readers a new and invaluable perspective on the scientific, bioinformatics, bioengineering, medicinal, aquacultural, and environmental value and exquisiteness of marine organisms.

**Keywords** Marine organisms · Sponge · Coral · Starfish · Crab · Jellyfish · Sea urchin · Ascidian · Nemertean · Stem cells · Regeneration



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**Part I**  
**Gametes, Maturation, Fertilization**  
**and Modes of Reproduction**

# Chapter 1

## Marine Nemertean Worms for Studies of Oocyte Maturation and Aging



Stephen A. Stricker

**Abstract** Many marine invertebrates are capable of providing an abundant supply of oocytes that are fertilized external to the female body, thereby making these specimens well suited for studies of development. Along with intensively analyzed model systems belonging to such groups as echinoderms, tunicates, mollusks, and annelids, various lesser-studied taxa can undergo an external mode of fertilization. For example, nemertean worms constitute a relatively small phylum of marine protostome worms whose optically clear oocytes are easily collected and fertilized in the laboratory. Thus, to help promote the use of nemertean oocytes as a potential model in embryological analyses, this chapter begins by describing general methods for obtaining adults and for handling their gametes. After presenting such protocols, this chapter concludes with some representative results obtained with these specimens by summarizing the roles played by adenosine monophosphate-activated kinase (AMPK) during oocyte maturation and by c-Jun N-terminal kinase (JNK) during oocyte aging and death.

### 1.1 Introduction

Both sea urchins and starfish normally spawn their gametes into the sea during the breeding season, a feature that has helped make these echinoderms popular model organisms for investigations of fertilization and development. Similarly, various other marine invertebrate taxa with species utilizing an external mode of fertilization such as tunicates, mollusks, and annelids have also been widely employed in embryological studies. Although each of these animals offers its own distinct set of benefits for analyses conducted by embryologists, such species share in common the ability to provide easy access to abundant gametes, simple culturing protocols, and optically clear preparations that collectively account for their widespread usage

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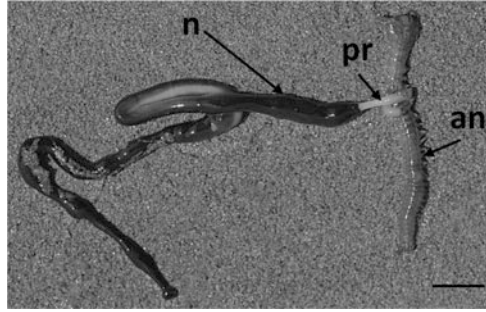
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**Fig. 1.1** An adult *Paranemertes peregrina* nemertean (n) using its eversible proboscis (pr) to capture an annelid worm (an). Scale bar = 1 cm



in investigations of development. Over the last two decades, many of the commonly analyzed marine invertebrate models have had their genomes sequenced by consortia of scientists that often included developmental biologists. However, with the advent of more economical genomic methods, individual laboratories are potentially capable of adding new under-studied species to the list of useful models, thereby supplementing more mainstream examples that have long been available.

One such group of less intensively analyzed invertebrates is the unsegmented worms that constitute the phylum Nemertea (Kvist et al. 2014). Of the ~1200–1500 currently described species, most nemerteans, or “ribbon worms,” live in intertidal or subtidal benthic habitats and actively feed on other invertebrates by means of an eversible proboscis that contains toxins for immobilizing prey (Fig. 1.1) (Stricker and Cloney 1983). Sexes are typically separate in nemerteans, and each ripe adult tends to form several dozen, to more than a hundred, saccular gonads down the length of its body during the breeding season (Stricker 1987; Stricker et al. 2001). Copulatory appendages are generally lacking, and thus when fully ripe, females and males usually discharge their gametes through short gonoducts freely into the surrounding seawater.

Given such features, nemerteans offer many of the same advantages provided by echinoderms and other commonly studied marine invertebrates with external fertilization. In addition, unlike deuterostomes such as echinoderms and tunicates, nemerteans belong to the protostome lineage of triploblastic animals and thus also represent an example of the most common type of extant animal.

To help promote the use of nemerteans in further embryological analyses, this contribution begins by considering the collection or purchase of North American species. Thereafter, practical tips for handling oocytes of an undescribed species of the nemertean *Cerebratulus* are provided, before ending with two examples of the kinds of data that can be obtained when using these oocytes to analyze mechanisms of maturation and aging.



## 1.2 Methods

### 1.2.1 Collection Procedures

Several nemerteans can be readily found at low tide either in rocky habitats or within soft substrates. For example, on the west coast of the United States, ripe specimens of such genera as *Amphiporus*, *Cerebratulus*, *Emplectonema*, *Maculaura* (formerly, *Micrura*), and *Tubulanus* are collectible at multiple sites from California through Washington, whereas *Cerebratulus lacteus* and several other nemerteans that are suitable for embryological studies occur on the east coast. To determine which species might be encountered in local habitats, various relevant guides to seashore life can be consulted. Similarly, such guides or dedicated keys for species classifications are often capable of providing trustworthy identifications for commonly gathered specimens.

After establishing which nemerteans are available for collection, chances of successfully finding specimens can be enhanced by checking areas away from direct sunlight (e.g., under rocks or within the sediment) and by beginning the search well before the tide is fully out. This is because many nemerteans are photonegative and tend to spend more time on or near the surface as tide levels recede.

For most nemerteans, spawning occurs during late spring or summer in response to increased day length and temperature. Such discharge of gametes from one animal may stimulate nearby cohorts to spawn, and thus within a relatively short timeframe, essentially all adults at a particular collecting site can be cleared of stored gametes for the year. Accordingly, in order to prolong the availability of oocytes and sperm, nearly ripe specimens can be obtained prior to natural spawnings in the field and subsequently maintained in the laboratory under more constant light and temperature regimes for several weeks beyond the normal end of the breeding season. Moreover, to avoid the possibility of triggering mass spawnings, collected specimens are best kept in separate containers and handled gently, particularly during the peak of the breeding season.

As an alternative to personally collecting specimens, it may be possible to buy nemerteans from several marine supply companies [e.g., Marinus Scientific ([marinusscientific.com](http://marinusscientific.com)), WestWind SeaLab Supplies (434 Russell St, Victoria, BC V9A 3X3, Canada, Phone: +1 250-386-8036), Gulf Specimen Marine Lab ([gulfspecimen.org](http://gulfspecimen.org)), and the Marine Biological Laboratory Animal and Tissue Supply (<http://www.mbl.edu/mrc/services/aquatic-animal-tissue-supply>)]. Regardless of whether specimens are personally collected in the field or purchased from supply houses, once obtained, adults can be kept in the laboratory and harvested for embryological analyses according to methods outlined in the following sections. Such protocols are known to work with an undescribed west coast species of *Cerebratulus* (*Cerebratulus* sp.), although it is likely that with minor modifications, similar methods can be applied to various nemerteans.

### ***1.2.2 Maintaining Adult Specimens in the Laboratory***

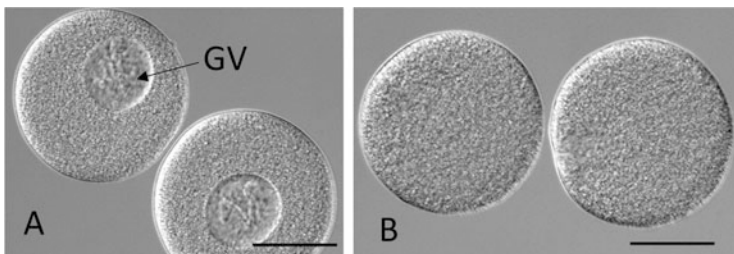
Intact adults and even some large body pieces can often be kept up to several weeks in the laboratory by adhering to the following common-sense guidelines for maintaining marine invertebrates. Specimens should be kept at the prevailing temperature range typically encountered in local waters. At coastal marine biology stations, such conditions are most easily met by a continuous supply of flowing seawater that is pumped into the laboratory, whereas inland operations will require incubators that can reach the required temperatures. Although store-bought refrigerators are generally incapable of elevating their cooling cycles to reach a useful range of 12–18°C, changing the factory-installed thermostat to one with higher settings can provide an inexpensive alternative to the purchase of a dedicated cooling incubator.

Storage containers useful for housing nemerteans can be made of either glass or plastic but ideally should be of embryological grade (“E ware”) that has not been subjected to fixatives or harsh solvents. If purchased new, it is best to condition the containers for several days in flowing seawater or in daily changes of seawater to add a beneficial microbial coating prior to the introduction of an adult worm. Thereafter, keeping only one adult or large body piece per each numbered container optimizes chances of prolonged survival while also helping to keep track of specific worms used in experiments.

Once specimens are placed in containers at suitable temperatures, the seawater surrounding each adult nemertean must be refreshed on a regular basis. If the worms are kept at coastal laboratories in mesh-enclosed containers that allow a constant circulation of seawater, the mesh should be cleaned occasionally in order to ensure adequate flow. Alternatively, when maintained in containers lacking continuous flow or aeration, the stagnant seawater needs to be replaced at least once or twice a day. Moreover, every few days, the container should be rinsed in fresh water to avoid contamination from adult waste products. Common signs of stress incurred during storage include the fragmentation of the body into smaller pieces. Once this begins, the remainder of the worm should be promptly transferred to another conditioned container, since detached body remnants rapidly degenerate and thereby cause further degradation of the worm.

### ***1.2.3 Obtaining Gametes and Generating Cultures of Embryos***

For relatively small adults (~1–5 cm long), such as those of *Maculaura alaskensis*, a sufficient supply of gametes can be obtained by repeatedly puncturing the entire body of ripe specimens with jeweler’s forceps to release sperm or oocytes from essentially all of the serially repeated gonads present in the worm. With larger specimens (e.g., *Cerebratulus*), small pieces of the body can be cut with fine scissors

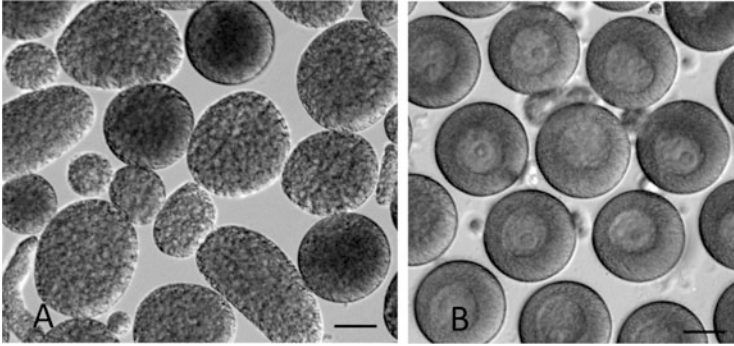


**Fig. 1.2** (a) Immature oocytes of the nemertean *Cerebratulus* sp., exhibiting a prominent germinal vesicle (GV). (b) Mature oocytes at 60 min post-stimulation of germinal vesicle breakdown. Scale bars = 50  $\mu$ m

to provide numerous gametes that in turn can correspond to up to several thousand oocytes per centimeter of ripe body piece. Accordingly, if collateral damage to the worm during such cutting procedures is kept to a minimum, a single worm can be used multiple times for various experiments.

In most nemerteans, fully grown oocytes isolated from ripe females range in diameter from  $\sim 70$  to  $200 \mu\text{m}$ , and each contains a prominent nucleus (=“germinal vesicle,” GV), indicating an arrest at the prophase I stage of meiosis (Fig. 1.2a). If allowed to incubate in either natural seawater or calcium-containing artificial seawater [e.g., “MBL artificial seawater” (Cavanaugh 1956) or Coralife marine salt (<http://www.drsfostersmith.com>)], such prophase-arrested specimens rapidly begin to mature within  $\sim 30$  to 60 min depending on the particular species and incubation temperatures that are used. The process of germinal vesicle breakdown (GVBD) signals the onset of such maturation, and following GVBD oocytes arrest at metaphase I of meiosis (Fig. 1.2b). Similarly, batches of immature oocytes rapidly react to various cAMP-elevating treatments by undergoing GVBD (Stricker and Smythe 2000, 2001). Alternatively, maturation can be routinely prevented if oocytes emanating from punctured or cut females are rapidly transferred to ice-cold calcium-free seawater (CaFSW) or seawater solutions containing various kinds of pharmacological agents such as antioxidants, protein kinase C inhibitors, stimulators of adenosine monophosphate-activated kinase (AMPK), or blockers of maturation-promoting factor (MPF) activity (Smythe and Stricker 2005; Stricker 2011, 2012; Stricker and Smythe 2006a, b; Stricker et al. 2010a, b, c).

When collecting gametes for inseminations, sperm should be quickly pipetted from the surface of punctured or cut males and kept relatively undiluted within a capped microcentrifuge tube in the refrigerator or cooling incubator. If stored in this way, sperm can remain capable of fertilizing oocytes for a day or more. As opposed to relatively long-lived sperm, however, an unseminated mature oocytes arrested at metaphase I of meiosis rapidly lose their ability to undergo normal fertilization within  $\sim 6$ – $10$  h post-GVBD, and such specimens typically show overt signs of degradation by about a day after maturing (Fig. 1.3a). Conversely, if fully grown oocytes isolated from adult females are maintained in an immature state by treatments such as those listed above, longevity can be stretched to several days, as



**Fig. 1.3** (a) Degrading mature oocytes of the nemertean *Cerebratulus* sp. ~1 day post-GVBD. (b) Intact immature oocytes ~1 day post-GVBD. Scale bars = 50  $\mu$ m

evidenced by the capacity of these aged oocytes to undergo maturation followed by fertilization and embryogenesis after more than a day of prophase I arrest (Fig. 1.3b).

Regardless of whether or not oocytes are immediately stimulated to mature after being collected from ripe females, each batch should be washed several times in either filtered seawater or CaFSW to remove debris released from the adult during the puncturing or cutting process. Such washes can be accelerated by using a hand-operated centrifuge for gentle sedimentation of the oocytes before decanting the bulk of the overlying fluid. Moreover, pelleting can be enhanced by removing the jelly coat surrounding the oocytes with the aid of a 60-ml plastic irrigation syringe (<https://www.vitalitymedical.com>) that had its tapered end sawed off to yield a cylinder. At the cut end of such syringes, a piece of Nitex<sup>®</sup> nylon screen ([www.dynamicaqua.com](http://www.dynamicaqua.com)) of appropriate mesh can be attached by means of a hot-melt glue gun. The actual size of the Nitex mesh should be ~5–10  $\mu$ m smaller than the diameter of the oocytes that are being handled, and oocytes washed with this device should be gently aspirated across the mesh several times while being kept submerged in order to remove jelly coats and surrounding debris without causing substantial lysis.

To provide baseline comparisons of fertilized specimens with unfertilized oocytes undergoing maturation and aging, mature oocytes are best inseminated within ~6 h of GVBD using freshly diluted sperm solutions that yield approximately 5–50 sperm around each viable oocyte. If in vivo imaging of membrane-impermeable probes is required during or after fertilization, oocytes are best microinjected either prior to GVBD or soon after maturation has begun (Stricker 1996; Stricker et al. 1998). Zygotes resulting from fertilizations should be kept in loosely arranged monolayers within culture dishes in order to avoid anoxia, and once cleavages begin, the overlying seawater should be changed on a daily basis. Depending on the species and incubation temperature, embryos typically remain stationary and thus easily observed for up to a day or so, at which point ciliated stages begin to swim toward the top of the culture dish and eventually develop into motile larvae.

### ***1.2.4 General Cell Biological Applications***

Once fully grown oocytes are obtained from ripe females, such material can be subjected to various cell biological protocols according to standard methods that are described in detail elsewhere. For example, microinjection of nemertean oocytes with calcium-sensitive fluorescent dyes and the subsequent monitoring of dye-loaded specimens by dual-channel confocal imaging are discussed by Stricker (1996, 1997, 2000). Similarly, the use of nemertean oocytes in either kinase assays or immunoblotting studies employing phospho-specific antibodies is covered by Stricker and Smythe (2003) and Escalona and Stricker (2014).

## **1.3 Results and Discussion**

### ***1.3.1 Using Nemertean Oocytes in Analyses of Maturation and Aging Processes***

In addition to general features shared with other externally fertilizing invertebrates, several other properties make nemerteans well suited for studies of oocyte maturation and aging. For example, fully grown oocytes can be rapidly triggered to mature simply by incubating prophase-arrested specimens in either calcium-containing seawater or various solutions that elevate intracellular cAMP levels, and, conversely, maturation is reliably blocked by a wide array of pharmacological agents (Stricker and Smythe, 2000, 2001; Stricker et al. 2013). Moreover, in many cases, drugs that block seawater-induced GVBD fail to prevent cAMP-stimulated GVBD (Smythe and Stricker 2005; Stricker 2011, 2012; Stricker and Smythe 2006a, b; Stricker et al. 2010a, b, c). This general ability of cAMP elevators to override the blockage of GVBD by various drugs dissolved in seawater in turn provides a helpful control ensuring that any observed failure to undergo maturation in the presence of an inhibitor is not simply due to general morbidity of the oocytes.

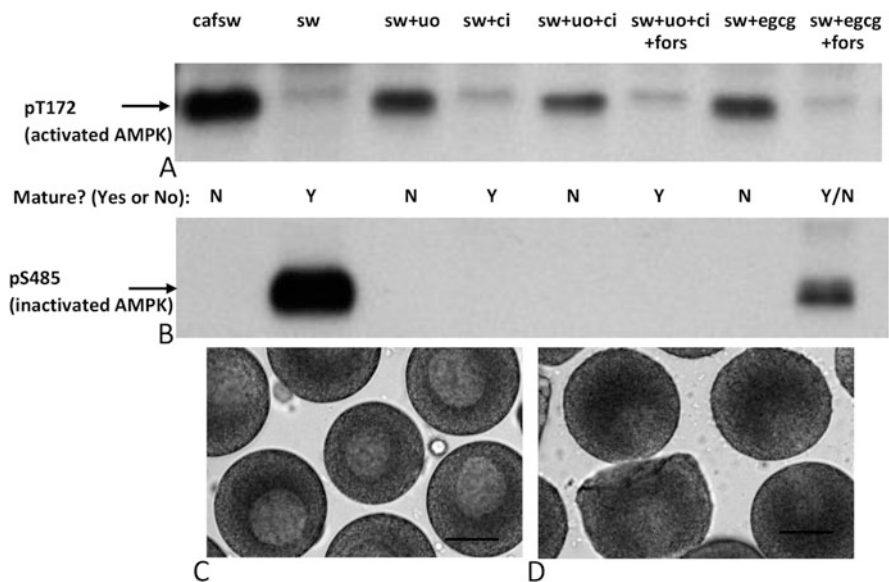
Another benefit of using nemertean oocytes for studies of maturation is that follicle cells are typically lacking (Stricker et al. 2001). Thus, indirect roles played by such accessory cells in the maturation process will not confound interpretation of experimental results. In addition, although these protostomes are distantly related to vertebrates, numerous phospho-specific antibodies raised against mammalian epitopes have been used in immunoblotting analyses to track protein activities in nemertean oocytes (Escalona and Stricker 2014), and the validity of such signals is supported not only by the proper molecular weights of the bands that are observed but also by the appropriate changes in signal strength that occur in response to relatively specific pharmacological modulators. In accordance with such cross-reactivity, oocyte maturation in nemerteans appears to share various signaling pathways in common with intensively analyzed mammalian systems such as mice. Nevertheless, fundamental differences are also evident. For example, in nemerteans,

cAMP elevators and AMPK stimulators serve to trigger versus block seawater-induced maturation, respectively, whereas in mice, the elevation of intraoocytic cAMP and the activation of AMPK cause opposite responses to those displayed by nemerteans (Stricker et al. 2010b, 2013).

Similarly, although the activities of such kinases as the Cdk1 (cyclin-dependent kinase 1) component of MPF and some types of mitogen-activated protein kinases (MAPKs) display the same general kinetics in aging nemertean oocytes as noted for mammals, some discrepancies are also evident. For example, aging nemertean oocytes degrade via a distinctly different morphological pattern of cell death than is observed in mammals and other deuterostomes (Stricker et al. 2016). Thus, studies of maturation and aging in nemertean oocytes may prove useful in identifying both common features and exceptional processes across the animal kingdom. In order to highlight some of the data obtained in studies using nemertean oocytes, the final two sections summarize selected results related to the deactivation of AMPK during oocyte maturation and the activation of JNK during the degradation of aged oocytes.

### ***1.3.2 AMP Kinase (AMPK) Deactivation During Oocyte Maturation***

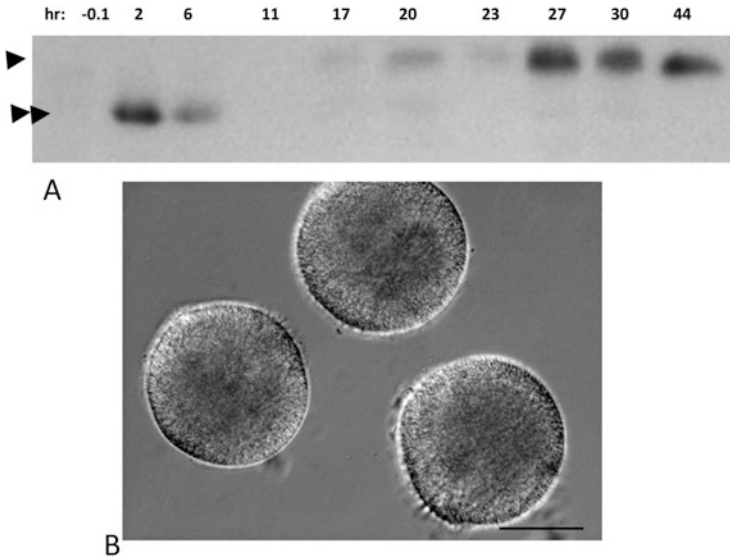
Prophase-arrested nemertean oocytes exhibit high levels of active AMPK as indicated not only by elevated phosphorylation of the stimulatory T172 site of AMPK (Fig. 1.4a) but also by correlative activation of upstream kinases that phosphorylate T172 as well as the phosphorylation of downstream targets known to be activated by AMPK (Stricker 2011; Stricker et al. 2010c). Upon stimulation by seawater, AMPK is deactivated in maturing oocytes by the dephosphorylation of the T172 residue and the concomitant phosphorylation of an S485 site that serves to inhibit AMPK activity (Fig. 1.4a, b). Such seawater-induced phosphorylation changes associated with AMPK deactivation can be blocked by several known AMPK activators, including the antioxidant EGCG (epigallocatechin gallate), as well as by the MAPK kinase inhibitor U0126 (Fig. 1.4a–c) (Stricker 2011; Stricker et al. 2010c). Conversely, adding cAMP elevators such as forskolin to seawater typically overrides the stimulatory effects of AMPK activators and allows both AMPK deactivation and oocyte maturation (Fig. 1.4a–d). Collectively, such findings indicate that AMPK activation is required for seawater-mediated, but not cAMP-induced, oocyte maturation in nemerteans (Stricker 2011; Stricker et al. 2010c).



**Fig. 1.4** Deactivation of adenosine monophosphate activated kinase (AMPK) during seawater-induced maturation. (a, b) Immunoblots of *Cerebratulus* sp. oocytes after 2 h incubation in various solutions (cafsw = calcium-free seawater; sw = seawater; uo = less specific MAPKK inhibitor U-0126; ci = more specific MAPKK inhibitor ci-1040; fors = cAMP elevator forskolin; egcg = AMPK activator epigallocatechin gallate), showing a decrease in the activating phosphorylation (pT172) and a concomitant increase in the inhibitory phosphorylation (pS485) of AMPK during normal sw-induced maturation of *Cerebratulus* sp. oocytes. (c, d) Blockage of GVBD by seawater solutions of the AMPK stimulator AICAR (5-Aminoimidazole-4-carboxamide ribonucleoside) (c) and the overriding of such inhibition by the addition of the cAMP elevator forskolin to sw + AICAR treatments (D). Scale bars = 50  $\mu$ m

### 1.3.3 *c-Jun N-terminal Kinase (JNK) Activation During Oocyte Degradation*

During maturation, nemertean oocytes activate a ~40-kD putative JNK isoform (Fig. 1.5a). If left unseminated, aging oocytes lose the 40-kD JNK signal by about 6 h and eventually activate a 48-kD JNK just prior to undergoing degradation (Fig. 1.4a). Such JNK activation occurs about a day after GVBD, at about the same time that MPF factor activity declines (Stricker and Ravichandran 2017). Accordingly, JNK inhibitors not only reduce the 48-kD JNK signal in aged oocytes (Stricker and Ravichandran 2017) but also retard MPF deactivation as well as the onset of degradation (Fig. 1.5b), collectively suggesting that JNK activation may promote the decline in MPF activity observed in degrading oocytes.



**Fig. 1.5** C-Jun N-terminal kinase (JNK) signals during oocyte maturation and aging in seawater-stimulated *Cerebratulus* sp. oocytes. (a) Immunoblot of phospho-JNK signals indicative of JNK activation at various timepoints, including: (i) before seawater stimulation ( $-0.1$  h), (ii) the conversion of immature oocytes into metaphase-I-arrested mature oocytes (2 h), and (iii) the aging-related death of uninseminated mature oocytes, which typically begins around 24 h post-maturation (note: single arrowhead marks p48 phospho-JNK bands; double arrowheads mark p40 phospho-JNK bands). (b) Intact oocytes at 1 day post-treatment in seawater solution of the JNK inhibitor SP600125. Scale bar = 50  $\mu$ m

## 1.4 Conclusions

Oocytes produced by protostome worms belonging to the phylum Nemertea represent a rich and relatively untapped resource for embryological analyses, especially those that focus on oocyte maturation and aging. With optically clear properties that lend these specimens to live imaging studies, nemertean oocytes can be readily triggered to mature via calcium-containing seawaters or cAMP-elevating agents. Conversely, maturation is reversibly inhibited by a wide array of pharmacological modulators. In addition, the aging of uninseminated specimens occurs over a comparatively short time span and involves a mode of degradation that is morphologically distinct from that described for deuterostome oocytes. Accordingly, although nemerteans resemble mammals and other deuterostomes in using the same basic signaling pathways to regulate oocyte maturation and aging, notable differences are also readily evident. Thus, further investigations of these worms may well yield novel and interesting insights into the underlying mechanisms of how oocytes across the animal kingdom mature and age.



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# Chapter 2

## Sperm Nuclear Basic Proteins of Marine Invertebrates



Anna Török and Sebastian G. Gornik

**Abstract** In this chapter, a short evolutionary history and comparative analysis of sperm nuclear basic proteins (SNBPs) in marine invertebrates are presented based on some of the most recent publications in the field and building upon previously published reviews on the topic. Putative functions of SNBPs in sperm chromatin beyond DNA packaging will also be discussed with a primary focus on outstanding research questions.

In somatic cells of all metazoans, DNA is packaged into tightly folded and dynamically accessible chromatin by canonical histones H2A, H2B, H3 and H4. Sperm chromatin of many animals, on the other hand, is organised by small yet structurally highly heterogeneous proteins called SNBPs, which can package sperm DNA on their own or in combination with each other. In extreme cases, sperm chromatin is condensed into a volume 6–10 times smaller than that of a somatic nucleus. SNBPs are classified into three major groups: H1 histone-type proteins (H-type SNBPs), protamines (P-type SNBPs) and protamine-like proteins (PL-type SNBPs). P-type SNBPs are mostly found in vertebrates, while PL-type SNBPs are ubiquitous in many invertebrate phyla. PL-type and P-type SNBPs evolved from histone H-type SNBP precursors through vertical evolution. Porifera, Ctenophora and Crustacea, Echinoidea (phylum Echinodermata) and Hydrozoa (phylum Hydrozoa) lack SNBPs. Echinoidea and Hydrozoa, however, evolved novel nucleosomal histone variants with specific roles during spermatogenesis. Seemingly, chromatin condensation plays a critical role in the silencing and tight packing of the genome within the sperm nucleus of most animals. However, the question of what necessitates the compaction of some sperm DNA beyond classical nucleosomal packaging while other sperm function using ‘normal’ histones remains unanswered to date.

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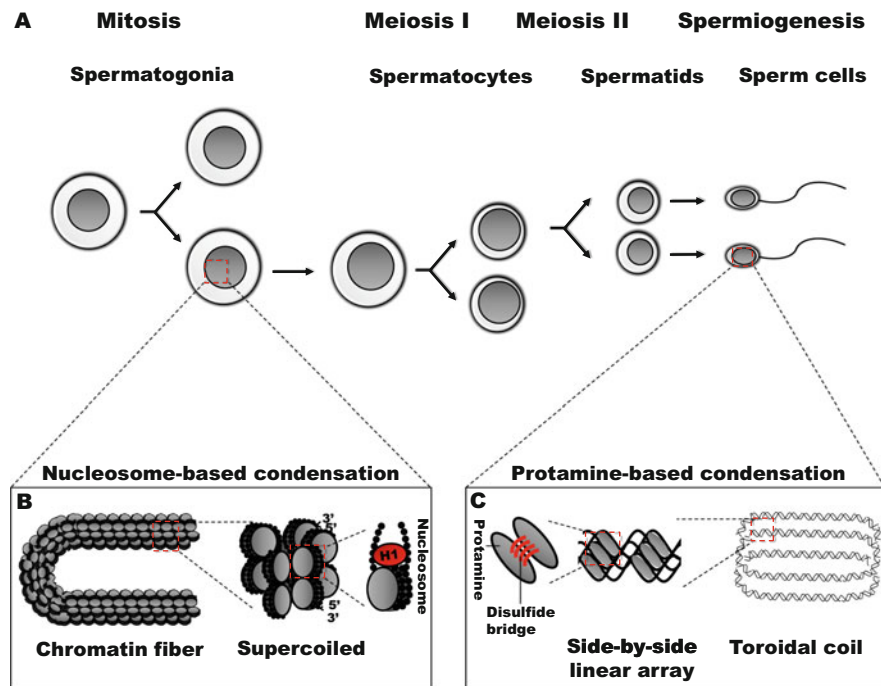
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**Fig. 2.1** (a) During the process of spermatogenesis, germ cells go through mitotic divisions followed by meiosis to produce haploid spermatids. These cells then differentiate into mature sperm cells. In most animals, this final differentiation is accompanied by major chromatin remodelling. (b) Spermatogonia utilise nucleosomes to package their DNA in much the same way than somatic cells. H1 linker histones aid DNA compaction. (c) In spermatids, during meiosis, nucleosomes are replaced by SNBPs, which further condense the sperm's chromatin into a toroidal coil based on the form of tightly packed side-by-side linear arrays, which in some animals are stabilised by disulphide bridges

## 2.1 Diversity of Sperm Nuclear Basic Proteins (SNBPs)

In somatic cells, DNA is packed into tightly folded and dynamically accessible chromatin by two pairs of histone H2A–H2B and H3–H4 dimers that form an octamer core around which the DNA is wound in a compact structure called the nucleosome (Talbert and Henikoff 2010) (Fig. 2.1). In addition, H1 linker histones aid DNA compaction (Hergeth and Schneider 2015) (Fig. 2.1b).

Spermatogenesis is a complex biological process of male haploid sperm production from diploid spermatogonial stem cells. Sperm chromatin undergoes major remodelling during this process and, in mature sperm, generally exhibits a high degree of condensation and stability compared to somatic cell chromatin (Ward and Coffey 1991; Sassone-Corsi 2002). Across different animal groups, sperm chromatin condensation and stability is organised by structurally highly heterogeneous proteins called sperm nuclear basic proteins (SNBPs). SNBPs can pack sperm DNA on their own or in combination with each other. In extreme cases, sperm

chromatin is condensed into a volume 6–10 times smaller than that of a somatic nucleus (Fuentes-Mascorro et al. 2000; Miller et al. 2010). SNBPs are classified into three major groups: H1 histone-type proteins (H-type SNBPs), protamines (P-type SNBPs) and protamine-like proteins (PL-type SNBPs) (Bloch 1969; Ausio 1999).

H-type SNBPs are a testis-specific, highly basic, lysine- and arginine-rich group of histone H1 variants. They mediate the condensation of DNA during male gametogenesis in similar ways to their somatic counterparts, preventing DNA damage, by stabilising nucleosomes and compact chromatin fibres into higher-order structures. H-type SNBPs are found across all invertebrates and are essential during spermatogenesis (Ausio 1999; Faraone Mennella et al. 2002; Chikhirzhina et al. 2011; Pan and Fan 2016; Roque et al. 2016). The differentiation between H-type SNBPs and somatic H1 histones took place early in metazoan evolution (Eirin-Lopez et al. 2006a) (Fig. 2.2).

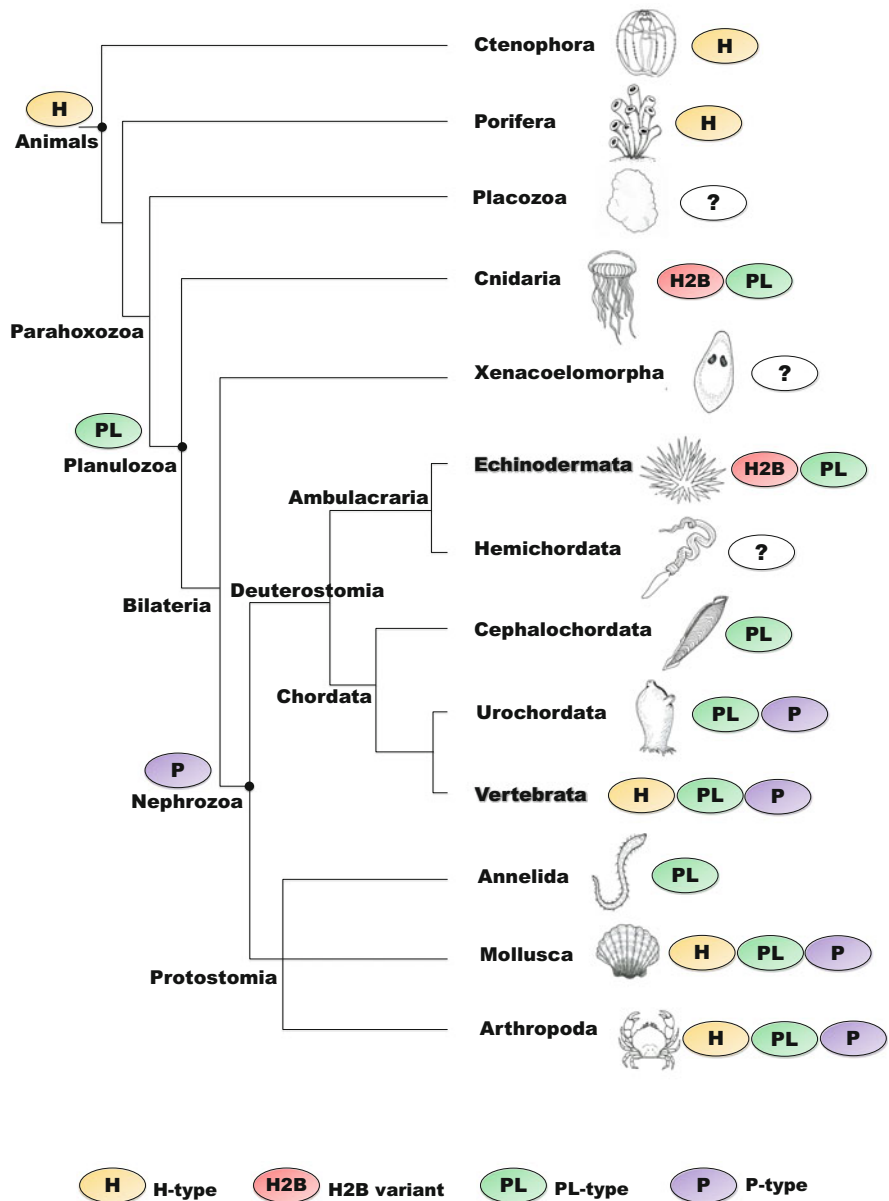
P-type SNBPs, such as protamines, are small, highly heterogeneous, arginine- and/or cysteine-rich proteins that assist or replace sperm histones during spermatogenesis of many animals, after chromatin remodelling (Ausio 1999; Braun 2001; Lewis et al. 2003). They bind to the major groove of DNA, neutralising the phosphodiester backbone and causing it to form a toroidal coil structure resulting in a linear side-by-side chromatin organisation (Brewer et al. 1999; Biegeleisen 2006; Balhorn 2007; Roque et al. 2011) (Fig. 2.1c). Many protamines also contain multiple cysteines that allow the formation of disulphide bridges between adjacent protamines to further stabilise DNA/protamine complexes (Bedford and Calvin 1974; Balhorn 1982, 2007). The most studied P-type SNBP is protamine P1, which occurs in sperm of all mammals and is essential for sperm function by stabilising sperm chromatin (Domenjoud et al. 1991).

PL-type SNBPs, like P-type SNBPs, associate with or replace most of the somatic histones during the final stages of spermatogenesis (Subirana et al. 1973; Ausio 1999). They are considerably larger than P-type SNBPs but equally heterogeneous. Unlike the arginine-rich P-type SNBPs, PL-type SNBPs are rich in both arginine and lysine residues, and this makes them a structurally intermediate group between H-type and P-type SNBPs (Ausio 1999).

It is worth noting that all three types of SNBPs pack DNA into highly condensed chromatin fibres, regardless of the particular structure of the individual DNA/protein complexes they form (Casas et al. 1993). Sperm chromatin packed with SNBP is considered extremely dense, highly inaccessible and transcriptionally inert (Ooi and Henikoff 2007).

## 2.2 Evolutionary Origin of SNBPs

P-type SNBPs, then called protamines, were first discovered in 1874 by Miescher in the spermatozoa of fish (Dahm 2010). They are also found in many other vertebrates such as mammals and reptiles and in a few invertebrates including urochordates, molluscs and terrestrial arthropods (Subirana et al. 1973; Olivares et al. 1986; Daban



**Fig. 2.2** Simplified animal phylogeny showing the origins, distribution and diversity of SNBPs and sperm-specific histone H2B variants across major phyla

et al. 1990; Chiva et al. 1992; Rocchini et al. 1995a) (Fig. 2.2). P-type SNBPs were first described in molluscs almost 100 years later (Subirana et al. 1973; Olivares et al. 1986). Since then, PL-type SNBPs have also been identified in many other

invertebrate phyla, including urochordates, cephalochordates, cnidarians, annelids, echinoderms, tunicates and some vertebrates (Saperas et al. 1992, 1994, 1997, 2006; Rocchini et al. 1995b; Ausio 1999; Eirin-Lopez et al. 2006b; Torok et al. 2016) (Fig. 2.2).

Two hypotheses have been advanced for the origin of P-type and PL-type SNBPs: the horizontal evolution hypothesis and the vertical evolution hypothesis (Ausio 1999). According to the horizontal evolution hypothesis, P-type SNBPs had a retroviral origin (Jankowski et al. 1986; Krawetz and Dixon 1988). This was based on the observation that protamines appeared to be randomly distributed within bony fish (Osteichthyes) and that their genes were flanked by repetitive regions showing a high degree of similarity to certain retroviral sequences (Jankowski et al. 1986). However, more detailed phylogenetic analyses revealed that the origin of P-type SNBP within bony fish can be traced to a common ancestor and that their extant distribution can be explained by multiple independent losses during bony fish radiation (Saperas et al. 1994). The horizontal hypothesis for P-type and PL-type SNBP origins through retroviral transmission has therefore not been widely accepted (Lewis et al. 2004; Eirin-Lopez et al. 2006b; Frehlick et al. 2006; Eirin-Lopez and Ausio 2009).

The alternative vertical evolution hypothesis postulates that P-type SNBPs evolved from a primitive histone H1 precursor via PL-type SNBP intermediates (Subirana et al. 1973; Ausio 1999). For this to be true, one should only find histones and PL-type precursors in early-diverging metazoans with more specialised PL-type and P-type SNBPs in later-diverging groups only. To date, no PL-type or P-type SNBPs have been found in Porifera or Ctenophora (Ausio 1999) (Fig. 2.2). In Cnidaria, only H- and PL-type SNBPs exist with P-type SNBPs being absent, also supporting the vertical evolution hypothesis prediction (Rocchini et al. 1996; Torok et al. 2016). Therefore, P-type SNBPs appear to have evolved in the last common ancestor of Bilateria before deuterostomes and protostomes split (Rocchini et al. 1996; Ausio 1999; Eirin-Lopez and Ausio 2009) (Fig. 2.2).

The vertical evolution hypothesis is bolstered by a number of studies which show that PL-type or P-type SNBPs of a wide variety of different animals such as molluscs, tunicates and fishes are structurally highly similar to H1 linker histone (Watson and Davies 1998; Watson et al. 1999; Lewis et al. 2004; Eirin-Lopez et al. 2006a, b). This preservation of structural properties is surprising, since histone H1 molecules are highly lysine-rich (25–30%), while PL-type SNBPs are rich in both arginine and lysine (35–50%), and P-type SNBPs are rich in arginine only (~30%) (Eirin-Lopez et al. 2006a). The vertical evolution hypothesis can explain how lysine-rich H1 proteins can transition to lysine- and arginine-rich P- and PL-type SNBPs by codon usage and protein structure analysis. In histone H1 genes, most lysines are encoded by an AAG codon, while in P-type SNBPs, arginine is often encoded by an AGA codon. The two point mutations required to change each lysine to arginine are highly unlikely to occur both in number and localization of changes to the C-terminal region of histone H1 in a relatively short amount of evolutionary time (Lewis et al. 2004). Instead, it has been suggested that the radical increase of arginine content is originated from a number of frameshift mutations. For example, the



**Fig. 2.3** Evidence for the proposed mechanisms of rapid lysine to arginine transitions found in the evolution from H1 precursors to P-type SNBPs. A putative frameshift mutation at position 342 in the PL-I gene of *Ciona intestinalis* would result in a new SNBP with a nearly doubled arginine content more similar to P-type SNBPs

deletion of a single nucleotide at a specific position in the C-terminus of a SNBP precursor of the P1 gene of the tunicate *Styela montereyensis* might have generated the current arginine-rich *Styela* P1 SNBP. This is bolstered by the fact that a putative in silico frameshift in PL-I of *Ciona intestinalis* generates a new PL-type-like protein with a nearly doubled arginine content and high similarity to *Styela* P1 (Fig. 2.3) (Lewis et al. 2004; Eirin-Lopez et al. 2006a). Furthermore, extant PL-type SNBPs that have putatively arisen from H1 genes through frameshifts still have structural features in common with both H-type and P-type SNBPs. Like histone H1, they possess a protease-resistant core domain and unstructured, basic C- and N-terminal flanks. Interestingly, post-translational cleavage of many extant PL-type SNBPs generates products highly similar to P-type SNBPs, such as in the bivalve genus *Mytilus*, the mollusc *Spisula solidissima* and the tunicates *Styela montereyensis* and *Ciona intestinalis* (Eirin-Lopez et al. 2006a, b). If such cleavage fragments evolved to become independent genes, they would produce bona fide P-type SNBPs. This seems to have happened in the genus *Mytilus*, where a PL-type cleavage fragment called PL-III likely became an independent gene (Carlos et al. 1993). Positive selection for these new and shorter proteins could also depend on the observation that a higher arginine content increases the affinity of proteins for DNA (Ausio et al. 1984; Eirin-Lopez et al. 2008; Piscopo et al. 2010).

Taken together, based on current knowledge, it can thus be concluded that PL-type and P-type SNBPs evolved from histone H1 precursors partly through lysine to arginine transitions, which were introduced by frameshift mutations and positively selected for through DNA-binding affinity. Further opportunity for SNBP diversity arose from the segregation of PL-type protein components from different regions of the above-mentioned histone H1 precursor molecule. Moreover, SNBP evolution was also aided by gene duplication that resulted in independent genes that exhibited subsequent parallel vertical evolution (Eirin-Lopez et al. 2008). However, frameshift mutations and segregation of components alone may not be the only factor in the origin of SNBPs, and it is expected that other as yet undefined mechanisms also contributed to histone H1 to P-type SNBP evolution (Saperas and Ausio 2013).



## 2.3 SNBPs in Marine Invertebrates

### 2.3.1 *P-Type and PL-Type SNBPs Contribute to Sperm Chromatin Compaction and Often Replace Somatic Histones Entirely During Spermatogenesis*

In many animals, P-type and PL-type SNBPs have been co-opted or have evolved to aid histones and histone variants to facilitate increased chromatin compaction and stability in sperm. For example, in mature sperm of the bivalve molluscs, *Swiftopecten swiftii* and *Glycymeris yessoensis*, a PL-type ‘S protein’ is found alongside nucleosomal histones (Odintsova et al. 1989). Sperm chromatin from *Mytilus galloprovincialis* and many other Mytilidae consists of three protamine-like proteins, PL-II, PL-III and PL-IV, in addition to a residual amount of the four core histones (Zalensky and Avramova 1984; Vassalli et al. 2015).

In many cases, bulk sperm nucleosomal histones can be entirely replaced by SNBPs (Ausio and Van Holde 1987). For example, *Mytilus edulis* sperm chromatin contains two PL-type SNBPs and one H-type SNBP, and nucleosomes were absent, as shown by micrococcal nuclease (MNase) digestion (Ausio and Subirana 1982). Likewise, in mature sperm of the cephalochordate *Branchiostoma floridae*, only PL-type proteins can be identified (Eirin-Lopez et al. 2008). Similarly, in the tunicate *Styela montereyensis*, the majority of histones are replaced by basic, lysine-rich PL-type proteins during spermatogenesis (Chiva et al. 1990, 1992). In sperm of the marine annelid worm *Chaetopterus variopedatus*, only two proteins have been identified: an arginine- and lysine-rich PL-type protein and a lysine-rich H1-like protein (Depetrocellis et al. 1983). The sperm chromatin of the marine annelid worm *Platynereis dumerilii* also lacks histones but contains two basic PL-type polypeptides called P-1 and P-2 (Sellos and Kmiecik 1985).

### 2.3.2 *Porifera, Ctenophora and Crustacea Exclusively Use Somatic Histones to Pack Sperm DNA*

Despite the fact that many animals utilise SNBPs to increase chromatin compaction and stability of sperm, in some animal groups, these proteins are missing entirely, and sperm chromatin seems to be packaged with somatic histones only via nucleosomes and linker histone H1. For example, analysis of the sperm nuclear proteins in the sponge *Neofibularia nolitangere* revealed only histones with no P- or PL-type SNBPs being identified (Ausio et al. 1997). Furthermore, transcriptome data generated from eight species representing all four extant sponge classes Hexactinellida, Demospongiae, Homoscleromorpha and Calcarea (Riesgo et al. 2014) did not reveal any SNBP sequences. Likewise, searching the transcriptome and genome data of the ctenophore *Mnemiopsis leidyi* (Ryan et al. 2013) does not identify any SNBPs and only canonical histones. In contrast, Cnidaria from the class Anthozoa possess

PL-type proteins (Torok et al. 2016). Thus, it appears that early-diverging animals, including sponges (Porifera) and ctenophores, exclusively use histones to pack sperm DNA and lack SNBPs, suggesting that SNBPs first appeared during the evolution of metazoans after ctenophores and sponges arose (Fig. 2.2).

Interestingly, crustaceans within the phylum Arthropoda seem to have lost SNBPs secondarily during evolution and now exclusively utilise somatic histones for sperm chromatin maturation (Kurtz et al. 2008). As a consequence, they have relatively loosely packed sperm chromatin, compared to sperm whose chromatin is condensed with SNBPs. Interestingly, this low level of condensation of sperm chromatin is essential for fertilisation of crustaceans (Kurtz et al. 2008). Crustacean sperm lack flagella and evolved a modified mechanism for oocyte penetration that does not rely on forces generated by sperm movement (Hinsch 1971). It is thus presumed that the lower degree of sperm chromatin condensation provides more flexible sperm nuclei that can pass through the acrosomal canal facilitating fertilisation by these unusual sperm cells (Gimenez-Bonafe et al. 2002). It is speculated that crustacean sperm chromatin, if packaged with P-type or PL-type SNBPs, would be too dense and compact for this function. It is also conceivable that an initial loss of P-type or PL-type SNBPs to create less densely packed chromatin enabled loss of the sperm flagellum.

### 2.3.3 *Echinoidea (Phylum Echinodermata) and Hydrozoa (Phylum Cnidaria) Lack SNBPs but Evolved Novel Nucleosomal Histone Variants with Specific Roles in Spermatogenesis*

Some members within the marine invertebrate phyla, Echinodermata and Cnidaria, lack SNBPs entirely and instead have evolved novel sperm-specific histone variants. These histones carry highly unusual and extensive N-terminal extensions that contain up to seven repeats of the DNA-binding SPK[K/R] amino acid motifs

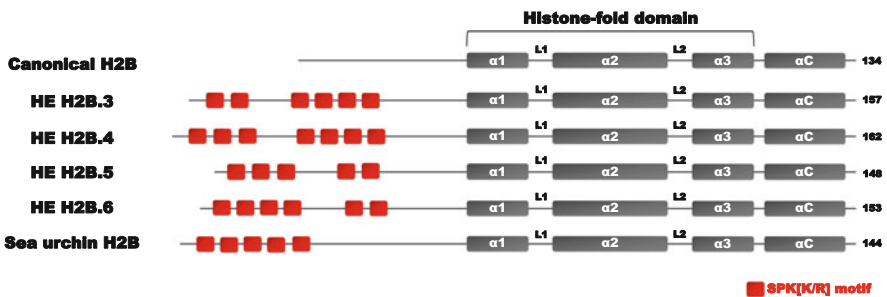


Fig. 2.4 N-terminal SPK[K/R] motif distribution in sperm-specific H2B variants of *Hydractinia echinata*

(Fig. 2.4). In the absence of SNBPs, the sperm-specific histone variants of echinoderms and hydrozoans play a more central role in packaging of sperm DNA than any other known sperm-specific histone variants (Strickland et al. 1977; Ausio 1999; Torok et al. 2016).

This contrasts with other known sperm-specific histone variants, which show only a few amino acid differences compared to their somatic counterparts (Malik and Henikoff 2003). Furthermore, typical sperm-specific histones are expressed at very low levels, have highly specific expression patterns and exhibit well-defined nuclear localisations. They also have novel functional properties that often affect histone post-translational modifications within their otherwise highly conserved protein sequences (Malik and Henikoff 2003; Kamakaka and Biggins 2005).

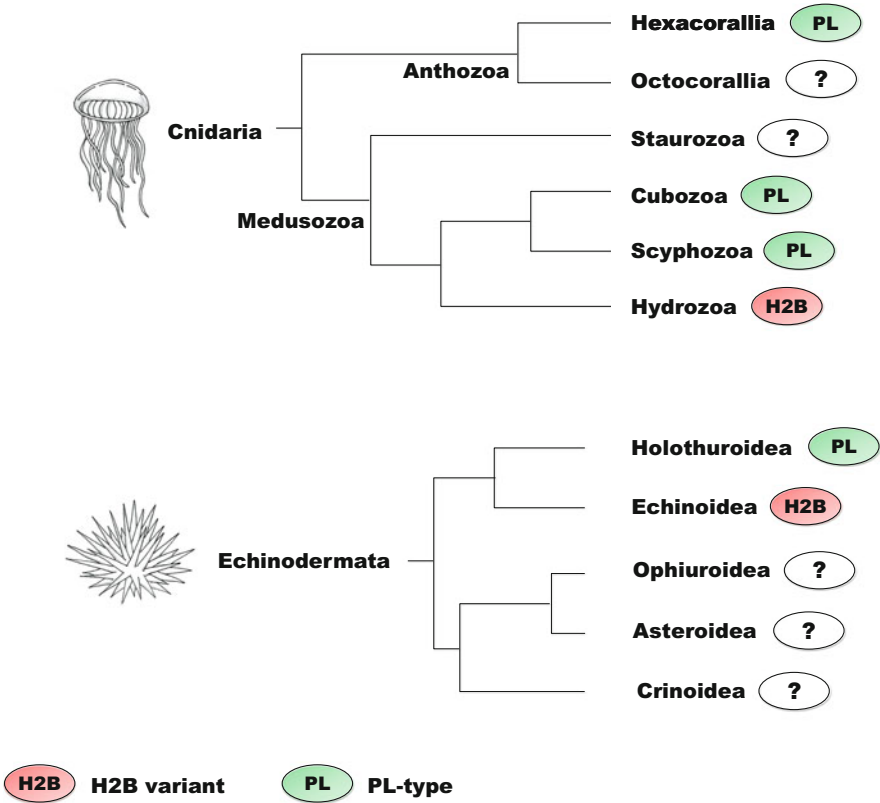
Echinoidea is a class of echinoderms comprising the sea urchins. Hydrozoa is a class of Cnidaria within the subphylum Medusozoa. In Echinoidea, variants of histones H1 and H2B possess the unusual extended N-terminal domains (Strickland et al. 1977; Poccia et al. 1984), while in Hydrozoa, the N-terminal extensions are limited to sperm-specific H2Bs (Torok et al. 2016; Reddy et al. 2017).

In both cases, nucleosomal organisation is retained in sperm chromatin, and no protamines or PL-type SNBPs are present (Easton and Chalkley 1972; Spadafora et al. 1976; Torok et al. 2016) (Fig. 2.5). The Echinoidea histone variants appear early in spermatogenesis and gradually substitute canonical histones during sperm cell proliferation (Poccia et al. 1987), whereas the timing of H2B variant expression during spermatogenesis of hydrozoans is not yet known.

It has been shown that the SPK[K/R] motifs bind to DNA in the minor groove with high affinity, with a footprint of two base pairs per motif repeat (Suzuki 1989; Suzuki et al. 1993). Furthermore, it has been shown that phosphorylation of the serine within the motif inhibits DNA binding (Green et al. 1993, 1995). However, the exact nature and identity of the kinases and phosphatases that dynamically control the phosphorylation status of SPK[K/R] motifs remains unknown.

In sea urchins, the serines within the SPK[K/R] motifs are known to be phosphorylated at early stages of sperm maturation, decreasing the affinities of these histones to the DNA backbone by weakening electrostatic interactions of SPK[K/R] motifs with DNA. Histones with phosphorylated SPK[K/R] motifs function as normal histones throughout spermatogenesis (Green and Poccia 1988). In later stages of sperm maturation after most or all of DNA condensation has taken place, a dephosphorylation event occurs (Poccia et al. 1987) to allow the SPK[K/R] motifs to bind to linker DNA (Lindsey and Thompson 1992) thus stabilising the chromatin of sperm (Poccia et al. 1987). It has also been observed that these SPK[K/R]-containing histones change the spermatid nuclear shape from a spherical to a conoid form (Hill et al. 1990; Vodicka et al. 1990).

Initially, the SPK[K/R]-containing N-terminal extensions of these histone variants were thought to facilitate the dense packaging of sperm chromatin, thus mimicking protamine function (Busslinger et al. 1982). However, further studies established that these variants do not play a role in sperm chromatin condensation directly but instead stabilise sperm chromatin, rendering it inaccessible for



**Fig. 2.5** Simplified phylogeny showing the distribution of sperm-specific histone H2B variants in Echinodermata and Cnidaria

transcription factors, transcription start sites and other *cis*-regulatory elements, without changing condensation status (Talbert and Henikoff 2010).

The constraints the sperm-specific histone variants impose onto sperm chromatin by inhibiting access to nuclear DNA are extreme, when compared to somatic nucleosomal packing, and the SPK[K/R] variant H2B histones need to be removed postfertilisation to allow access to DNA for transcription factors and other *trans*-regulatory proteins. This ensures normal embryogenesis and development (Concha et al. 2005). In postfertilisation sea urchin eggs, inhibition of a specific protease called sperm histone protease (SpHp) belonging to the cathepsin-L subtype of cysteine proteases that degrade sperm histones halts embryogenesis by disrupting DNA replication and cell cycle progression (Concha et al. 2005; Morin et al. 2012). This protease is present as inactive form in unfertilised sea urchin eggs, is activated at fertilisation and rapidly degrades the sperm histones from the male pronucleus, leaving the maternal histone variants in the female pronucleus unaffected (Morin

et al. 2012). Without male H2B variant histone clearance, male and female pronuclei fuse, but cell division cannot commence (Concha et al. 2005).

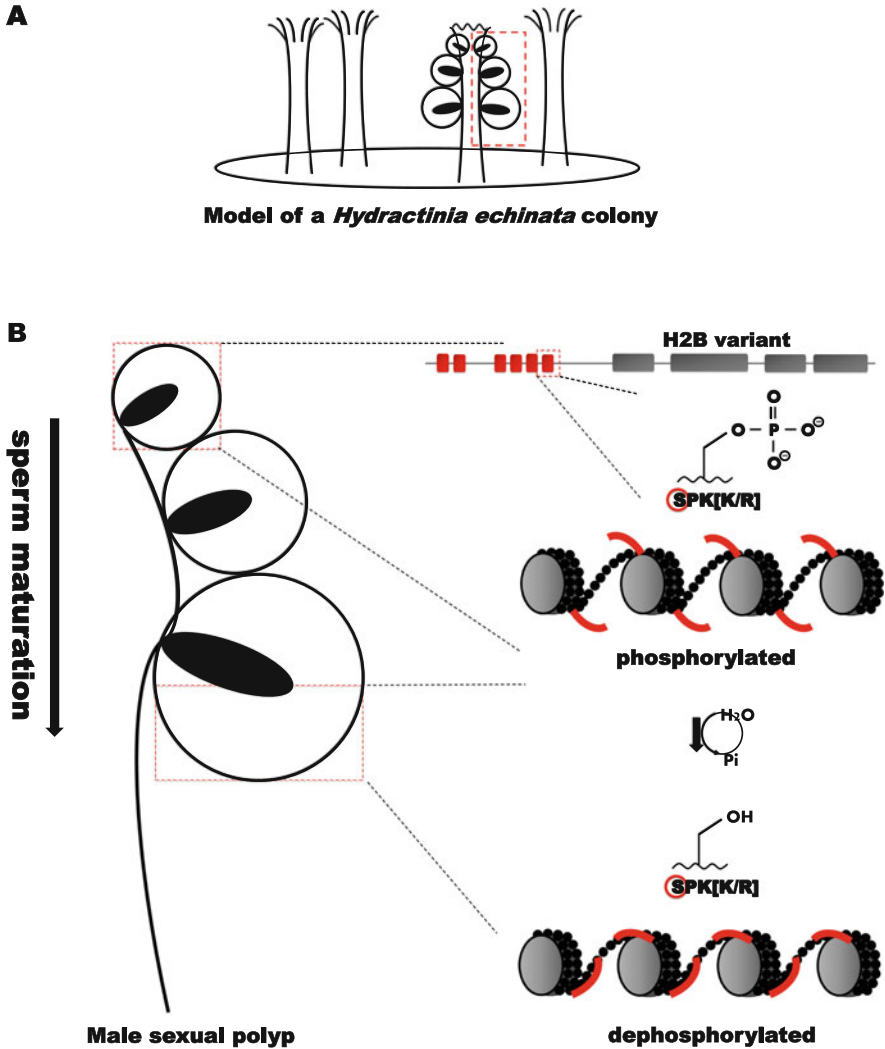
Most observations for these N-terminally extended H2Bs have been carried out in sea urchins, so the specific role and timing of expression of these variants in hydrozoan male gonads have not been confirmed to date. It is hypothesised that the N-terminal tails of hydrozoan sperm-specific H2B variants occupy and protect linker DNA to limit accessibility and increasing sperm chromatin stability in similar ways as their sea urchin counterparts (Fig. 2.6). Another open question is whether these SPK[K/R]-containing histone variants evolved independently in these two invertebrate phyla or if they evolved from a common, ancient histone H2B variant that was lost in other lineages.

## 2.4 Conclusion, Discussion and Outstanding Questions

Different types of sperm nuclear basic proteins and various mechanisms exist to enable sperm chromatin packaging, and many species have modified them to their own needs. Some species utilise only somatic histones, others use PL- and P-type SNBPs to pack their sperm DNA, while others have evolved completely novel histone variants for the same task. P-type SNBPs have likely evolved from common H1 precursors through vertical evolution via PL-type SNBPs. Moreover, in a few animals, novel sperm-specific histone variants have evolved, extending the repertoire of sperm chromatin condensing proteins even further.

The answer to the question what necessitates the compaction of some sperm DNA beyond classical nucleosomal packaging while other sperm exists that exclusively use 'normal' histones for compaction is currently unknown. Several synergistic functions for the presence of SNBPs in the sperm of animals have been proposed, but data on this are sparse for marine invertebrates, and most hypotheses are derived from well-studied terrestrial model systems such as mouse, fruit fly and human. The main proposed functions of SNBPs can be summarised as:

1. Sperm chromatin condensed with SNBPs results in a more compact and hydro-dynamically more efficient nucleus. This could indirectly affect sperm shape, stability and fitness, resulting in a more streamlined sperm with superior migration, egg penetration and fertilisation capabilities (Utsuno et al. 2014; Luke et al. 2014; Dogan et al. 2015).
2. SNBPs preserve DNA integrity and protect the paternal haploid genome against external environmental factors during sperm to egg migration. There is some evidence that protamine-induced DNA compaction provides radioprotection by reducing the ability of radiolytic reactive oxygen species to access DNA and thus hinders double-stranded breaks (Suzuki et al. 2009). In mice, it was found that protamine P2 deficiency leads to sperm DNA damage and embryo death (Cho et al. 2003), and in humans, protamine deficiency is closely related with increased sperm DNA damage and male infertility (Ni et al. 2016). Intriguingly, it has been



**Fig. 2.6** (a) Schematic of a *Hydractinia echinata* colony showing feeding polyps and one sexual polyp. (b) Spermatogenesis takes place in the male sexual polyps over several days. During this process, it is hypothesised that the serines in the N-terminal SPK[K/R] repeats of the sperm-specific H2B variants are phosphorylated to act like canonical histones. In the later stages of sperm maturation, these serines are dephosphorylated allowing the extended N-termini to bind to linker DNA and stabilise

shown in mice and human that there is also a transient appearance of DNA strand breaks during spermatogenesis that probably function to eliminate free DNA supercoils formed during histone removal, prior to or accompanying protamine deposition (Marcon and Boissonneault 2004; Laberge and Boissonneault 2005).

These transient DNA breaks are then repaired, although it is unclear how they form and how they are repaired (Rathke et al. 2014).

3. SNBPs could have a function in paternal imprinting and developmental epigenetics. In many vertebrate and invertebrate species, where SNBPs have a major contribution to sperm chromatin, a small amount of nucleosomal DNA is retained despite global re-packaging with SNBPs (Gusse and Chevaillier 1980; Zalensky and Avramova 1984; Gatewood et al. 1987). It has been shown in human sperm that such histone-bound paternal DNA is less efficiently condensed than its SNBP-bound counterpart and thus gains access to the ooplasm in a more open state upon fertilisation (Hammoud et al. 2009). This could have important developmental significance. Paternal non-SNBP-bound sperm chromatin could act as a template, establishing the epigenome of early pluripotent embryonic cells.
4. SNBPs could be indirect egg activation via CK-II upon fertilisation. It was shown that casein kinase II (CK-II) plays an important role in the completion of meiosis after fertilisation (Russo et al. 2004). It was also shown that the activity of CK-II is greatly increased by the presence of poly-arginine-containing proteins such as PL- and P-type SNBPs (Ohtsuki et al. 1996).

Irrespective of the individual variety and function of SNBPs in a given species of animal, all SNBPs serve the fundamental purpose of adapting sperm cells to facilitate effective egg fertilisation, thus guaranteeing long-term survival of the animal species.

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# Chapter 3

## Fertilization in Starfish and Sea Urchin: Roles of Actin



Jong Tai Chun, Filip Vasilev, Nunzia Limatola, and Luigia Santella

**Abstract** Marine animals relying on “external fertilization” provide advantageous opportunities to study the mechanisms of gamete activation and fusion, as well as the subsequent embryonic development. Owing to the large number of eggs that are easily available and handled, starfish and sea urchins have been chosen as favorable animal models in this line of research for over 150 years. Indeed, much of our knowledge on fertilization came from studies in the echinoderms. Fertilization involves mutual stimulation between eggs and sperm, which leads to morphological, biochemical, and physiological changes on both sides to ensure successful gamete fusion. In this chapter, we review the roles of actin in the fertilization of starfish and sea urchin eggs. As fertilization is essentially an event that takes place on the egg surface, it has been predicted that subolemmal actin filaments would make significant contributions to sperm entry. A growing body of evidence from starfish and sea urchin eggs suggests that the prompt reorganization of the actin pools around the time of fertilization plays crucial regulatory roles not only in guiding sperm entry but also in modulating intracellular  $\text{Ca}^{2+}$  signaling and egg activation.

### 3.1 Introduction

Conceptually, fertilization is a simple event: union of sperm and egg from the same species to produce an offspring. The actual fertilization process, however, involves a series of preparatory changes that have to be exquisitely coordinated in both gametes. For example, before reaching the egg, spermatozoa should gain their cytological competence by undergoing acrosome reaction and acquire apt motility for successful fertilization to occur. The substance on the egg surface may induce these changes and emit the cues for chemotaxis. On the other hand, the oocytes

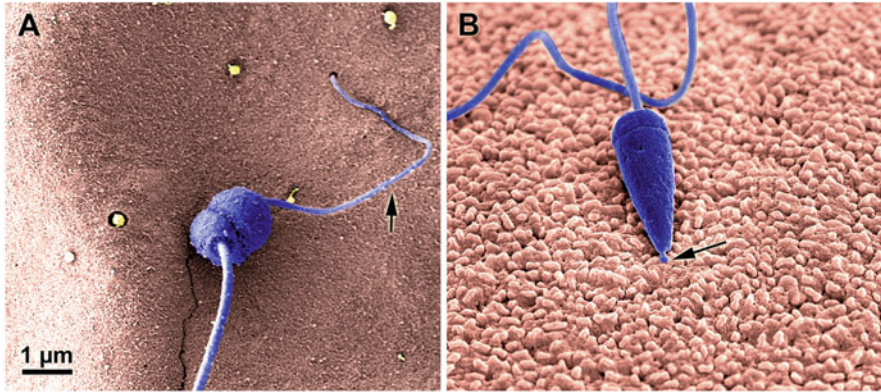
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should be matured into fertilization-competent eggs before fertilization. The sequential programs that lead to gamete maturation, chemotaxis, acrosome reaction, and the binding of sperm to eggs may fundamentally vary depending on the phyla and species, as has been reviewed elsewhere (Trimmer and Vacquier 1986; Hirohashi et al. 2008; Santella et al. 2012). Upon sperm's binding and fusion, the fertilized egg, which should be now called a zygote, undergoes a series of changes. The first detectable change is a swift shift of the membrane potential, which is accompanied by other cytological modifications such as cytoskeletal alteration and the increase of cytosolic pH and  $\text{Ca}^{2+}$  that are known to play important roles for the resumption of cell cycle and subsequent embryonic development. The intracellular  $\text{Ca}^{2+}$  increase in fertilized eggs is achieved mainly by two mechanisms, i.e., influx from the extracellular sources through voltage-gated ion channels and the ligand-gated release from the intracellular stores. The contribution and pattern of these two modes of  $\text{Ca}^{2+}$  increase may also differ widely depending on the species (Jaffe 1993; Miyazaki et al. 1975; Miyazaki 2006; Stricker 1999; Santella et al. 2004, 2012, 2015; Whitaker 2006). Hence, fertilization involves fine regulation of ion channel activities and cytoskeleton, as well as other cell signaling events that usher in a new life cycle in the zygote. In this chapter, we review how the basic cytoskeletal protein actin is utilized in the fertilization of starfish and sea urchin eggs.

### 3.2 Actin in Sperm Acrosome Reaction

In the research of fertilization, sea urchin and starfish have traditionally served as important animal models since sperm's entry into eggs was first viewed in the echinoderm species: *Echinus esculentus* (Dufossé 1847), *Paracentrotus lividus* (Hertwig 1876), and *Asterias glacialis* (Fol 1879). While sperm in animal kingdom conspicuously display vast interspecific diversities in their morphology and the strategy to reach and fuse with the eggs (Pitnick et al. 2009), echinoderm spermatozoa generally tend to use filamentous projection from the head. Owing to this characteristic morphological feature and the timely development of phase-contrast microscopy, acrosome reaction was convincingly demonstrated in starfish sperm (Dan 1952). When exposed to the homologous egg water, sperm from diverse species of starfish (*Asterina pectinifera*, *Asterias amurensis*, and *Astropecten scoparius*) readily extended long, slender, and rigid filaments (Dan et al. 1954). This so-called acrosomal process of starfish sperm can reach 25  $\mu\text{m}$  in length, which is much longer than the one produced by sea urchin sperm (Fig. 3.1), and may serve as a tether to draw the sperm into the fertilized egg (Dan et al. 1954; Puppo et al. 2008). The activated sperm of another echinoderm, sea cucumber can extend even longer acrosomal process than starfish sperm. In the case of *Thyone briareus*, the extending acrosomal process can reach over 70  $\mu\text{m}$  in length within just 7 s. The exceedingly fast extension of the acrosomal process turned out to be due to the rapid polymerization of actin monomers that are reserved between the acrosomal vesicle and the nucleus of the sperm (Tilney et al. 1973; Tilney and Inoué 1982). This



**Fig. 3.1** Fertilizing sperm on the egg surface viewed by scanning electron microscopy. (a) Starfish (*A. pectinifera*). Several well-like holes are open in the egg vitelline coat. Sperm acrosomal process (arrow) pierces into the hole and gains access to egg microvilli. (b) Sea urchin (*Paracentrotus lividus*). Note that the acrosomal process extending from the sea urchin sperm head (arrow) is much shorter than that of the starfish sperm. Image (b) adapted from *Embryogenesis Explained* (Gordon and Gordon 2016), Copyright©2016 World Scientific Publishing, Singapore

phenomenon was one of the first examples suggesting that actin polymerization per se can provide protrusive force to push the cell boundary (Footer et al. 2007) and rendered insights into the mechanisms of cell motility and plasticity through the changes in specialized actin-based subcellular structures such as lamellipodia, filopodia, microspikes, and growth cones (Welch et al. 1997; Luo 2002; Svitkina 2018). Owing to this long acrosomal process, which traverses the egg jelly coat, starfish spermatozoa can trigger the initial responses in the fertilized eggs, such as swift membrane depolarization, and  $\text{Ca}^{2+}$  increases while the sperm head still remains outside of the jelly coat (Puppo et al. 2008).

In contrast to the sperm head, sperm tail (flagellum) is predominantly enriched with microtubules that are organized in the highly conserved “9 + 2” arrangement together with the molecular motor dyneins to constitute the axoneme. While the flagella of echinoderm sperm do not contain detectable level of actin (Tilney et al. 1973), F-actin has been intermittently visualized in the sperm of some other species including crane fly and mammals (Behnke et al. 1971; Dvorková et al. 2005; Cohen et al. 2004; Correa et al. 2007). It has been reported that actin inhibitors such as cytochalasin B and D can deform the tail of macaque sperm and moderately diminish sperm motility in a dose-dependent manner (Correa et al. 2007). However, the presence of actin in the sperm tail has not been clearly demonstrated for echinoderms, and accordingly its contribution to sperm motility has not been much explored.

### 3.3 Actin in Oocyte Maturation

Toward the end of oogenesis, fully grown oocytes in the ovary are arrested in certain phases of meiotic cell cycle until they are utilized. For fertilization, these oocytes should be converted to fertilization-competent eggs. In starfish, this transition (release from the arrest at the first prophase) is initiated by the oocyte maturation hormone, which was identified as 1-methyladenine (1-MA) (Kanatani et al. 1969). Upon binding to its yet unidentified receptor, which is presumably present in the plasma membrane (Kanatani and Hiramoto 1970), 1-MA induces a series of biochemical and cytological modifications in the oocytes, such as intracellular  $\text{Ca}^{2+}$  increase (within 1–2 min), reorganization of the actin cytoskeleton, and changes in protein phosphorylation and membrane potential. With the progression of these changes, the nucleus of the oocyte (germinal vesicle, GV) loses its envelope (GV breakdown), and the nucleoplasm intermixes with cytoplasm eventually to render the cell physiologically suitable for fertilization (Moreau et al. 1978; Dale et al. 1979; Dorée et al. 1981; Schroeder and Stricker 1983; Meijer and Guerrier 1984; Lim et al. 2001). It has been shown that, following GV breakdown, the nuclear zone of the starfish oocyte forms a contractile actin network that captures the chromosomes and delivers them within the reach of microtubule asters (Lénárt et al. 2005). Thus, actin is expected to assist in the subsequent progression of meiotic progression.

The surface of oocytes is covered with countless projections of F-actin bundles called microvilli and with the meshwork of subplasmalemmal actin filaments. Both microvilli and the cortical actin meshwork undergo drastic reorganization during the meiotic maturation of oocytes. It is quite conspicuous that the 1-MA-induced reorganization of the actin cytoskeleton, visualized by the structural changes of the microvilli on the surface of oocyte, is as prompt as the  $\text{Ca}^{2+}$  increase (Schroeder and Stricker 1983; Santella and Kyozuka 1994; Kyozuka et al. 2008). Thus, in response to 1-MA, the number of microvilli inserting into vitelline coat becomes significantly reduced (Hirai et al. 1971), leading to the selective loss of microvilli-residing  $\text{K}^+$  channels and the consequent shift in membrane potential during the course of meiotic maturation (Dale et al. 1979; Moody and Bosma 1985).

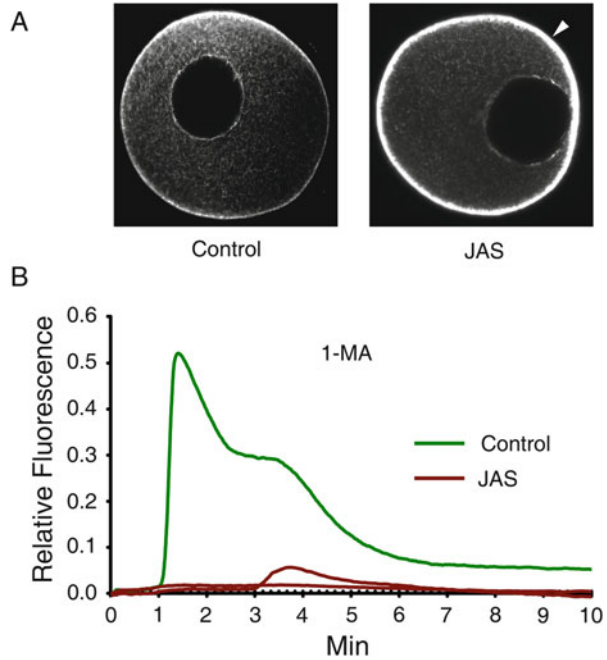
One crucial cytoplasmic change that takes place during meiotic maturation is the translocation of cortical granules from the inner cytoplasm to the subplasmalemmal zone. This process appears to be heavily dependent upon actin dynamics both in starfish and sea urchin, as judged by its inhibition by the agents interfering with actin dynamics (Santella et al. 1999; Wessel et al. 2002). Cortical granules that are now apposed to the egg plasma membrane are more readily exocytosed and elevate the vitelline layer when the  $\text{Ca}^{2+}$  wave arrives during fertilization. In addition, both GV and endoplasmic reticulum (ER) appear to be bound and modulated by the actin cytoskeleton (Stricker and Schatten 1991; Terasaki 1994). ER is the major  $\text{Ca}^{2+}$  store in the egg, which is studded with ion channels such as  $\text{InsP}_3$  receptor and ryanodine receptor. The actin-based rearrangement of ER in the maturing oocytes may in part contribute to the progressive sensitization of the maturing oocytes to  $\text{InsP}_3$  (Chiba



et al. 1990; Lim et al. 2003). This phenomenon, in which the same amount of exogenous  $\text{InsP}_3$  evokes a much larger  $\text{Ca}^{2+}$  increase in the matured eggs than in the immature oocytes, can be precluded by latrunculin A (LAT-A), a drug promoting actin depolymerization (Lim et al. 2003).

Starfish oocytes reentering meiotic cell cycle by the action of 1-MA manifest intracellular  $\text{Ca}^{2+}$  increases at two different time points: the early response readily induced by the hormone and the late  $\text{Ca}^{2+}$  increases at the time of GV breakdown. The earlier response takes place within 1–2 min after the hormone addition, and it comprises spatiotemporally coordinated  $\text{Ca}^{2+}$  release from internal stores that starts from the vegetal hemisphere and propagates as a single wave along the subplasmalemmal zone (Moreau et al. 1978; Santella and Kyojuka 1994; Dorée and Kishimoto 1981; Kyojuka et al. 2008). The few minutes' time lag required for the  $\text{Ca}^{2+}$  wave to respond to 1-MA implies that the signal transduction involved in this pathway is quite efficient. Experimental evidence has indicated that 1-MA-induced meiotic resumption is mediated by  $\beta\gamma$  subunit of heterotrimeric G-protein (Chiba et al. 1993; Jaffe et al. 1993). Indeed, metabolically stable GTP ( $\text{GTP}\gamma\text{S}$ ) microinjected into starfish oocytes mimicked 1-MA by readily inducing a similar  $\text{Ca}^{2+}$  wave, while  $\text{GDP}\beta\text{S}$  inhibited the GV breakdown, the hallmark of meiotic maturation of oocytes (Kyojuka et al. 2009). Interestingly, it has been shown that alteration of the actin cytoskeleton by LAT-A, jasplakinolide (JAS), or heparin suppresses the 1-MA-induced  $\text{Ca}^{2+}$  increase (Kyojuka et al. 2008, 2009). For example, pretreatment of *A. pectinifera* oocytes with 12  $\mu\text{M}$  JAS nearly blocked the 1-MA-induced  $\text{Ca}^{2+}$  wave (Fig. 3.2). However, these oocytes retained intact activities of  $\text{InsP}_3$  receptor, as judged by the peak amplitude of the  $\text{Ca}^{2+}$  increase triggered by  $\text{InsP}_3$  uncaging. On the other hand, oocytes of *A. pectinifera* are known not to respond to cyclic ADP-ribose with a  $\text{Ca}^{2+}$  increase (Nusco et al. 2002). These and other observations led to the suggestion that the  $\text{Ca}^{2+}$  increase induced by 1-MA may be mediated by a mechanism distinct from  $\text{InsP}_3$  receptor or ryanodine receptor (Kyojuka et al. 2008). The late-stage  $\text{Ca}^{2+}$  increases in maturing oocytes of starfish are due to the influx from outside through the L-type  $\text{Ca}^{2+}$  channels, which is detected as a train of  $\text{Ca}^{2+}$  spikes taking place around the time of GV breakdown (Limatola et al. 2015) when the oocytes undergo continual depolarization and swift shift of membrane potential (Dale et al. 1979). This late stage of  $\text{Ca}^{2+}$  response to 1-MA, which had not been previously reported, was also shown to be dependent upon the status of cortical actin cytoskeleton. Exposure of the maturing oocytes to 1  $\mu\text{M}$  LAT-A 8 min after the hormone addition (thus after the early  $\text{Ca}^{2+}$  wave has subsided) led to the significant reduction in the frequency of the  $\text{Ca}^{2+}$  spikes, while the average amplitude of the  $\text{Ca}^{2+}$  spikes was significantly enhanced by the LAT-A-induced actin depolymerization (Limatola et al. 2015). Taken together, these observations suggest that the reorganization of the cortical actin cytoskeleton and microvilli readily induced by 1-MA is instrumental in fine-tuning other structural and functional changes that take place in maturing eggs, in particular the optimization of the intracellular  $\text{Ca}^{2+}$  stores and ion channels.

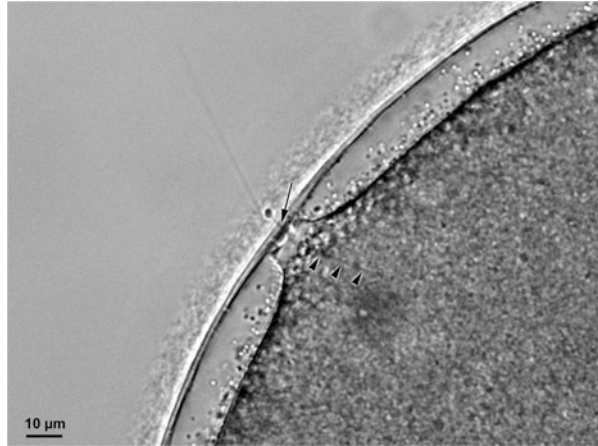
**Fig. 3.2** The  $\text{Ca}^{2+}$  increase induced by 1-MA in the starfish (*A. pectinifera*) oocytes is suppressed by JAS. (a) Actin filaments visualized by Alexa Fluor 568 phalloidin in live oocytes with or without (control) preincubation with 12  $\mu\text{M}$  JAS. Note that the subplasmalemmal actin is hyperpolymerized by the incubation with JAS (arrow). (b) Quantified  $\text{Ca}^{2+}$  responses after 1-MA stimulation. Adapted from Kyojuka et al. (2009) PLoS One 4: e6296



### 3.4 Actin in Fertilized Eggs

Fertilization requires successful gamete recognition, adhesion, and fusion. As aforementioned, the egg and sperm influence each other to induce morphological and physiological changes in a sequential manner, which involves multiple bilateral receptor-ligand couplings (Ohlendieck and Lennarz 1996; Vacquier 2012). In starfish, the egg jelly coat provides at least three signaling substances for homologous spermatozoa to induce acrosome reaction: (1) acrosome reaction-inducing substance (ARIS), which is a sulfated proteoglycan-like molecule with >1000 repeating units; (2) Co-ARIS, sulfated steroidal saponins; and (3) asterosap, a peptide. The cognate receptors on sperm mediate a cascade of biochemical changes affecting acrosomal reaction (Matsumoto et al. 2008). Similarly, sea urchin spermatozoa possess the specific receptors (e.g., suREJ1) for egg jelly protein that are activated to trigger the acrosome reaction (Moy et al. 1996). Exocytosis of the acrosomal vesicle as a result of the  $\text{Ca}^{2+}$  increase in the sperm may now expose species-specific adhesion/fusion molecules such as bindin on the surface of the acrosomal process (Vacquier and Moy 1977). Two bindin receptors have been identified in sea urchin eggs, which might provide protease activity as well (Kamei and Glabe 2003; Mah et al. 2005). Because an earlier study showed that the 350-kDa bindin receptor is predominantly localized in the tip of microvilli (Ohlendieck et al. 1994), it seems probable that the microvilli may play a crucial role in intercellular fusion during fertilization, as well as in the pathogenic processes and in the immune responses (Wilson and Snell 1998). Indeed,

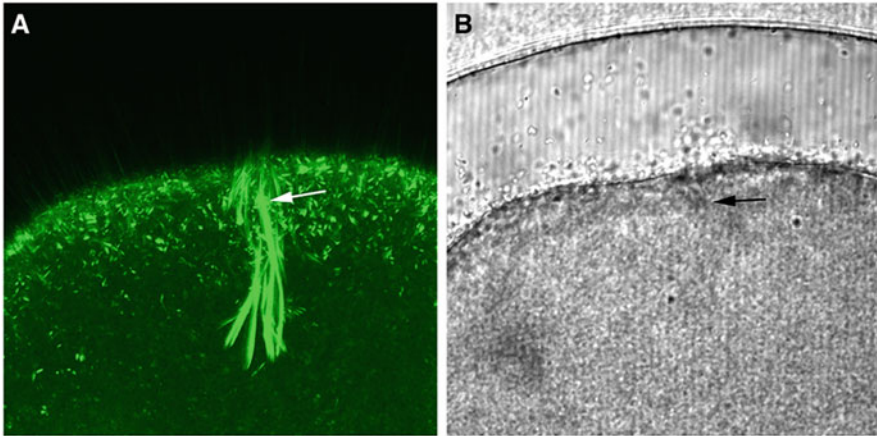
**Fig. 3.3** A moment before sperm entry into the fertilized egg of starfish (*A. aranciacus*). The acrosomal process of sperm pierces the vitelline coat, which is about to elevate (arrow), and is adjoined by the egg microfilaments in the fertilization cone. Apparently, the connection continues to a tubular structure in the inner cytoplasm (arrowheads). Adapted from Puppo et al. 2008 PLoS One 3: e3588



lymphocytes are also covered with oocyte-like microvilli (Majstoravich et al. 2004). The actin-filled protrusions from both gametes, i.e., the acrosomal process of activated sperm and the microvilli of eggs, are likely to subserve sperm-egg fusion and the subsequent entrance of sperm into eggs. Analyses of the ultrastructure of sea urchin eggs at fertilization suggested that several microvilli at the sperm fusion site may undergo lateral association to produce a fertilized cone (Tilney and Jaffe 1980). Adjoining of the acrosomal process of incoming sperm with the fertilization cone apparently forms a thick “tether” that can be seen even in the light microscope due to its characteristic optical density (Fig. 3.3). After entering the egg, the sperm head is now attached to several thick bundles of actin filaments in the egg cytoplasm (Fig. 3.4, also see Santella et al. 2016).

Following fertilization, starfish and sea urchin eggs elongate their microvilli as the fertilization envelope is formed. The elongated microvilli traversing the entire depth of the perivitelline space may mechanically contribute to the elevation of the vitelline layer or to the accommodation of the plasma membrane whose area is greatly increased as a result of cortical granule exocytosis and the membrane fusion (Carron and Longo 1982; Chun et al. 2010). Below microvilli, portions of subplasmalemmal actin filaments in the fertilized eggs undergo centripetal translocation (Terasaki 1996; Vasilev et al. 2012), while other filaments remain locally and become reorganized to form a new surface boundary of the zygote (Chun et al. 2010). Although precise roles of the inwardly translocating actin filaments are not known, it is conceivable that their centripetal migration might also assist in the concomitant sperm entry into the egg cortex. Although it is known that the migration of the male and female pronuclei toward each other is mediated by microtubule-based movement, the centrosome separation has been attributed to the actin filaments (Schatten et al. 1988; Holy and Schatten 1991).

Being heavily involved in gamete fusion and sperm entry, actin is thought to play an important role in controlling the number of sperm incorporated into the egg. In line with earlier observation that interference with actin dynamics using cytochalasin



**Fig. 3.4** A moment after sperm entry into the fertilized egg of starfish (*A. aranciacus*). Actin filaments were visualized by Alexa Fluor 568 phalloidin in live eggs at fertilization. (a) Confocal image. (b) Bright field view. While microvilli of the fertilized egg prolong and traverse the perivitelline space, thick actin bundles are now formed and attached to the egg-incorporated sperm head (arrow)

caused polyspermy in the fertilized eggs of clams (Ziomek and Epel 1975), starfish and sea urchin eggs treated with actin-inhibiting drugs manifested either increased polyspermy rates or failure of sperm entry. Hyperpolymerization of subplasmalemmal actin induced by JAS and heparin in starfish egg (*A. aranciacus*) led to supernumerary sperm-egg interactions that induced multiple initiations of  $\text{Ca}^{2+}$  release (Puppo et al. 2008). The same effect was observed when endogenous depactin, an actin-depolymerizing protein, was inhibited in the eggs of *A. aranciacus* by a specific function-blocking antibody (Chun et al. 2013). In sea urchin (*P. lividus*), pretreatment of eggs with moderate dose of cytochalasin B (400 nM) or phalloidin (3  $\mu\text{M}$ ) increased the rate of polyspermy, but sperm entry was suppressed by higher dose of the same drugs (10 and 30  $\mu\text{M}$ , respectively). Likewise, 10 nM of LAT-A and 12  $\mu\text{M}$  of JAS severely inhibited sperm entry, as judged by Hoechst-33342-stained spermatozoa (Chun et al. 2014). Taken together, these observations suggested that fine regulation of egg surface actin filaments is a prerequisite for correct gametes interaction and fusion.

The egg actin cytoskeleton also appears to contribute to the regulation of exocytosis. In the early studies utilizing the “cortical lawn” prepared from fractured sea urchin eggs, negative data showing no effect of actin or tubulin inhibitors on exocytosis led to the suggestion that  $\text{Ca}^{2+}$  is the sole modulator of cortical granule exocytosis (Whitaker and Baker 1983; Whitaker 1994). However, later studies with intact eggs led to a different conclusion. Pretreatment of starfish eggs with an actin drug JAS did not apparently affect the intracellular  $\text{Ca}^{2+}$  increases in response to  $\text{InsP}_3$  uncaging or fertilizing sperm, but the elevation of the vitelline layer was severely inhibited as a sign of impaired cortical granule exocytosis (Kyozyuka et al.

2008; Puppo et al. 2008; Santella and Chun 2011). Similarly, sea urchin eggs pretreated with JAS or other actin drugs often failed to elevate the vitelline layer at fertilization, although the  $\text{Ca}^{2+}$  response at fertilization was unaffected or comparable with that in the control eggs (Chun et al. 2013). Hence, in live eggs, the subplasmalemmal actin cytoskeleton surrounding cortical granules also seem to serve as a decisive factor controlling exocytosis.

### 3.5 Modulation of Intracellular $\text{Ca}^{2+}$ Signaling by the Actin Cytoskeleton

The subplasmalemmal actin filaments also affect the spatiotemporal pattern of intracellular  $\text{Ca}^{2+}$  signaling. Whereas  $\text{Ca}^{2+}$  signals affect structural organization and function of the actin cytoskeleton through the action of  $\text{Ca}^{2+}$ -dependent actin-binding proteins such as gelsolin, the status of actin filaments can also influence the  $\text{Ca}^{2+}$  signals in return. This reciprocal relationship may comprise a feedback loop and contribute to  $\text{Ca}^{2+}$  homeostasis inside cells (Santella et al. 2008; Chun and Santella 2009a, b). Actin may influence intracellular levels of  $\text{Ca}^{2+}$  at least in two different ways. Firstly, actin is a highly abundant protein, and the monomeric actin displays an unusually high binding affinity to free  $\text{Ca}^{2+}$  with the  $K_d$  value being estimated around  $2 \times 10^{-9}$  M (Gershman et al. 1986; Carrier et al. 1986). This implied that actin can serve as a highly efficient  $\text{Ca}^{2+}$  buffer inside the cell and led to the hypothesis that polymerizing actin can absorb excess  $\text{Ca}^{2+}$  ions into the microfilament, which can serve as a different kind of  $\text{Ca}^{2+}$  store. At depolymerization, these filaments can now release free  $\text{Ca}^{2+}$  (Lange 1999). For the same reason, monomeric actin and the treadmilling actin filaments can serve as a diffusion barrier for free  $\text{Ca}^{2+}$ . Indeed, the diffusion of individual  $\text{Ca}^{2+}$  ions is known to be conspicuously limited in cells like oocytes (Allbritton et al. 1992). All these physical parameters of actin may serve as a direct mechanism to regulate intracellular  $\text{Ca}^{2+}$  levels. Secondly, the subplasmalemmal and cortical actin filaments may set the tone to the microenvironment of the ion channels that are studded in the plasma membrane or on the membranes of organelles and vesicles such as ER. For these reasons, polymerization status of the actin filaments and the dynamics of their rearrangements were expected to influence the efficiency of ion channel activities. It has long been known that the  $\text{InsP}_3$  receptor binds to actin filaments, which may modulate its  $\text{Ca}^{2+}$ -transmitting activities (Fujimoto et al. 1995; Fukatsu et al. 2004; Bose and Thomas 2009). In addition to the  $\text{Ca}^{2+}$  release through  $\text{InsP}_3$  receptor, it has been shown that  $\text{Ca}^{2+}$  uptake through the pump on ER is under the regulation of the actin cytoskeleton in neurons (Wang et al. 2002).

Starfish and sea urchin eggs are supposed to be suitable in addressing the questions regarding the causal relationship between the actin cytoskeleton and intracellular  $\text{Ca}^{2+}$  signaling. Certainly, large eggs with resilient surface and transparent cytoplasm have an advantage in tackling these questions in live cells by

microinjection of fluorescent dyes and imaging technology. Indeed, the first experimental models in which intracellular  $\text{Ca}^{2+}$  wave was demonstrated were medaka fish and sea urchin eggs at fertilization (Ridgway et al. 1977; Steinhardt et al. 1977). The biochemical and electrical signals transmitted by a fertilizing sperm have to be transduced at the plasma membrane/cytoskeleton interface in the egg which is covered by microvilli and is crowded with actin filaments. With starfish eggs, several methodological strategies have been adopted to alter the F-actin structure and dynamics: microinjection of actin-binding protein cofilin (Nusco et al. 2006), metabolically stable guanidine nucleotides (Kyojuka et al. 2009), recombinant protein sequestering PIP2 (Chun et al. 2010), and so on. In each case, certain aspects of  $\text{Ca}^{2+}$  influx (cortical flash) and intracellular  $\text{Ca}^{2+}$  release have been observed to be significantly changed by the alteration of the actin cytoskeleton. For example, starfish eggs microinjected with human cofilin, which binds and severs actin filaments, led to enhancement of the  $\text{Ca}^{2+}$  wave at fertilization with the concomitant suppression of the cortical flash (Nusco et al. 2006). Corroborating this observation, inhibiting the endogenous activity of depactin (structural homologue of cofilin in starfish) with microinjection of the function-blocking antibody suppressed the  $\text{Ca}^{2+}$  wave in starfish eggs at fertilization (Chun et al. 2013). The modulation of intracellular  $\text{Ca}^{2+}$  signaling by the actin cytoskeleton is not a peculiar phenomenon restricted to starfish. When sea urchin eggs (*P. lividus*) were pretreated with actin drugs prior to fertilization, the spatiotemporal pattern of the  $\text{Ca}^{2+}$  increase was changed. For example, two actin drugs LAT-A and cytochalasin B, which promote actin depolymerization but by different mechanisms, displayed similar effects. In both cases, the fertilized eggs required longer time lag to generate the  $\text{Ca}^{2+}$  wave after firing cortical flashes (Chun et al. 2014). The exact physiological significance of this interval between the cortical flash (sperm-induced egg membrane depolarization) and the onset of the  $\text{Ca}^{2+}$  wave at fertilization is not clear (Dale and De Santis 1981; Santella et al. 2015). It might represent the time required for the egg to receive the sperm-borne signal and convert it into the synthesis of  $\text{Ca}^{2+}$ -mobilizing second messengers. While the exact mechanism by which a fertilizing sperm initiates  $\text{Ca}^{2+}$  wave in the egg is not fully established and probably varies depending on the animal species (Parrington et al. 2007), it is noteworthy that disassembly of subplasmalemmal actin filaments with LAT-A or cytochalasin induces both fertilization-like  $\text{Ca}^{2+}$  waves and depolarization of the membrane potential in starfish (*A. aranciacus*) eggs (Lim et al. 2002; Moccia 2007). Our recent finding that the  $\text{Ca}^{2+}$  increase induced by LAT-A is caused by de novo synthesis of  $\text{InsP}_3$  by stimulating  $\text{PLC}\gamma$  (Vasilev et al. 2018) suggests that the subplasmalemmal actin filaments also modulate the activity of a key enzyme involved in intracellular  $\text{Ca}^{2+}$  signaling.

### 3.6 Concluding Remarks

The old and new experimental data obtained from the oocytes and eggs of starfish and sea urchin that we have reviewed here suggest that actin is functionally involved in nearly all aspects of fertilization both in sperm and eggs: maturation, interaction, and fusion of the gametes. Actin provides both rigidity and plasticity for the membranes of sperm and eggs. The actin cytoskeleton is highly dynamic as exemplified by the elongating acrosomal process of sperm and the microvilli extending into the perivitelline space of the fertilized eggs. The actin pools in the sperm head or in the egg cortex maintain the spatiotemporal balance between F-actin and G-actin, which can be swiftly shifted as needed. In this review, our emphasis has been focused on the role of actin filaments in the subplasmalemmal space, which has also been referred to as “ectoplasm” (Just 1939). Conceptually, the actin filaments in the sperm acrosomal process and egg microvilli also fall into this category in a sense that they are subjacent to the plasma membrane. The actin filaments located in this tight zone of plasma membrane/cytoskeleton interface bear utmost importance not only for fertilization but also for all intercellular signaling. Although fertilization takes place on the egg surface, the subplasmalemmal zone is the locus where all the signal-transducing events take place. Whereas many aspects of fertilization may considerably vary among species, what we have reviewed here for echinoderms, and the other findings reported in the literature, support the idea that the actin meshwork in the subplasmalemmal zone may serve as a scaffold on which receptors, signaling molecules such as G-proteins, and protein kinases, as well as pertinent enzymes and ion channels, are functionally and perhaps physically interlinked together through the self-remodeling framework of the actin cytoskeleton.

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# Chapter 4

## Starfish as a Model System for Analyzing Signal Transduction During Fertilization



Emily Wiseman, Lauren Bates, Altair Dubé, and David J. Carroll

**Abstract** The starfish oocyte and egg offer advantages for use as a model system for signal transduction research. Some of these have been recognized for over a century, including the ease of procuring gametes, *in vitro* fertilization, and culturing the embryos. New advances, particularly in genomics, have also opened up opportunities for the use of these animals. In this chapter, we give a few examples of the historical use of the starfish for research in cell biology and then describe some new areas in which we believe the starfish can contribute to our understanding of signal transduction—particularly in fertilization.

### 4.1 Introduction

Echinoderms have long served as model systems for the study of oocyte maturation, fertilization, and early development. These animals offer multiple advantages for the investigation of cellular and molecular processes. These advantages include the large number of gametes that can be collected, the fact that the oocytes, eggs, and early embryos are synchronized, the large size of the oocytes and eggs, and the ease of working with the gametes *in vitro* (Fig. 4.1) (Strathmann 1987; Wessel et al. 2010). In this chapter, we will describe some of the historical uses of echinoderms, starfish in particular, for the study of signal transduction processes and offer suggestions for how they may be useful in the future.

The relevance of echinoderms to understanding vertebrate processes lies in the fact that they are closely related, based upon phylogenetic characteristics and molecular phylogeny. They are the basal deuterostomes and, as such, offer insight into the relationship between invertebrates and vertebrates (Swalla and Smith 2008).

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Emily Wiseman and Lauren Bates contributed equally.

E. Wiseman · L. Bates · A. Dubé · D. J. Carroll (✉)

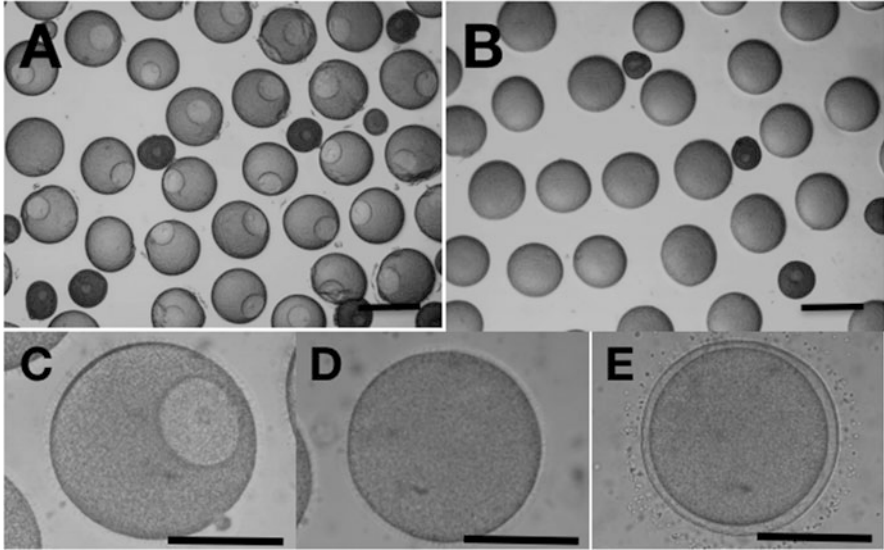
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**Fig. 4.1** Starfish oocytes, eggs, and zygotes offer advantages for the study of signaling pathways for multiple reasons. Starfish oocytes and eggs are remarkably synchronized. As shown in these light micrographs of *Patiria miniata* oocytes (a) and eggs (b), over 90% of the full-grown cells are arrested in prophase I of meiosis, while 2/26 have undergone spontaneous maturation. Also visible are very small oocytes (<100  $\mu\text{m}$ ) that are sometimes present. These are easy to separate by filtration through nitex mesh. (c–e) Higher magnification view of a single oocyte, egg, and zygote, respectively, to illustrate the clarity of the cells. In (c), the nucleolus is clearly visible within the germinal vesicle. (a, b) Scale bar = 200  $\mu\text{m}$ ; (c–e) scale bar = 100  $\mu\text{m}$

The genome of the sea urchin, *Strongylocentrotus purpuratus*, has an estimated size of 800 Mb and approximately 23,000 genes, which is similar to vertebrates (Sodergren et al. 2006). Sequencing and assembly of the *Patiria miniata* (starfish) genome are ongoing, and the data is available on GenBank at the National Center for Biotechnology Information (NCBI) website (Accession PRJNA49323). The availability of these genomes will be invaluable for exploring the genome expansion that occurred in the basal vertebrate genome (Sodergren et al. 2006). This genome expansion led to increased complexity in vertebrates, making the relatively less complicated echinoderm genomes a useful tool for comparing basic physiological processes in deuterostomes.

The usefulness of echinoderms (both sea urchins and starfish, primarily) as a model system in signaling began perhaps with the discovery that fertilization and the creation of the new individual was the result of the fusion between sperm and egg. While the exact discoveries of this event are somewhat clouded in history [see (Briggs and Wessel 2006) for an excellent discussion of the history of this discovery], the critical understanding that fertilization was between one sperm and one egg was discovered in the starfish (Fol 1879). This revolutionized the study of genetics because it clearly defined the maternal and paternal contribution to inheritance. This

also stimulated research into the mechanisms and importance of meiosis—how do the gametes prepare their DNA for fertilization?

## 4.2 Understanding the Regulation of Meiosis at the Cellular Level

The molecular signals that control oocyte maturation and regulate meiosis are certainly still under investigation today. The most active area of research on this question has been on intercellular signaling in the mammalian ovary, where an incredible story has developed on the movement of signals from the follicle cells into the oocyte via gap junctions (Jaffe and Egbert 2017). However, there is much left to be discovered, including the signals within the oocyte that lead to the resumption of meiosis. Starfish oocytes that are arrested at prophase I can be easily obtained by dissection and then stimulated to undergo maturation *in vitro* by the addition of the hormone 1-methyladenine (Wessel et al. 2010). The oocytes reach metaphase within 30–40 min of hormone exposure and can be fertilized immediately, making them a very convenient model system. The starfish oocyte will undoubtedly serve as a useful model for these continued studies on the cell cycle, much as it contributed to our understanding of the role and function of maturation-promoting factor (MPF) over 40 years ago.

MPF was discovered in the cytoplasm of maturing *Rana pipiens* (frog) eggs and stimulated a fundamental shift in the understanding of the cell cycle (Masui and Markert 1971). In these pivotal experiments, cytoplasm that was transferred from progesterone-treated oocytes into separate non-treated oocytes stimulated the initiation of oocyte maturation (Masui and Markert 1971). This maturation-promoting substance was present beginning approximately 6 h following progesterone treatment. Furthermore, cytoplasm transferred from this second oocyte into a third demonstrated that the maturation-inducing substance was also being stimulated in the recipient oocytes. As mentioned above, starfish oocytes are arrested at prophase I and resume meiosis in response to the hormone 1-methyladenine. A maturation-inducing factor similar to that observed in frogs was discovered in starfish (Kishimoto and Kanatani 1976). Furthermore, frog MPF (initially from *Bufo*) also induces oocyte maturation in starfish oocytes, demonstrating the universal nature of the maturation substance (Kishimoto et al. 1982). The identity of MPF was discovered over the course of several years with the identification of CDC2, now known as cyclin-dependent kinase CDK1 in most cells, in yeast, and the cyclins in the sea urchin (Nurse and Bissett 1981; Evans et al. 1983). Not only were starfish oocytes also useful for understanding the regulation of MPF during meiosis, they likewise contributed to our knowledge of the role of MPF during mitosis (Kishimoto 2015). Properties of the starfish oocyte, including the large and easily manipulable oocytes, methods for microinjection, and the availability of large numbers of synchronized cells, made it the perfect system for complementing the studies in frogs.

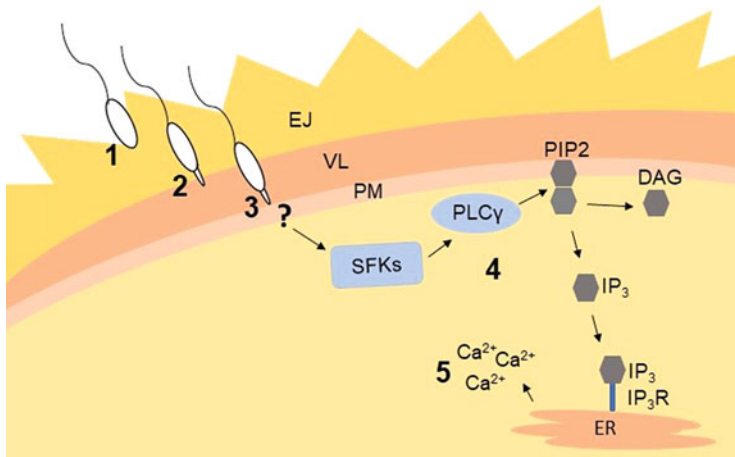
The combination of microinjection with biochemistry has proven useful in understanding the molecular mechanisms of how MPF is regulated and how this contributes to mitosis, not just meiosis. It soon became clear that MPF was more complicated than the simple interaction between cyclin and CDK-MPF it is not active under certain conditions, even when active cyclin-CDK is present in the cell (Kishimoto et al. 1982). For example, microinjection of cytoplasm from 1-MA-treated, enucleated starfish oocytes into recipient oocytes fails to stimulate MPF activity or nuclear envelope breakdown (an indicator of oocyte maturation), suggesting that the germinal vesicle is critical for initiating meiosis (Kishimoto et al. 1981). Furthermore, maturation can be stimulated without detectable MPF (Picard et al. 1988). These results suggest the presence of an unidentified substance that contributes to the maturation-promoting activity. This unidentified substance needed for full MPF activity was discovered to be Greatwall kinase (GWL) by using a combination of techniques amenable to the starfish system (Hara et al. 2012). Microinjection of antibodies against GWL inhibits entry into mitosis even though cyclin-CDK is active (Hara et al. 2012; Okumura et al. 2014; Kishimoto 2015). Therefore, studies in the starfish have enriched the story of the cell cycle and suggest the novel idea that complete MPF activity is not due to cyclin-CDK alone but is a combination of cyclin-CDK and GWL (Kishimoto 2015).

### ***4.2.1 Future Contributions of the Starfish***

Starfish oocytes and eggs provide an excellent model for studying signaling pathways involved in maturation, fertilization, and cell cycle regulation because of the historical advantages mentioned above and as a result of the growing ability of investigators to utilize modern genomic and proteomic methods. Through combinations of biochemistry, cell biology, and molecular biology techniques, some of the signaling pathways involved in maturation, fertilization, and cell cycle regulation have already been partially elucidated. In this chapter, we work our way from the outside to the inside, describing aspects of fertilization signaling that are actively being studied in the starfish model system, focusing on the contribution of the egg (see Fig. 4.2 as a guide).

### ***4.2.2 Signaling at Cell Surface***

The proteins on the surface of eggs that are responsible for gamete binding, fusion, and the initiation of intracellular signaling are poorly understood across all species, although candidate molecules are being discovered (Jahromi and Shamsir 2013; Inoue 2017). There has not been much progress in developing new ways to study these molecules until very recently. Much of this is due to advances in genomic resources in the starfish, since the ovary genome and ovary and mature egg

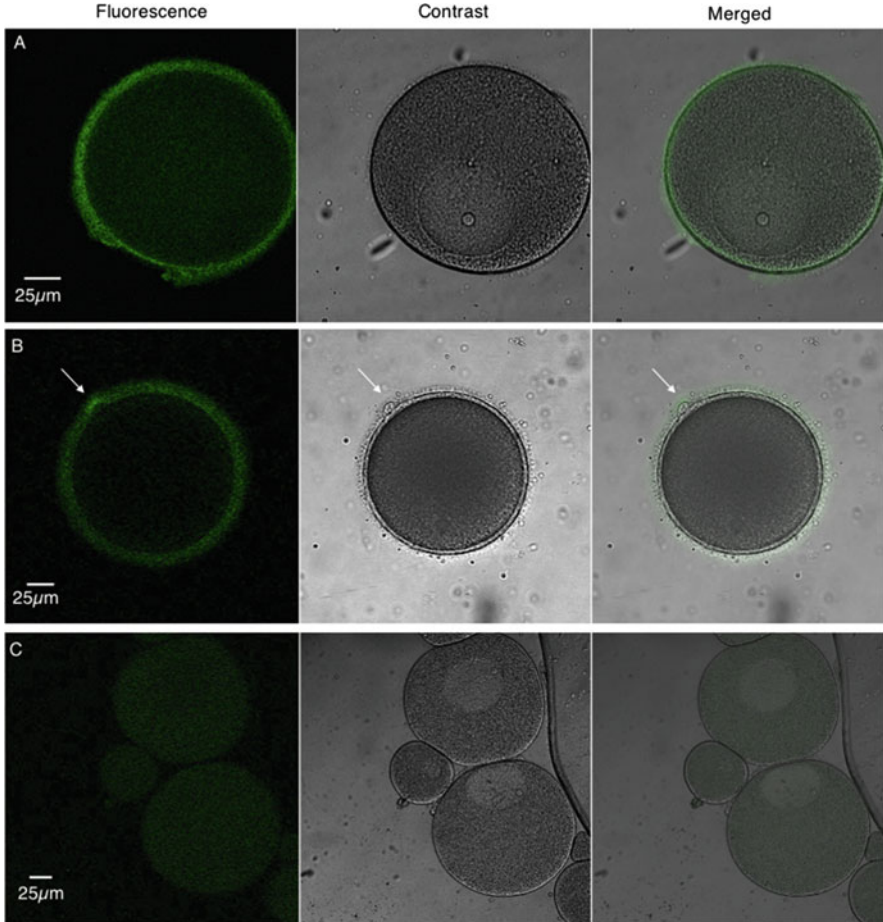


**Fig. 4.2** Summary of the cellular events taking place during fertilization. When a sperm contacts the egg jelly (EJ), it encounters a species-specific peptide which induces the sperm to undergo the acrosome reaction: (1). During the acrosome reaction, the sperm releases proteases into the external environment to degrade proteins in the vitelline layer (VL), as well as exposing new proteins on the surface of its newly formed cytoplasmic extension called the acrosomal process (2). Next, the sperm binds to and fuses with the proteins on the egg plasma membrane (3), releasing its nuclear contents into the egg and activating the signaling pathway (4) leading to internal  $\text{Ca}^{2+}$  release (5), which causes the permanent block to polyspermy and resumption of meiosis. The “?” at the plasma membrane denotes that we do not know the proteins involved in activating this pathway. Combinations of biochemistry, molecular biology, and microscopic techniques were utilized to discover the different components of this fertilization process. For more information on the techniques used to study the interactions at layers surrounding the egg and cell surface see Sect. 4.2.2. To learn more about the techniques used to study the internal signaling events and  $\text{Ca}^{2+}$  release, refer to Sects. 4.2.3 and 4.2.4, respectively. At the end of the chapter, new genomic resources are discussed for their use in furthering our knowledge of fertilization

transcriptome have been sequenced (see below). Part of the reason it has been difficult to identify these cell surface proteins may be due to the rapid evolution of gamete proteins. Comparison of the orthologues of three genes that are required for gamete recognition in mice and humans shows divergence of up to 70% in the protein sequences (Swanson and Vacquier 2002). To address this large divergence in the sequences of gamete proteins, new methods are being developed to isolate and identify the crucial proteins or protein complexes that function at the moment of fertilization.

One traditional method that has been refined to help look for integral proteins is the use of protein labeling by biotinylation. Biotinylation is a useful method as you can label all of the proteins on a cell surface without crossing the membrane (see Fig. 4.3). This method is particularly useful when looking for any peptides that may be cleaved or released from the surface of the egg during fertilization, which could provide a handle for finding the entire integral membrane protein. To identify proteins release from the egg surface, sea water samples are taken after fertilization, and the biotin-labeled proteins are isolated by affinity chromatography with avidin.





**Fig. 4.3** Confocal view of biotinylated oocytes and egg demonstrate that the biotin does not cross the cell membrane. Samples were biotinylated and then probed with streptavidin conjugated with Dylight488 and viewed under a confocal microscope. (a) Immature oocyte labeled with biotin. Labeling evident in the egg jelly as well as across vitelline coat. (b) Mature egg that was fertilized under the microscope. Arrow indicates the fertilization cone. (c) Immature eggs that were not biotinylated and then probed with Dylight488 as a control

The affinity between biotin and avidin is one of the strongest known (Lazaridis et al. 2002). In 1941, Edmond Emerson Snell observed that when chicks ate raw egg whites, they developed “egg white injury”—a disease caused by a deficiency in vitamin b7, otherwise known as biotin. To counteract this disease, the chicks were given biotin supplements; however, the egg white injury did not abate. Snell was able to determine that there is a protein in the egg white that was sequestering the biotin making it metabolically unavailable. He aptly named the protein avidin, because of its avidity for binding biotin (György et al. 1941).

Despite the difficulty in discovering proteins that function on the surface at fertilization, two proteins have recently been identified in mammals that participate in the binding between the two gametes (Chalbi et al. 2014). The sperm protein Izumo1 and egg protein Juno have been shown to be binding partners; and while necessary for binding, it seems they do not contribute to egg activation (Chalbi et al. 2014). In *Xenopus*, Uroplakin III, an egg cell surface protein in the tetraspanin family, has been proposed as a molecule necessary for fertilization (Sakakibara et al. 2005). This protein, which localizes to an egg membrane lipid raft, may become phosphorylated by a Src-family kinase at fertilization, although it does not appear to be linked directly to phospholipase C gamma (PLC $\gamma$ )-mediated calcium release (Hasan et al. 2005; Sato et al. 2006). Egg cell surface proteins necessary for fertility have also been discovered in a genetic screen in *Caenorhabditis elegans* (Kadandale et al. 2005). These molecules, EGG1 and EGG2, contain multiple LDL receptor extracellular domains, a transmembrane domain, and a very small cytoplasmic domain, suggesting a limited role in signaling (Kadandale et al. 2005; Oren-Suissa and Podbilewicz 2007). Therefore, it is very likely that the interaction and fusion between sperm and egg, in any species, involve multiple cell surface molecules on both gametes. Some may be specialized for binding, others for fusion, and yet others for initiating the signaling process to start development. Overall, however, the mechanism behind sperm and egg binding, fusion, and the initiation of development is poorly understood in any model.

In echinoderms, two proteins have been identified as essential during fertilization. A sperm protein named bindin and an egg cell surface protein named ERB1 are critical for sperm binding to sea urchin eggs (Vacquier and Moy 1977). Bindin is located on the acrosomal process of the sperm. The acrosome reaction is a process that allows the sperm to pass through jelly layer and the vitelline envelope to bind with the egg cell surface. Once the sperm comes in contact with the egg jelly, the acrosomal process begins to grow from the nose of the sperm. This protuberance is supported primarily through the growth of actin filaments (Tilney et al. 1973). The acrosomal process extends from the body of the starfish sperm, which is different than mammals and amphibians (Hoshi et al. 2012). To identify the receptor for bindin on the egg cell surface, sea urchin eggs were treated with a lysyl endoproteinase C (Lys C), which caused a 70-kDa fragment from the surface to be released (Giusti et al. 1997). The fragment was found to be part of a larger protein that was about 350 kDa (Kamei and Glabe 2003; Vacquier 2012). Fertilization was greatly reduced when eggs were pretreated with Lys C prior to fertilization indicating the possibility that this 350 kDa protein is integral for fertilization but may not necessarily be the partner for bindin (Giusti et al. 1997). Using cDNA subtraction between two different sea urchin species, a different 350 kDa protein was discovered. This protein showed species specificity, and when isolated and radiolabeled, it only bound to conspecific bindin. As a result, it was named egg bindin receptor 1 (EBR1) (Kamei and Glabe 2003; Vacquier 2012). No evidence has been presented to suggest this molecule (EBR1) could initiate the signaling pathway that leads to the calcium wave, nor facilitate sperm-egg fusion. Rather, it appears that this molecule functions as an egg cell surface-binding protein for sperm.

A similar strategy is proposed to examine the role of starfish egg cell surface proteins in binding, fusion, and signal transduction at fertilization. A number of hypotheses exist concerning these events (Klinovska et al. 2014); however, much remains to be learned in all species. In starfish, trypsin-like proteases have been shown to activate starfish eggs, stimulating the  $\text{Ca}^{2+}$  wave as well as the initiation of DNA synthesis, suggesting the presence of a protease-sensitive receptor on their surface (Carroll and Jaffe 1995). In ongoing current studies, protease inhibitors have been shown to effectively inhibit fertilization (unpublished observations). One hypothesis is that proteases responsible for the proteolytic activity on the egg cell membrane may be localized on the head of the sperm as well as released into the surrounding environment. Pretreatment of starfish eggs with trypsin to “pre-cleave” the protease-sensitive egg surface proteins does not inhibit fertilization (unpublished observations). It is possible that sperm binds to the remaining membrane-bound portion of the cleaved protein, and this interaction facilitates the fusion of the two cells. One of the next steps in trying to determine which protein, or protein complex, may be acting on the surface is to look exclusively at the surface without the jelly or the vitelline coat. This is one of the many benefits of the starfish eggs. Once the vitelline coat is removed, the eggs still maintain their shape and ability to be fertilized (Schroeder and Stricker 1983).

While the proteins that activate the egg have not yet been found, there has been progress in understanding the lipid makeup of the egg cell surface, which could serve a new direction in ascertaining the proteins, or more likely the protein complex, which activates the egg at fertilization. For instance, specialized membrane structures called lipid rafts have been discovered in sea urchin eggs and may be critical for successful fertilization (Belton et al. 2001). Lipid rafts are areas of lipid order that are enriched with cholesterol and composed of sphingolipids and signaling proteins and are insoluble in nonionic detergents (Levental and Veatch 2016). In sea urchins, Src-type proteins,  $\text{PLC}\gamma$ , and other uncharacterized proteins were found in the insoluble section of membrane isolation, which further supports the idea that lipid rafts are functional in fertilization (Simons and Toomre 2000; Belton et al. 2001; Pike 2006).

### ***4.2.3 Moving Inside the Egg During Fertilization***

The starfish has also contributed to our understanding of the initial intracellular signals that occur at fertilization. In starfish, the known signaling proteins of the fertilization pathway leading to calcium release were discovered through microinjection of dominant-interfering recombinant proteins which also served as binding partners in affinity interactions.

In *P. miniata*,  $\text{PLC}\gamma$  was first shown to be necessary at fertilization when recombinant bovine  $\text{PLC}\gamma\text{SH2}$  domains [known to specifically inhibit the intact  $\text{PLC}\gamma$  protein—(Rotin et al. 1992)] were microinjected into starfish eggs and prevented  $\text{Ca}^{2+}$  release at fertilization (Carroll et al. 1997). Additionally, through

affinity interaction experiments, a ~58-kDa protein was found to associate with these bovine PLC $\gamma$ SH2 domains, possess kinase activity, and was recognized by a polyclonal Src antibody (Giusti et al. 1999a, b), suggesting a Src family kinase (SFK) associates with PLC $\gamma$  at fertilization. Microinjection of chicken Src SH2 domains (inhibitors of Src) resulted in a delay in Ca<sup>2+</sup> release after fertilization (Giusti et al. 1999a, b); and, microinjection of human Src elicited a rise in Ca<sup>2+</sup> release similar to that at fertilization, partial or complete fertilization envelope elevation, and stimulated DNA synthesis (Giusti et al. 2000), providing further evidence this Src kinase plays an important role at fertilization. To determine whether the Src kinase was activated either upstream or downstream of PLC $\gamma$  in this signaling cascade, eggs preinjected with PLC $\gamma$ SH2 domains were injected with active Src (Giusti et al. 2000). These eggs still exhibited a delay in Ca<sup>2+</sup> release after fertilization demonstrating that this SFK was activated upstream of PLC $\gamma$ . In 2004, using a newly available *P. minata* cDNA library, the starfish PLC $\gamma$ SH2SH2 domains were cloned to produce a GST-tagged fusion protein which was microinjected into starfish oocytes (Runft et al. 2004). These domains completely blocked Ca<sup>2+</sup> release at fertilization and interacted with the same Src family kinase as described above, confirming the function of starfish PLC $\gamma$  and its interaction with Src at fertilization (Runft et al. 2004). Furthermore, screening of the *P. miniata* cDNA library revealed three SFKs (AmSFK1, AmSFK2, and AmSFK3) existed in *P. miniata* oocytes and eggs (O'Neill et al. 2004). To determine the function of these SFKs in the fertilization pathway, the dominant interference strategy described above was applied using GST-SH2 domain fusion constructs of each SFK. The eggs injected with either AmSFK1 or AmSFK3 SH2 domains showed delayed or no Ca<sup>2+</sup> release at fertilization, indicating a role for these kinases in signaling pathways leading to egg activation (O'Neill et al. 2004).

There are undoubtedly more signaling partners and mechanisms to be discovered at fertilization and during early development in the starfish. The physiological responses to egg activation are highly varied, including the calcium wave, cortical granule exocytosis, the transition from meiosis to mitosis, the fusion between the sperm and egg pronuclei, and the initiation of DNA synthesis, remodeling the cytoskeleton and preparing for cell division. New methods for discovering and investigating these molecules are developing that take advantage of the echinoderm system (Roux et al. 2006; Roux-Osovitz and Foltz 2014). These methods include using affinity interaction with known signaling molecules or fusion proteins followed by proteomics to identify novel interacting proteins (Roux-Osovitz and Foltz 2014).

#### 4.2.4 Understanding Calcium Signaling

The starfish oocyte is also an excellent model system for the study of calcium signaling and dynamics. Furthermore, the calcium wave that occurs at fertilization serves as an excellent assay for the study of the upstream signaling pathways present

in the egg (Whitaker 2006; Ramos and Wessel 2013). The immature oocyte, which can bind and fuse to sperm but does not begin development, lacks sensitivity to IP<sub>3</sub>. However, the response to IP<sub>3</sub> develops over a 30-min period during oocyte maturation, thus providing an interesting model for the establishment of a calcium release pathway in a living cell (Chiba et al. 1990). The starfish oocyte has also been used to discover novel aspects of calcium regulation (Ramos and Wessel 2013; Ramos et al. 2014). In this study, a two-pore channel (TPC) and an ADP-ribosyl cyclase (ARC), proteins that have been suggested to regulate calcium dynamics via NAADP, were identified in the relatively newly available *P. miniata* ovary transcriptome. Microinjection of antisense morpholino oligos was used to demonstrate a role for these proteins in embryo development (Ramos et al. 2014).

The large size and relatively clear cytoplasm of the starfish oocyte and egg make them good candidates for fluorescent calcium imaging dyes, such as Ca green dextran (Stricker 1995). During fertilization, starfish eggs exhibit a cortical flash, presumably due to calcium influx from the surrounding seawater, followed by a single, larger calcium wave that originates at or near the sperm fusion site (Stricker et al. 1994; Carroll et al. 1997). The size of the eggs allows for coupling the microinjection and imaging of the eggs/zygotes with downstream biochemical assays because they can be removed from the microinjection chamber and further processed (Carroll and Hua 2009).

#### **4.2.5 Microinjection of the Live Oocyte and Egg**

The starfish oocyte, egg, and embryo have long been a favorite model for microinjection, based in part on some of the advantages mentioned above (easy of procuring gametes, synchronized cells, simple culture medium—seawater, and size of the gametes). The oocytes and eggs are also very resilient—investigators have injected proteins, pharmacological agents, DNA, and RNA, for a variety of reasons, for example, to block or accelerate function—and the cells typically survive and produce useable data.

One of the first examples of microinjection of a function-blocking antibody into a starfish embryo occurred over 40 years ago when antibodies against myosin were injected into one blastomere of a living two-cell starfish embryo to block cleavage (Mabuchi and Okuno 1977). They were using methods developed in sea urchins for injecting spermatozoa or manipulating molecules (Hiramoto 1962, 1974). The movement of liquid in the microinjection needle is controlled physically, by either constricting the needle or by inclusion of mercury as a brake. This method allowed for the ability to inject liquids of different viscosity in a quantitative manner (Jaffe and Terasaki 2004).

### 4.3 Genomics in the Starfish (the Exciting Future!)

The availability of genomic resources has come relatively late to the starfish model system. The initial foray was a part of the sea urchin genome project and the production of high-density arrayed cDNA libraries (Sodergren et al. 2006). These libraries were arrayed on large nitrocellulose sheets (22 cm<sup>2</sup>) and were screened with probes that were initially developed against orthologues of the gene of interest. To generate this arrayed cDNA library, mRNA isolated from starfish oocytes was used to produce a random-primed library in the pSport plasmid which was then arrayed onto the nitrocellulose support from over 110,000 clones (Kalinowski et al. 2003). The initial use of this library was to clone an adenosine-like G-protein coupled receptor (Accession number AF521907) using degenerate RT-PCR with primers based upon mammalian adenosine-type receptors. When this starfish adenosine-type receptor cDNA was expressed in *Xenopus* oocytes, it was able to respond to adenosine (Kalinowski et al. 2003). This same high-density cDNA library was used to identify three different Src family kinases (SFKs) expressed in the starfish egg, again using degenerate RT-PCR based upon primers designed against human Src (P12931) (O'Neill et al. 2004). While extremely useful, the high-density arrayed cDNA library required the use of probes designed against known genes, usually from non-echinoderm species. Furthermore, the time from developing the probes to having the cloned cDNA in hand could be quite extensive. The lack of genomic resources in the starfish is slowly being resolved with the publication and easy availability of echinoderm genomes, including the sea urchin genome (Sodergren et al. 2006). This led to interest in sequencing other genomes of other echinoderms, including this prevalent model system *P. miniata*, which was included in the first publicly available database specifically for echinoderms Echinobase (Kudtarkar and Cameron 2017), initially called SpBase for the sea urchin *Strongylocentrotus purpuratus* (Cameron et al. 2009). Of great interest is the relatively recent appearance of transcriptome data for several starfish, including *P. miniata* and *Astropecten aranciacus* (Wong et al. 2007; Musacchia et al. 2017). This fantastic resource only became possible due to the development of technologies for massively parallel sequencing of cDNAs prepared from total cell mRNA (Morin et al. 2008; Wang et al. 2009). With this advancement, it is now possible for individual labs to generate RNA sequence information from cells or tissues of choice for individualized gene expression profiling. As a result, the genomic and transcriptomic information for the starfish has been deposited in GenBank of the National Center for Biotechnology Information, making it publically available and easy to use (NCBI Resource Coordinators 2017).

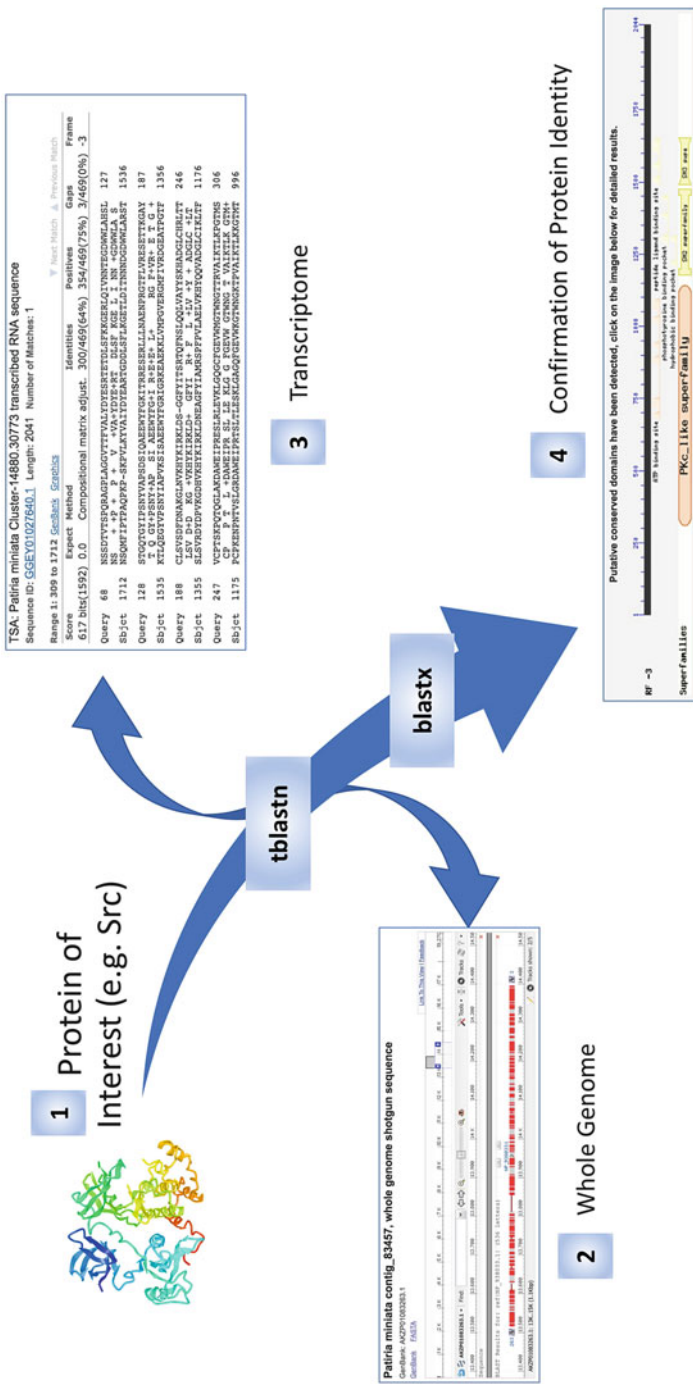
It is expected that the availability of the genome and transcriptome sequences in GenBank will open up many useful possibilities for signal transduction research, including the ability to rapidly clone genes-of-interest and to develop tools for manipulating these genes and their protein products. Quick searches of the existing starfish ovary transcriptome database show evidence for orthologues of many signaling proteins (see below).

### 4.3.1 Validation of the Existing Starfish Transcriptome Data

Four non-receptor protein tyrosine kinases have been identified in the starfish *P. miniata*. Three are the Src family kinases AmSFK1, AmSFK2, and AmSFK3 and one is a C-terminal Src kinase (O'Neill et al. 2004). The validation for the presence of these proteins is made possible by searching the whole genome and mature egg transcriptome for the presence of the specific mRNA (O'Neill et al. 2004). The mRNA for AmSFK1 and AmSFK2 matched to transcriptome-expressed RNA sequences that coded for the entire open reading frame of the SFK, while only partial RNA sequences were present in the ovary for AmSFK3 and AmCSK.

### 4.3.2 Finding Novel Signaling Genes and Their RNA

The transcriptomes of the starfish *P. miniata* and *Astropecten aranciacus* will prove useful for the “discovery” of other signaling molecules for future study. As mentioned above, a simple strategy using the basic local alignment search tool (BLAST) on the NCBI website will quickly show evidence for the presence or absence of any gene of interest (Fig. 4.4). Several examples are shown here. In these examples, the human orthologue for the protein-of-interest was used as the “query” to search the *P. miniata* ovary transcriptome using tblastn, which uses a protein sequence to search a translated nucleotide database. This strategy will identify one or more starfish ovary transcriptome records that could produce a protein that shares significant homology to the human orthologue (Table 4.1). To demonstrate how this might be employed, we searched for evidence of several different signaling molecules, from the cell surface to intracellular effectors, in the existing transcriptome. Examples of RNAs that could code for each protein were found. For example, the starfish ovary transcriptome shotgun assembly (TSA) has an mRNA with 54% identity to a human acetylcholine receptor (NP\_001160166), 68% identify to *X. laevis*, 80% to *S. purpuratus*, and 95% to the starfish *Acanthaster*. Another interesting surface receptor with RNA expressed in the starfish ovary is a folate receptor-like protein similar to Juno, the oocyte receptor for the sperm protein Izumo (Ohto et al. 2016; Inoue 2017). Not surprisingly, this RNA encodes a protein with 82% identity to a putative folate receptor in *Acanthaster*, 59% identity to *S. purpuratus*, and 45% identity to both *Xenopus* and human records (Fig. 4.5 and Table 4.1). These results demonstrate the absolute need for generating species-specific genomic information. With 54% and 45% identify for the human GPCR and Juno, respectively, it is enough to align the proteins and to conclude that they are orthologues. However, this level of identity is not high enough for functional use in the identification of the RNA, or protein, by mass spectrometry or reverse transcriptase PCR. Having the starfish ovary transcriptome, or even an oocyte or egg transcriptome, will greatly expand the options for those studying these molecules in the starfish.



**Fig. 4.4** Strategies for the discovery of signaling proteins in the starfish transcriptome. To identify the starfish orthologues, *Homo sapiens* protein sequences were used as the queries. Initially, tblastn searches of the starfish in silico transcribed nucleotide database (either the genome or transcriptome) with the protein sequence of interest were used to identify the starfish DNA or mRNA that produces the putative starfish orthologue of the human protein. To confirm this result, blastx is used with the identified starfish DNA or mRNA to search different protein databases to confirm suspected identity of the discovered mRNA/protein by homology



**Table 4.1** Evidence for signaling proteins expressed as mRNA in starfish ovary transcriptome

Protein name	Starfish TSA	% identity <i>Acanthaster</i>	% Identity <i>S. purpuratus</i>	% identity <i>Xenopus</i>	% identity human
G alpha(i)	HP135300	99	98	85	89
Acetylcholine GPCR	GAWB01013895	95	80	68	54
Integrin alphaV/8	GAWB01070883	78	45	37	37
Integrin $\beta$ 1	GAWB01035543	83	66	46	46
Grb2	HP116058	69	66	53	53
SFK1	GAWB01035222	89	66	62	59
PLC $\gamma$	GAWB01041886	92	68	50	49
H-Ras	HP096636	99	nd	85	85
FGF receptor	GAWB01064780	76	49	44	44
Juno (folate receptor like)	GAWB01010976	82	59	45	45

Accession numbers of proteins used for comparison: (1) Gai, XP\_022103947, NP\_001001475, NP\_001079549, NP\_002060; (2) Acetylcholine GPCR, XP\_022105189, XP\_786790, XP\_018119614, NP\_001160166; (3) Integrin  $\alpha$ V/8, XP\_022083625, XP\_011682491, XP\_018122789, NP\_003629; (5) Integrin  $\beta$ 1, XP\_022079781, XP\_011679515, XP\_018124144, NP\_391988; (6) GRB2, XP\_022107052, XP\_788430, XP\_018089705, NP\_002077; (7) SFK1, XP\_022091012, NP\_001135851, NP\_001008186, NP\_005424; (8) PLC $\gamma$ , XP\_022091935, XP\_011667789, XP\_017950677, NP\_877963; (9) H-Ras, XP\_022105925, *S. purpuratus* not detected, XP\_018083670, NP\_002515; (10) FGFR, XP\_022080788, XP\_011672178, XP\_018082829, AKS36876. Nd, not detected

### 4.3.3 Applying CRISPR Technology to Studying Signaling in the Starfish

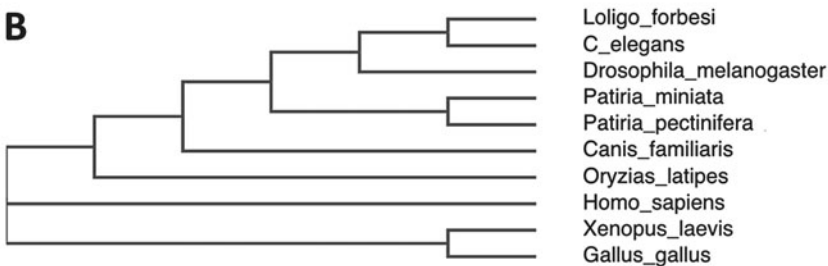
Of great interest will be the application of this new genomic knowledge to understanding signaling processes in different physiological systems. In most cases, antisense or morpholino oligos have been used to knock down expression of specific proteins in echinoderms. These techniques have been used successfully, but they can have issues with effectiveness or specificity (Angerer and Angerer 2004; Cui et al. 2017). The recent applications of the CRISPR technology to sea urchins (Lin and Su 2016; Shevidi et al. 2017) offer a roadmap for use in the starfish. When combined with the ease of identifying novel sequences in the available starfish transcriptomes, the possibility for developing complete signaling pathways is exciting.

**A**

guanine nucleotide-binding protein G(i) subunit alpha-1 isoform 1 [Homo sapiens]  
 Sequence ID: [NP\\_002060.4](#) Length: 354 Number of Matches: 1

Range 1: 1 to 354		<a href="#">GenPept</a>	<a href="#">Graphics</a>			▼ Next Match	▲ Previous Match
Score	Expect	Method	Identities	Positives	Gaps	Frame	
659 bits(1700)	0.0	Compositional matrix adjust.	314/354(89%)	326/354(92%)	0/354(0%)	+1	
Query	265	MGCATSAEDKAAAERSKAIDRNLRIDGEKAAREVLLLLGAGESGKSTIVKQMKIHEEG					444
Sbjct	1	MGC SAEDKAA ERSK IDRNL R DGEKAAREVLLLLGAGESGKSTIVKQMKIHE G					60
Query	445	YSEEDCKQYKPVVYSNTIQSMIAIIRAMGSLKVDGDRDTRDARQLFALAGQAEEGELS					624
Sbjct	61	YSEE+CKQYK VVYSNTIQS+IAIIRAMG LK+DFGD R DDARQLF LAG AEEG ++					120
Query	625	PELAAVMKRLWADGGVQGCFSRSREYQLNDSASYLNLDRLAAPGYIPTQQDVLRTRVK					804
Sbjct	121	ELA +KRLW D GVQ CF+RSREYQLNDSA+YYLN LDR+A P YIPTQQDVLRTRVK					180
Query	805	TTGIVETHFTFKDLHFKMFVGGQSRERKKWIHCFEGVTAIIFCVALSAYDLVLAEDEEM					984
Sbjct	181	TTGIVETHFTFKDLHFKMFVGGQSRERKKWIHCFEGVTAIIFCVALS YDLVLAEDEEM					240
Query	985	NRMHESMKLFDISICNNKWFETSTIILFLNKKDLFEKIKTSPLTICFPEYTGSNTYEEAA					1164
Sbjct	241	NRMHESMKLFDISICNNKWF+TSIILFLNKKDLFEKI KSPLTIC+PEY GSNTYEEAA					300
Query	1165	AYIQMFEDLNKRKDKKEIYTHFTCATDTNNIQVFVDAVTDVLIKNNLKDCGLF					1326
Sbjct	301	AYIQ QFEDLNKRKDK KEIYTHFTCATDT N+QVFVDAVTDVLIKNNLKDCGLF					354

**B**



**Fig. 4.5** Identification of signaling molecules in the starfish transcriptome. Alignment of putative starfish proteins with their human orthologues using blastx (a) shows the remarkable conservation between these species, using Galpha(i) as an example. As expected, phylogenetic analysis shows that the starfish protein is most highly conserved with proteins from other echinoderms (b)

**4.4 Connections to Human Health**

Utilizing starfish as a model system to advance the understanding of human reproduction and fertilization may at first seem disjointed. However, many advances, such as the first human IVF that resulted in the birth of Louise Brown in 1978, would not have been possible without the study of more simplified model systems (Stephoe and Edwards 1978). Although the goals of studying reproduction and fertilization are to advance knowledge and apply that to improving human health, mammals are not always the best model system for studying reproductive biology. Mammalian oocytes generally have low yields, with the mouse model only yielding 10–50 oocytes during ovulation (Brayboy and Wessel 2016). In addition, the oocytes

collected from humans and studied in the lab are often discarded oocytes, which can be of poor quality or not synchronized, making it difficult to understand the molecular mechanisms influencing fertilization (Brayboy and Wessel 2016). Moreover, the ethical issues of donating human oocytes for research can make research directly on human oocytes problematic. Although the human oocyte is necessary for the clinical approach to solving issues of reproduction, the study of the oocytes of a model system like the starfish is critical before clinical applications can be developed.

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# Chapter 5

## Toward Multiscale Modeling of Molecular and Biochemical Events Occurring at Fertilization Time in Sea Urchins



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**Abstract** We review here previous theoretical and experimental works, which aim to model major events that occur at the time of fertilization in the sea urchin. We discuss works that perform experiments and develop hypotheses that link different scales of biological systems such as the intracellular  $\text{Ca}^{2+}$  concentration oscillations and the swimming behavior of sperm, the  $\text{Ca}^{2+}$  wave propagation and the fertilization membrane elevation of the egg, and the mRNA translational activation and the completion of the first mitotic division of the early embryo. The aim of this review is on one hand, to highlight the value of systems biology for understanding the mechanisms associated with fertilization and early embryonic development in sea urchins. On the other hand, this review attempts to illustrate, for mathematicians and bioinformaticians, the potential that represent these molecular and cellular events for modeling clear physiological processes.

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**Keywords** Sea urchin · Fertilization · Sperm swimming · Egg activation ·  $\text{Ca}^{2+}$  signaling pathway · mRNA translation · Systems biology

## 5.1 Introduction

The events that occur at fertilization and during the early embryonic development of the sea urchin represent, for biologists, a fascinating system of study to understand the molecular and biochemical mechanisms that preside at the beginning of the life of a metazoan. The succession of molecular, biochemical, and cellular events that follows fertilization of the sea urchin egg gives the opportunity to approach their integration into different spatiotemporal contexts.

Sea urchin embryos have been extensively used in laboratories for more than one century and are particularly useful for studying fertilization, the crucial process by which most sexually reproducing individuals begin (Briggs and Wessel 2006; Santella et al. 2012). Sea urchins are gonochoric, and gametes are very easy to obtain in large quantities. The sea urchin fertilization process is external, and sperm swimming plays a key role for the navigation toward the egg so that the two mature and competent gametes may fuse. The size of the sea urchin egg ( $\approx 100 \mu\text{m}$  diameter) and their optical characteristics make them particularly interesting for manipulation, microinjection, and observation under optical microscopy (Stepicheva and Song 2014; Angione et al. 2015).

Therefore, it is easy to observe the elevation of the fertilization envelope first described in 1847 by Derbès as a “circular line” encompassing the egg following fertilization (Wong and Wessel 2008; Derbès 1847). In advanced high school, community college, and university biology laboratory courses, fertilization envelope elevation serves classically as the first indication of sperm-mediated egg activation (Vacquier 2011). The induction by the sperm and the spread of the fertilization envelope elevation around the egg is triggered by a calcium signaling process (Whitaker 2006; Ramos and Wessel 2013; Costache et al. 2014). Calcium first increases at the point of sperm-egg interaction and crosses the egg as a wave to the antipode (Hafner et al. 1988). This calcium wave is responsible for sea urchin egg activation, which can be characterized in part by the fertilization envelope elevation, the increases of protein synthesis, and the entry into the cell cycle (Steinhardt and Epel 1974; Poenie et al. 1985).

Unfertilized sea urchin eggs have completed their meiotic maturation and are physiologically blocked at the G1 stage of the cell cycle. Fertilization triggers entry into S-phase and completion of the first mitotic division of the embryonic division. Thanks to the large amount of eggs recovered and the synchrony of fertilization and cell cycle events, these gametes have been useful material for biochemical investigations of calcium-mobilizing second messengers, cell cycle actors, and protein translation control triggered by fertilization (Evans et al. 1983; Cormier et al. 2001; Morgan and Galione 2014). The sea urchin embryo is also a major system for studies on the mechanisms of gene regulation in early development (Davidson et al. 1998).



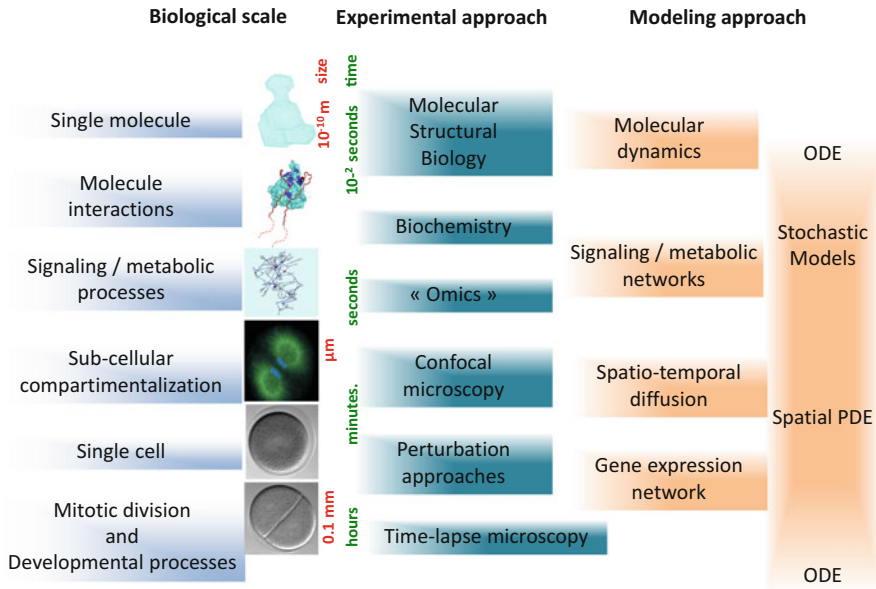
Because of the remarkable usefulness of the early embryos as a research model system for modern molecular, cell, and developmental biology, the genome of the sea urchin was sequenced (Sea Urchin Genome Sequencing C et al. 2006). This sequencing gave researchers the opportunity to learn a great deal about the components of key signaling pathways induced by fertilization (Bradham et al. 2006; Byrum et al. 2006; Fernandez-Guerra et al. 2006; Morales et al. 2006; Song et al. 2006). Furthermore, the sea urchin genome sequence provides strong support for the “bilaterian regulatory toolkit” and gives new insight into the evolution of gene interactions that form the so-called gene regulatory network regulating embryonic development embryogenesis (Arnone et al. 2016; Howard-Ashby et al. 2006).

The complex nature of the vast networks activated by fertilization that allow the beginning of the embryonic development and successive cell divisions represents an important challenge to understanding their dynamics, regulations, and functions. The propagation of the calcium wave and its biological implication in terms of the activation of the egg involve several mechanisms and sub-processes that connect intracellular molecular interactions to the scale of a cell. The quantitative modeling of such a multiscale system depends upon mathematical tools and the extensive use of computational simulations (Meier-Schellersheim et al. 2009).

We review here previous theoretical and experimental works, which aim to model three of the main molecular, metabolic, subcellular, and cellular events triggered by the sperm and the egg at the time of fertilization and during the first mitotic division of the sea urchin embryo. Providing a nontechnical overview of mathematical/bioinformatic models using the sea urchin early developmental system, in different context of scales of time and space, we wish to demonstrate that the sea urchin represents an interesting system for multiscale modeling analyses (Fig. 5.1).

## 5.2 From $\text{Ca}^{2+}$ Signaling Pathway to Sperm Navigation

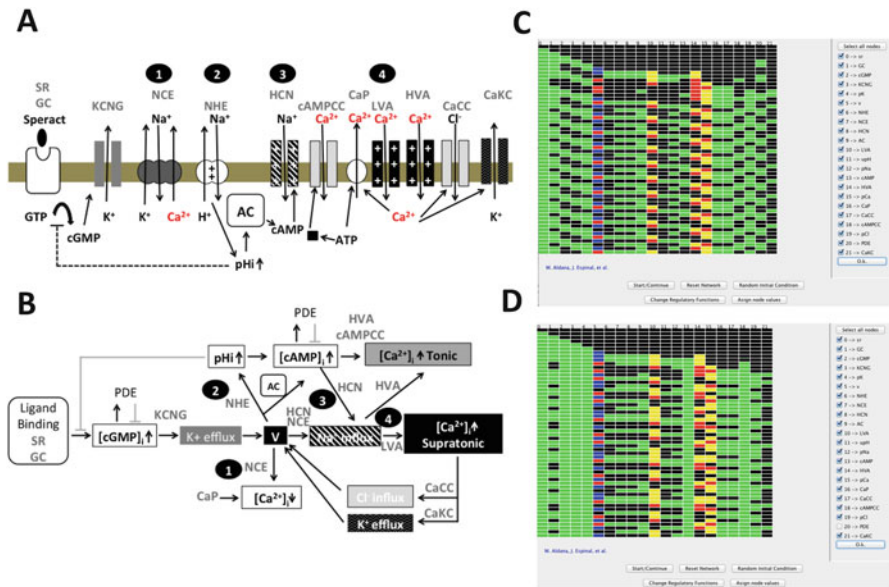
In many species eggs secrete chemoattractants from surrounding jelly layers to influence sperm swimming toward their source. The chemotactic behavior of sperm toward the egg is controlled by flagellar curvature alterations, which depend on intracellular  $\text{Ca}^{2+}$  concentration  $[\text{Ca}^{2+}]_i$  oscillations (Bohmer et al. 2005). In *Strongylocentrotus purpuratus*, speract a decapeptide, which is present in and diffuses from the external layer of the egg, binds to a receptor located in the flagellar membrane of the sperm. It activates a finely regulated signaling pathway, which produces a train of intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) oscillations. The ( $[\text{Ca}^{2+}]_i$ ) response is characterized by a sustained (tonic) increase in ( $[\text{Ca}^{2+}]_i$ ) followed by superimposed fluctuations of that response (supratonic) [review in Darszon et al. (2008)]. When speract binds the extracellular side of the receptor (SR), it activates latent guanylyl cyclase (GC) activity in the cytoplasmic side of the receptor, which produces cGMP (Galindo et al. 2000). The increase of cGMP triggers the opening of a cGMP-regulated  $\text{K}^+$  channel (KCNG) leading to a hyperpolarization of the membrane potential (V), which triggers the following



**Fig. 5.1** Graphic representation of different biological scales occurring at fertilization time and during early developmental embryogenesis in sea urchin. From left to right are illustrated the different biological scales, the time scales, the experimental approaches, and their associated modeling techniques. ODE, ordinary differential equation; PDE, partial differential equation [Adapted from Meier-Schellersheim et al. (2009) for sea urchin early developmental system]

four main processes: (1) The  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCE) is activated and consequently induces a decrease in the  $[\text{Ca}^{2+}]_i$  level. (2) The  $\text{Na}^+/\text{H}^+$  pump is activated, and consequently the intracellular pH ( $\text{pH}_i$ ) increases. The  $\text{pH}_i$  elevation inhibits the GC activity and activates a soluble Adenylate Cyclase (AC), which produces cAMP. (3) The elevation of cAMP activates a hyperpolarization-activated and cyclic nucleotide-gated channel (HCN) and a cAMP-dependent calcium channel, which tends to repolarize the membrane potential. (4) Repolarization removes the inactivation of the high- and low-voltage-activated  $\text{Ca}^{2+}$  channels (HVA and LVA), triggering the opening of these channels and consequently depolarization and an increase of the  $[\text{Ca}^{2+}]_i$  level. The oscillation of  $[\text{Ca}^{2+}]_i$  requires a new hyperpolarization that is achieved by a  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channel (CaCC) and a  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channel (CaKC) that are opened when the  $[\text{Ca}^{2+}]_i$  level is high. The processes are cyclically repeated to produce a train of  $[\text{Ca}^{2+}]_i$  oscillations. In parallel, basal  $[\text{Ca}^{2+}]_i$  levels are maintained by a constant passive  $\text{Ca}^{2+}$  extrusion through the calcium pump (CaP) and the NCE. The cyclic alternation of hyperpolarization and repolarization generates the train of oscillations in  $[\text{Ca}^{2+}]_i$  that control the pattern of the sea urchin sperm swimming (Espinal et al. 2011).

Based on the regulatory logic of the interactions rather than on their kinetic details, the group of Martinez-Mekler modeled the speract-activated  $\text{Ca}^{2+}$  signaling



**Fig. 5.2** Dynamic of the signaling pathway induced by speract in sea urchin sperm. (a) Schematic diagram illustrating the components involved in the speract signaling pathway. (b) Cascade events induced by the binding of speract to its receptor. (c) Representation of the logical signaling network without deletions (all nodes present). (d) Representation of the logical signaling pathway after the elimination of PDE [Adapted from Espinal et al. (2011) with permission of Martinez-Mekler G]

pathway network that controls the sperm motility (Espinal et al. 2011). They adopted a discrete dynamics formalism acting on a logic representation for the pathway. The logical signaling network consists of about 20 nodes representing the principal elements involved in the pathway (Fig. 5.2a, b). Each node has its own regulatory function, and for the construction of these regulatory functions, they used all biological knowledge of an electrophysiological nature available in the literature and their laboratory. In order to study the dynamics of the system, they implemented a discrete formulation derived from a Boolean approach. The behavior of most of the nodes is binary, meaning inactive or active, present or absent, and closed or open. A few nodes are required to be represented by three-state variables such as the  $[Ca^{2+}]_i$  (basal 0, tonic 1, and supratic 2) and the membrane potential (hyperpolarized 0, resting 1, and depolarized 2). An example of the time evolution representation of the network is illustrated in Fig. 5.2c, d. They validated their network model by comparing the outgoing dynamics of the system with previously available experimental data as well as with new experiments suggested by predictions. In all cases, the numerical simulations of the network model were in good agreement with experimental determinations. Interestingly, model prediction helped clarify the role of different elements in the network. In this context, the model was used to predict

that the  $\text{Ca}^{2+}$  CatSper channel is the main  $\text{Ca}^{2+}$  channel involved in the regulation of sea urchin sperm motility (Espinal-Enriquez et al. 2017).

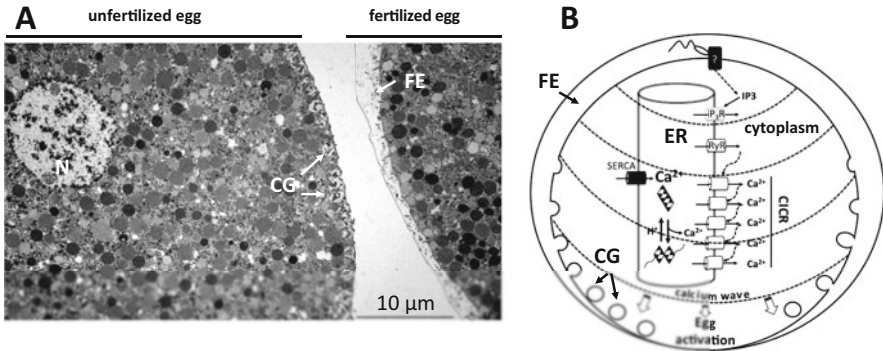
### 5.3 From $\text{Ca}^{2+}$ Wave to Fertilization Envelope Elevation

The view that calcium is essential for egg activation in metazoans and more specifically in marine invertebrate deuterostome has been extensively reviewed (Whitaker 2006; Ramos and Wessel 2013; Costache et al. 2014; Swann and Lai 2016). The hypothesis of  $\text{Ca}^{2+}$  release following fertilization of sea urchin was first tested in the mid-twentieth century (Mazia 1937). Using the luminescent calcium sensor aequorin, calcium release triggered by ionophore treatment and fertilization was demonstrated in sea urchin eggs (Steinhardt and Epel 1974; Steinhardt et al. 1977).

In sea urchin eggs, two independent types of  $\text{Ca}^{2+}$  increases have been observed following fertilization. The first one is a small initial cortical flash, which is a consequence of an action potential-mediated influx of extracellular  $\text{Ca}^{2+}$ . This is followed by a second major cytosolic wave due to the release of  $\text{Ca}^{2+}$  from the intracellular stores beginning from the sperm interaction site and traveling through the depths of the cytoplasm across the egg (Parrington et al. 2007; Whitaker and Steinhardt 1982). The initial cortical flash does not initiate the subsequent  $\text{Ca}^{2+}$  wave, which is a distinct process triggered by the sperm. Furthermore, it is noteworthy to mention that in sea urchin a single wave of  $\text{Ca}^{2+}$  crosses the eggs, whereas the sperm triggers  $\text{Ca}^{2+}$  oscillations in ascidians and mammals (Whitaker 2006; Sardet et al. 1998; Dupont and Dumollard 2004).

Accumulation of experimental data gives insights on how sea urchin eggs form a calcium wave, but some points still remain to be clarified (Whitaker 2006; Ramos and Wessel 2013; Swann and Lai 2016; Runft et al. 2002). It is generally accepted by the scientific community that just following fertilization, the  $\text{Ca}^{2+}$  rise occurs as a result of inositol 1,4,5-triphosphate ( $\text{IP}_3$ )-mediated release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (Terasaki and Sardet 1991). However, studies show that in sea urchin eggs, other intracellular second messengers, including nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic guanosine monophosphate (cGMP), cyclic ADP-ribose (cADPR), and nitric oxide (NO), increase at fertilization and could trigger  $\text{Ca}^{2+}$  release (Kuroda et al. 2001). However, in contrast to  $\text{IP}_3$ , none of these second messengers is indispensable to the fertilization wave in the sea urchin egg.

According to their velocity, one can distinguish three different main classes of intracellular  $\text{Ca}^{2+}$  waves (Jaffe 1993). At 20°C, the fast waves move at about 5–30  $\mu\text{m/s}$ , the slow waves move at 0.1–1  $\mu\text{m/s}$ , and the ultrafast ones accompany electrical propagation processes such as action potential in many neurons and muscles. The  $\text{Ca}^{2+}$  wave at fertilization in sea urchin crosses the egg (100  $\mu\text{m}$  in diameter) over a period of 20 s and is consequently classified as fast wave (Jaffe 1993; Galione et al. 1993). Cytosolic  $\text{Ca}^{2+}$  waves can be broadly classified into two types: type 1 waves propagate as sharp bands throughout the cell, with velocities and



**Fig. 5.3** Mechanisms of egg activation and fertilization envelope elevation. (a) Transmission electron micrograph showing partial views of two *Sphaerechinus granularis* sea urchin eggs, one before fertilization (on the left) and one after fertilization (on the right). Nu, nucleus; CG, cortical granules; FE, fertilization envelope. (b) Schematic drawing illustrating the main mechanisms involved in the fertilization envelope (FE) elevation. Sperm entry triggers, by a still unidentified process, the increase of cytosolic  $IP_3$ , which induces the release of  $Ca^{2+}$  from the endoplasmic reticulum (ER) to the cytoplasm. CICR propagates the wave outside of the reticulum. The drawing of the ion-releasing macromolecular complex (black and white diamonds) represents casequestrin as shown in Fig. 1 of Jaffe (2004) and Fig. 6 of Wang et al. (1998). The  $Ca^{2+}$  wave triggers egg activation and initiates the exocytosis of cortical granules (CG) and elevation of the fertilization envelope

frequencies that are high enough to permit the propagation of several fronts at the same time (Dupont and Goldbeter 1994). Type 2 waves correspond to the progressive increase of the cytosolic level of  $Ca^{2+}$  throughout the cell, followed by a return to the basal level in a quasi-homogeneous way (Dupont and Goldbeter 1994; Tsien and Tsien 1990). The  $Ca^{2+}$  wave at fertilization in sea urchin corresponds to a type 2 wave (Galione et al. 1993).

The propagation of the cytosolic calcium wave through a diverse range of cells prompted biologists and mathematicians to model the system. Such a modeling requires a thorough knowledge of the propagation mechanism of the wave and of the different molecular actors and organelles involved. The observation of the sea urchin egg by electron microscopy makes it possible to visualize the actual complexity of the spatial distribution of diverse organelles (Fig. 5.3a). Several organelles are present on the cortex of unfertilized eggs, including cortical granules (Vacquier 1975), acidic vesicles (Sardet 1984; Morgan 2011), and the endoplasmic reticulum (Sardet 1984). The endoplasmic reticulum is the site of ATP-dependent calcium sequestration and  $IP_3$ -induced calcium release, and experiments have shown that it plays a major function in the solitary calcium wave triggered by fertilization of sea urchin eggs (Terasaki and Sardet 1991). At the molecular level, the sea urchin “calcium toolkit” has been extensively described in the genome (Sea Urchin Genome Sequencing C et al. 2006; Roux et al. 2006). At any given time, the level of intracellular  $Ca^{2+}$  is the result of a balance between the “on” reactions that induce the introduction of  $Ca^{2+}$  into the cytoplasmic compartment and the “off” reactions

that remove free intracellular  $\text{Ca}^{2+}$  via a combination of buffers, pumps, and exchangers (Briggs and Wessel 2006). The  $\text{IP}_3$ -receptor channel ( $\text{IP}_3\text{R}$ ) and the ryanodine receptor/channel (RyR), which is activated by cGMP or cADPR, are present in the endoplasmic reticulum membrane (Fig. 5.3b). The involvement of the two molecules acting together or separately is always a point of discussion, and it is still not clear which specific one would begin the cytosolic  $\text{Ca}^{2+}$  wave (Swann and Lai 2016). The classic process of calcium-induced calcium release (CICR) contributes to the explosive release during the fertilization wave (Galione et al. 1993; McDougall et al. 1993). CICR is a reaction-diffusion mechanism in which  $\text{Ca}^{2+}$  release is mediated by RyR and  $\text{IP}_3\text{R}$  present in the endoplasmic reticulum. The CICR theoretical model was originally proposed for  $[\text{Ca}^{2+}]_i$  oscillations (Friel 1995; Goldbeter et al. 1990). The RYR and the  $\text{IP}_3\text{R}$  are sensitive to and consequently open in the presence of low  $\text{Ca}^{2+}$  concentration, whereas higher millimolar concentrations inhibit them. In a widely used model, calcium waves are propagated by a reaction-diffusion process in which calcium ions progress outside the endoplasmic reticulum network tubes and trigger further  $\text{Ca}^{2+}$  release from the ER by binding to an  $\text{IP}_3$ -bound  $\text{IP}_3$  receptor and promoting further CICR. The SERCA pump (smooth endoplasmic reticulum calcium ATPase) drives the accumulation of  $\text{Ca}^{2+}$  in the endoplasmic reticulum (Berridge et al. 2003). The SERCA is present in sea urchin eggs (Roux et al. 2006).  $\text{Ca}^{2+}$  induces conformational changes in calsequestrin (Wang et al. 1998). However these changes do not directly trigger  $\text{Ca}^{2+}$  release from this buffer protein, and in most models, free  $\text{Ca}^{2+}$  in the reticulum is assumed not to be limiting. A complementary tandem model has been proposed, in which a process called proton-induced proton release (PIPR) propagates the  $\text{Ca}^{2+}$  wave within the reticulum, while CICR propagates the  $\text{Ca}^{2+}$  wave outside the endoplasmic reticulum (Jaffe 2004). In this model,  $\text{H}^+$ -induced conformational changes in calsequestrin would induce calcium release. Finally, the rise of calcium to more than  $0.2 \mu\text{M}$  in the cytoplasm is the primary cause of the activation and the elevation of the fertilization membrane in the sea urchin egg at fertilization (Hamaguchi and Hiramoto 1981).

A large variety of mathematical models have been developed for cellular  $\text{Ca}^{2+}$  oscillations (Schuster et al. 2002). Indeed, the reaction-diffusion processes mentioned above gave rise to an extensive research domain pertaining to the theory of partial differential equations (PDE). Such equations study the evolution of quantities depending upon several variables of interest, which can be for our focus the time and a two-dimensional or three-dimensional space variable (Desvillettes et al. 2017; Desvillettes 2007). It is widely accepted that chemical and biochemical reactions are particularly well represented by reaction-diffusion equations and systems as long as there is a great number of agents involved (mean-field limit). A realistic theoretical model for repetitive  $\text{Ca}^{2+}$  wave generation and propagation has been developed and gives insights into the origin of the pacemaker in mature ascidian eggs (Dupont and Dumollard 2004). Other models have focused on the rather typical single wave that is triggered at fertilization in *Xenopus* and sea urchin eggs (Duran and Santamaria-Holek 2014; Wagner et al. 2004). Interestingly, in sea urchins, it has been developed a multiscale biophysico-chemical model, which simulates the events

from the  $\text{Ca}^{2+}$  wave propagation induced by fertilization to the elevation of the fertilization membrane that represents a physical barrier against polyspermy (Duran and Santamaria-Holek 2014). Recently, taking the calcium wave triggered by fertilization of the sea urchin egg, exclusive mathematical approaches have been developed in order to know if traveling wave solutions of the reaction-diffusion equation can generate a traveling wave for the diffusion equation (Moundoyi et al. 2017). The next step is to describe, with mathematical tools, how such a  $\text{Ca}^{2+}$  wave generates the observed diffusion of the released contents of cortical granules between the cell membrane and the vitelline membrane.

#### 5.4 From Translation Regulation to the First Mitotic Division Following Fertilization

The fertilization-induced transitory increase in cytoplasmic free intracellular  $\text{Ca}^{2+}$  concentration and the sustained rise in intracellular pH trigger the activation of the sea urchin eggs (Epel 1990). Under the term “egg activation” are brought together, many characteristic changes, both morphological and biochemical, that happen in a period of time comprised between the penetration of the sperm into the egg and the onset of the mitotic cell cycle of the embryo (Costache et al. 2014; Swann and Lai 2016; Runft et al. 2002). Sea urchin eggs are haploid cells blocked in G1, and fertilization triggers entry into S-phase and completion of the first mitotic division of the embryonic development. De novo protein synthesis is dispensable for the S-phase but is required for the onset of M-phase and subsequent embryonic cell cycles (Dube 1988; Wagenaar 1983). The mitotic cyclins A and B were first discovered in sea urchin as key proteins, which are synthesized and degraded during the mitosis phase at each cell division (Evans et al. 1983). Cyclins and their catalytic kinase partners CDKs (cyclin-dependent kinases) control cell cycle progression (Malumbres 2014).

The protein synthesis rate of unfertilized sea urchin eggs remains remarkably low, and fertilization is the starting signal, which triggers their awakening and induces a dramatic increase of mRNA translation [review in Cormier et al. (2016)]. This increase of mRNA translation is independent of mRNA transcription and ribosome biogenesis (Epel 1967; Brandhorst 1976). Therefore, sea urchin egg activation provides an example of regulated gene expression at the translational level using maternal mRNAs that does not follow the traditional scheme of gene regulation governed at the transcription level (Mathews et al. 2000).

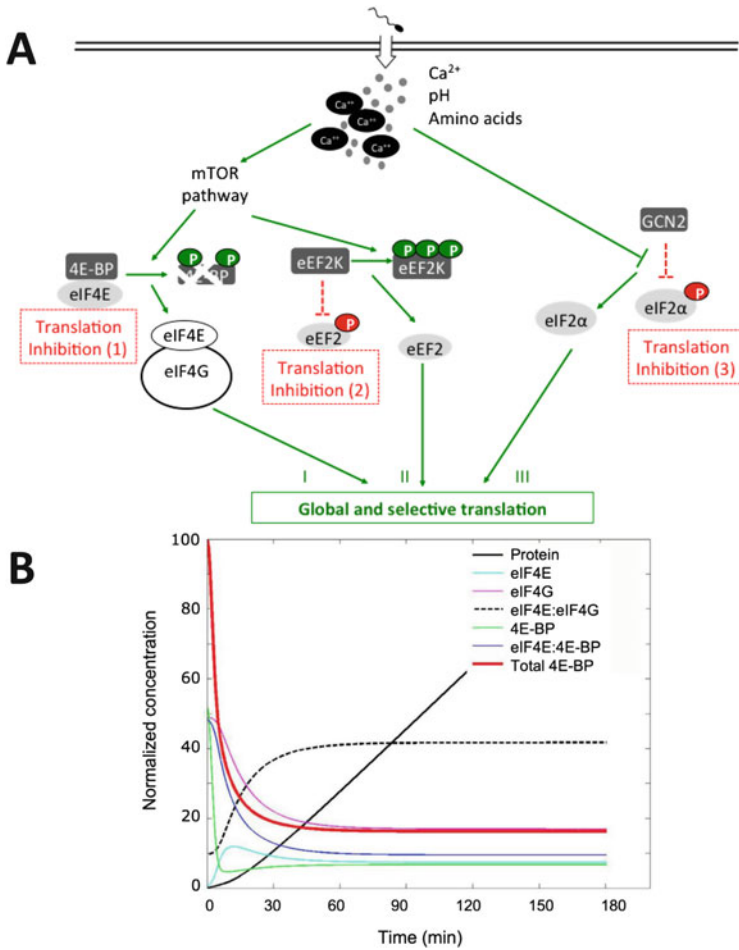
Sea urchin eggs are relevant models that have helped to address the control of gene expression at the translational level in relation to cell cycle regulation in physiological conditions (Cormier et al. 2003, 2016). While the large and rapid increase in the rate of protein synthesis following fertilization of the sea urchin egg has long been a paradigm of translational control, the mechanisms by which mRNA translation is increased following fertilization and how those controls contribute to

proper development have only started to be understood at the beginning of 2000. The multiple ways in which the overall rates of mRNAs translation can be controlled in response to the fertilization of sea urchin eggs have been reviewed recently (Cormier et al. 2016). The availability of the sea urchin genome has allowed the identification of the “translational toolkit” in this model system (Sea Urchin Genome Sequencing C et al. 2006; Morales et al. 2006). Thanks to accumulated knowledge on cap-dependent initiation of translation, we have obtained an integrative model for translation initiation using a programming environment for system biology coupled with model validation [named BIOCHAM (the BIOChemical Abstract Machine)] (Belle et al. 2010). This model is described by abstract rules and contains 51 reactions involved in 74 molecular complexes. The model proved to be coherent with the biological knowledge as judged from specification tests and simulations performed with computational tree logic (CTL) as well as Boolean simulations. The numerical simulation from BIOCHAM showed that cap-dependent translation was almost undetectable in unfertilized egg and strongly increased following fertilization, after a delay corresponding to the time to initiate the translational machinery, in agreement with biological observations. Furthermore, we showed that the combination of our model of translation regulation with the BIOCHAM version (Calzone et al. 2006) of the cell cycle regulation (Tyson 1991) can be used for analysis of the cap-dependent translational machinery in relationship with the cell cycle control. However, while this model constitutes a basis to which new information can be progressively supplemented, this type of model is not actually usable for driving hypotheses for further experimentations. Furthermore, the BIOCHAM model contains too many reactions and protein interactions, for which kinetics parameters remain undetermined, to allow quantitative simulations of dynamic changes occurring following sea urchin egg fertilization. It is therefore important to investigate the strategy of extracting a minimal core model containing a small number of actors playing a crucial role in the process.

Three translation factors have been experimentally shown to be activated *in vivo* in response to egg fertilization leading to an increase of protein synthesis (Fig. 5.4a): one elongation factor, eEF2 (eukaryotic elongation factor 2), and two initiation factors, eIF2 (eukaryotic initiation factor 2) and the cap-binding protein eIF4E (eukaryotic initiation factor 4E).

The protein eEF2 is considered to be the major regulatory target for controlling elongation rate (Proud 2007). The activity of this elongation factor is inhibited by phosphorylation. The phosphorylation/activation status of eEF2 depends on the activity of an unusual calcium-calmodulin kinase, which targets specifically eEF2 and is consequently called eEF2 kinase (Proud 2015). Multiple phosphorylation sites positively or negatively regulate eEF2 kinase activity. PKA (protein kinase A) and AMPK (AMP-activated protein kinase) activate, while mTOR (mechanistic target of rapamycin) and ERK (extracellular signal-regulated kinase) inhibit the kinase (Proud 2007, 2015). The kinase that phosphorylates eEF2 is therefore considered as an integrating target of several signaling pathways that leads to protein synthesis regulation through the phosphorylation of its unique substrate eEF2. We reported the existence of a new isoform of eEF2 protein whose phosphorylation is required





**Fig. 5.4** Signaling pathways and translation factors showed to be involved in the translational control triggered by fertilization. **(a)** Schematic diagram illustrating the components involved in translation activation induced by fertilization. Sperm entry induces the  $Ca^{2+}$  wave, a sustained rise in intracellular pH and an entry of amino acids.  $Ca^{2+}$  activates the mTOR pathway, which induces the phosphorylation and degradation of 4E-BP, allowing eIF4E to associate with eIF4G and consequently to engage the global cap-dependent translation of mRNAs. The activated mTOR phosphorylates and inhibits eEF2 kinase. The inhibition of eEF2 kinase allows eEF2 activation and the elongation step of the mRNAs translation. GCN2 is inhibited by the increase in amino acids; consequently eIF2 $\alpha$  is dephosphorylated and active for the initiation step. **(b)** Simulation of the concentration modifications of translation factors following fertilization of the sea urchin egg. Using the minimal model involving “eIF4E,” “eIF4G,” and “4E-BP,” the parameters determined in the unfertilized eggs and the changes predicted in the fertilized eggs of the kinetic of concentration changes of all the parameters were simulated as described in Laurent et al. (2014)

for the completion of the first cell cycle of early development in sea urchin eggs (Belle et al. 2011). Interestingly, the eEF2 isoform is rephosphorylated with a cell cycle-dependent behavior. We proposed that eEF2 is phosphorylated in a spatio-temporal context at the M-phase stage and that a localized pool of eEF2 would be involved in the elongation pauses in sea urchin (Belle et al. 2011; Monnier et al. 2001). However, no kinetic parameter is yet available to integrate this molecular actor in a minimal core model of the translation activity occurring at fertilization in sea urchin.

At the initiation level, we showed that eIF2 plays a key role in the increase of mRNA translation triggered by fertilization in sea urchin eggs (Costache et al. 2012). The translation factor eIF2 is a heterotrimeric complex composed of three subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). When phosphorylated, the  $\alpha$  subunit of eIF2 sequesters eIF2B and consequently inhibits the translational initiation and global protein synthesis while inducing the translation of specific mRNAs (Jackson et al. 2010). We demonstrated that eIF2 $\alpha$  phosphorylation contributes to the regulation of protein synthesis required for the first mitotic division in sea urchin embryos (Costache et al. 2012). GCN2, the most ancestral eIF2 $\alpha$  kinase, is an attractive candidate for the phosphorylation regulation of eIF2 $\alpha$  in sea urchin eggs. Active GCN2 in unfertilized eggs would phosphorylate and consequently inhibit eIF2 $\alpha$ . Fertilization would inhibit GCN2 allowing eIF2 $\alpha$  to contribute to the protein synthesis increase (Cormier et al. 2016; Costache et al. 2012). The regulation of translation initiation by eIF2 and its phosphorylation have been integrated in our model of translation regulation with the BIOCHAM version (Belle et al. 2010), but again the lack of kinetic data in the sea urchin does not allow us to integrate eIF2 $\alpha$  in a model for quantitative simulations.

Finally, the earlier event resulting in the dramatic increase in the rate of global protein synthesis triggered by fertilization in sea urchin eggs was reported to be due to the release of eIF4E from its repressor 4E-BP (eIF4E-binding protein) (Cormier et al. 2001; Salaun et al. 2003). In all eukaryotic cells, eIF4E recognizes and binds to the 5' cap structure ( $m^7GpppN$ , where N is any nucleotide). When available, eIF4E associates with eIF4G (eukaryotic initiation factor 4G), a large scaffolding protein that interacts with eIF4A (eukaryotic initiation factor 4A), an RNA-dependent ATPase and RNA helicase (Gingras et al. 1999). We showed that, in the sea urchin *Sphaerechinus granularis*, fertilization allows rapid eIF4E association with eIF4G, and we provided evidence that eIF4E/eIF4G complex formation is functionally important for the completion of the first mitotic division following fertilization, implicating cap-dependent translation in this process (Oulhen et al. 2007). A still intriguing discovery is that fertilization triggers dramatic posttranslational eIF4G modifications, which seem to be independent of the mTOR kinase but dependent on the  $Ca^{2+}$  signaling pathway (Oulhen et al. 2007, 2010).

4E-BP competes with eIF4G and consequently 4E-BP inhibits cap-dependent translation. A single 4E-BP protein ortholog exists in sea urchin (Sea Urchin Genome Sequencing C et al. 2006; Morales et al. 2006), while the vertebrate genome duplications gave rise to three classes of 4E-BPs, 4E-BP1, 4E-BP2, and 4E-BP3 in mammals (Gillespie et al. 2016). 4E-BPs bind to eIF4E through a bipartite interface

that consists of the eIF4E-binding domain YXXXXLΦ and a “noncanonical” eIF4E-binding motif (Peter et al. 2015). Using small-angle X-ray scattering (SAXS), we showed that while sea urchin 4E-BP is intrinsically disordered in the free state, it undergoes a dramatic compaction of 4E-BP when it interacts with eIF4E (Gosselin et al. 2011), in agreement with the dynamic model of human 4E-BP2 associations with eIF4E (Bah et al. 2015). The binding of 4E-BPs to eIF4E is controlled by the phosphorylation state (Pause et al. 1994; Gingras et al. 1998). Hypophosphorylated 4E-BPs bind to eIF4E, whereas hyperphosphorylated forms do not. The mTOR kinase phosphorylates 4E-BP and leads to its dissociation from eIF4E (Fonseca et al. 2014; Merrick 2015). While phosphorylation may result in electrostatic repulsion with the negative surface of eIF4E (Marcotrigiano et al. 1999), using phosphomimetics mutants of sea urchin 4E-BP unable to weaken 4E-BP/eIF4E binding, we hypothesized that additional posttranslational modifications are required (Oulhen et al. 2009). Finally, it has been demonstrated that multisite phosphorylation induces folding on the intrinsically disordered human 4E-BP2, resulting in a dramatic decrease of 4E-BP2 affinity for eIF4E (Bah et al. 2015).

By monitoring 4E-BP following egg fertilization and during embryonic development of sea urchin, we were the first to describe down- and upregulation of the 4E-BP protein level in physiological conditions (Salaun et al. 2003, 2005). We demonstrated that the 4E-BP degradation triggered by the fertilization of sea urchin eggs is under the control of  $\text{Ca}^{2+}$  and pH-dependent events (Oulhen et al. 2010). The control of 4E-BPs stability is now recognized as a supplementary means to regulate eIF4E availability and consequently to control cap-dependent translation activity (Salaun et al. 2003; Lin et al. 1995; Elia et al. 2008; Yanagiya et al. 2012). However, it is unlikely that the mechanism of 4E-BP degradation observed in sea urchin corresponds to the one observed in mammalian cells [reviewed in Cormier et al. (2016)]. The 4E-BP degradation induced by fertilization of sea urchin eggs is inhibited either by rapamycin treatment or PP242, a novel and specific ATP-competitive inhibitor of mTOR kinase (Salaun et al. 2003; Chasse et al. 2016). Taken together, these data indicate a major role of mTOR-mediated regulation of translation initiation at the early embryonic development.

The concentrations of 4E-BP and eIF4E in unfertilized eggs were determined by immunoblotting followed by densitometric quantification, and the kinetic parameters for 4E-BP/eIF4E interaction were determined by surface plasmon resonance (Laurent et al. 2014). These different data allowed us to build a highly simple reaction model for the regulation of protein synthesis following fertilization in sea urchin (Laurent et al. 2014). The model simulates the physiological changes in the total 4E-BP amount observed during the time after fertilization, and we demonstrated that a reduced model involving 4E-BP, eIF4E, and eIF4G is satisfactory to fully simulate the cap-dependent translational changes occurring at fertilization (Fig. 5.4b). Furthermore, the model postulates that two changes occurring at fertilization are required to fit with experimental data: an increase of 4E-BP/eIF4E complex dissociation and an important activation of the 4E-BP degradation mechanism. While, as expected, the model predicts that 4E-BP/eIF4E complex destabilization is controlled by the kinase mTOR; surprisingly the mechanism of 4E-BP

degradation itself seems also strongly affected by the kinase. Therefore, it would be of great interest to characterize the 4E-BP degradation mechanism occurring at fertilization and to search whether similar mechanisms exist in other organisms and other physiological regulations.

For a great number of proteins neo-synthesized from the different available maternal mRNAs, the rise in protein synthesis occurs at a constant rate following fertilization. Cyclin B protein translation follows a kinetic curve that is different from the global protein synthesis, and consequently we developed a mathematical model to analyze the dissimilarity in the biosynthesis kinetic patterns (Picard et al. 2016). We hypothesized that two forms of *cyclin B* mRNA, distinct by their immediate or not immediate availability to be translated, are present in sea urchin eggs. The model predicts that translation of the not immediately available form of mRNA is largely dominant compared to the immediately available form of cyclin B mRNA. This model should help provide insights into the signaling utilized for the control of the biosynthesis and accumulation of cyclin B protein triggered by fertilization and required for the completion of the first mitotic division of the sea urchin embryo (Chasse et al. 2016; Mulner-Lorillon et al. 2017).

## 5.5 Conclusions and Perspectives

The models that we have presented in this review simulate the basic features of three independent events that accompany fertilization in the sea urchin system. The different mathematical approaches allow the unveiling of processes, which are hard to detect only by experimental procedures. The most important challenges are now to develop the multi-scale implications of these modelings.

The discrete dynamics model for the speract-activated signaling network in sea urchin sperm allowed the prediction of novel characteristics of the signaling pathway (Espinal-Enriquez et al. 2017). During chemotaxis, sperm swim in a chemoattractant gradient by accommodating the flagellar waveform and consequently the swimming path. The rate of change in  $[Ca^{2+}]_i$  regulates chemotaxis (Alvarez et al. 2012). Therefore, the model proposed by Martinez et al. represents a solid basis of knowledge and a possible simulation to understanding how the temporal synchronization of the signaling pathway controls the fine-tuning required for influencing the movement of the sperm in response to the spatiotemporal gradient of chemoattractant secreted by the egg.

Fertilization offers the opportunity to study  $Ca^{2+}$  waves and their mechanisms of propagation in the context of a clear physiological function. Mathematical models have been developed for cellular  $Ca^{2+}$  waves propagation triggered by fertilization. Generally speaking, wave phenomena in the cell are ubiquitous, and their mathematical modeling is a growing field of research. This type of mathematical construction could be useful to better understand the dynamics of the fertilization membrane elevation induced by the  $Ca^{2+}$  wave. Protein kinases C (PKCs), multifunctional calmodulin-dependent protein kinases (CaMKs), and the phosphatase calcineurin

are present in the sea urchin egg (Roux et al. 2006). Now, a cross talk between simulation and experimentation should help us to predict which among these  $\text{Ca}^{2+}$  transducers represent potential candidates to link the  $\text{Ca}^{2+}$  wave to subsequent translational regulations and more general cellular events that occur at fertilization time in sea urchin.

The mTOR-signaling pathway plays a crucial role in the fine-tuning regulation of *cyclin B* mRNA translation following fertilization in sea urchin. Translatome analysis, by achieving polysome profiling in combination with high-throughput sequencing technologies (Chasse et al. 2017), should allow us to identify the mRNAs that are specifically translated under the mTOR pathway triggered by fertilization and consequently should help in establishing translational regulatory networks that regulate the early events of the sea urchin embryo. As is the case for modeling and analysis of gene regulatory networks (Karlebach and Shamir 2008), computational and mathematical methods should allow us accurate predictions of the behavior of the translational regulatory network following fertilization in sea urchin.

Finally, fine-scale spatial and temporal control of translation regulation could play a major role in relation to the cell cycle in general and with regard to spindle formation in particular (Mulner-Lorillon et al. 2017; Cormier 2017; Jansova et al. 2017; Susor et al. 2016). Taking advantages of the quasi-spherical shape and constant volume of the cell and the integration of knowledge about the translational machinery at different scales (molecular, structural, and intracellular compartments), the sea urchin egg system opens up a promising field of research for systems biology that focuses on translational regulation in relationships with cell cycle control.

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# Chapter 6

## Monosex in Aquaculture



Tomer Ventura

**Abstract** Monosex refers to the culture of either all-male or all-female populations, a sought after approach in aquaculture. This chapter reviews the advantages of monosex population culture and details the mechanisms to achieve it based on different modes of sex determination and sexual differentiation. A recent case study for an aquaculture biotechnology based on sexual manipulation in crustaceans serves in this chapter to identify the key elements for a successful application. This application which makes use of RNA interference with a key regulating hormone opens the pathway toward environmentally friendly applications in fish and additional aquacultured species. This chapter portrays the state of the art in sexual manipulations in aquacultured species, starting with vertebrate species, followed by the case study of the crustacean species and discussion on how the techniques used in this study are applicable for other species.

### 6.1 What Is Monosex?

Sexual dimorphism (or gonochorism, where two predominant forms exist with different characteristics between males and females) is a common phenomenon in many sexually reproducing animals. One of the most extreme cases of sexual dimorphism is evident in the triplewart seadevil anglerfish (*Cryptopsaras couesii*) where the mature female reaches up to 30 cm in length, while the parasitic male is as small as 1 cm, born with no digestive system and with an extraordinary sense of smell. The male anglerfish perceives the pheromones secreted by a reproductively active female as food and rushes to feed on her flesh and while doing so secretes enzymes which consume both him and part of the female, leaving only the testes intact, connected by anastomoses to the female's body (<http://www.fishbase.org/>

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[summary/3098](#)). If this species had been used in aquaculture production for human consumption, production of all-female populations would be orders of magnitudes more beneficial than mixed population culture. While sexual dimorphism is not as extreme in many commercially valuable species, there is still advantage for monosex culture of either all-male or all-female populations in many species, for different reasons, depending on the species and the market.

## 6.2 Why Monosex?

Monosex population culture is desired for several reasons. In most species there are sexually dimorphic patterns of favorable traits that are more accentuated in either males or females. In the more established poultry industry, for example, the production of all females for eggs and the production of all males for meat are desirable. So is the case with milk and meat in cows and bulls. In general, most fish species show dimorphic growth rates. Males grow faster in some species (like tilapia and catfish), while females grow faster in other species (like grass carp, several salmonids, and cyprinids). Monosex population culture of the faster growing gender therefore increases the production rate. When culturing one sex, the culture conditions are tailored to better suit the energetic demands of the cultured gender. Also, since there is no unwanted breeding, the genetic breeding program is managed in separate compartments and is more controlled. If unplanned reproduction was to occur in grow-out ponds, it would result in crowding and higher densities than intended and also lead to energy waste due to sexual activity on the expense of growth. The end product of monosex culture will therefore be more uniform as a consequence, a crucial marketing advantage. Introducing culture of advantageous exotic species is met with resistance or is not accepted in some countries due to the ecological risk (Ventura and Sagi 2012). One such example is Mozambique tilapia (*Oreochromis mossambicus*) that is an important aquacultured species worldwide, which invaded Queensland waterways and is considered there a noxious species. The ability to introduce one gender could mitigate the risk of leakage to natural waterways. Even if leakage should occur, it will not be viable for more than one generation. Last, theft of genetically improved lines is not optional when only one gender is marketed.

## 6.3 How to Generate Monosex?

Today monosex culture is being practiced in many fish species as well as in several invertebrate species either by manual segregation or, primarily by sex reversal, directly to the desired sex, or the other way around, through sex reversal to the undesired sex which will produce the desired sex in its progeny. While manual segregation requires the least technology, it is tedious, wasteful, and inefficient. Direct sex reversal requires administration of substantial amount of hormones to

skew the ratio in the population toward the desired sex. Indirect intervention is most beneficial and involves hormone administration to the parents, while the progeny product is not treated.

What prior knowledge is needed to generate monosex populations?

First, the genetic background needs to be clarified: Are male heterogametic with two non-similar sex chromosomes designated XY, or are they homogametic with two similar sex chromosomes designated ZZ (where females are the heterogametic sex with ZW sex chromosomes)? What is the preferred gender? The latter factor is not only species-related but also market-related, as for some species certain markets will prefer males and other markets will prefer females (Ventura and Sagi 2012). What are the molecular mechanisms of sex determination and sexual differentiation? These mechanisms vary between species of the same genus, making it hard to trace key effectors that can be manipulated. Still, there are key elements that are somewhat conserved across species that can be targeted. What is the timing for appearance of sex traits and what is the timing for successful sex reversal? Usually, the appearance of sexual traits marks the stage at which it is too late to intervene and enable a functional sex change. For this reason, sex markers are essential to discriminate between a successful sexually reversed individual and a false-identified one (Ventura et al. 2012).

## 6.4 Sex Determination and Sexual Differentiation

Sex determination and sexual differentiation are defined as two overlapping processes. These processes are usually governed by a master regulator that regulates a major effector that initiates an entire cascade of genetic network that leads to the onset of the characteristic phenotypes. Sex determination is set genotypically (with the exception of temperature or other environmental cues such as social structure that govern sex determination in some species), while sexual differentiation is the onset of the phenotypic changes which follow the effect of the sex determination process. These are overlapping processes which can be manipulated using the correct timing and tools, if available. Sex determination and sexual differentiation have evolved numerous times throughout evolution. For this reason many mechanisms are in place to reach similar outcomes in sexually reproducing species. In fact, sex is quite plastic in the animal kingdom with some species that start as males and then turn into females (protandry) or vice versa (protogyny). Additionally, some species are capable of self-fertilizing and have both testes and ovaries (hermaphrodites). The aquaculture-relevant fish species that relate to each form of sex determination were extensively described (Devlin and Nagahama 2002; Pandia 2012). To simplify the discussion into monosex applications, we will focus on genetic sex determination and species with two distinct sexes (gonochoristic).

### 6.4.1 Sex Determination: Heterogamecy

Sex determination starts right at the beginning with the fertilization. In case of a genetic sex-determining system (as opposed to environmental regulation), the sex chromosomes contributed by the gametes define the sex. In many aquacultured species, the sex of the progeny is defined by the Y or X sex chromosome carried by the sperm cell, like in humans. This means that males are the heterogametic sex (XY), while females are the homogametic sex (XX). In many other aquacultured species, sex of the progeny is defined by the W or Z sex chromosome carried by the egg. This means that females are the heterogametic sex (ZW), while males are the homogametic sex (ZZ). In many phyla it has been shown that species of the same genus could have either XX/XY or ZZ/ZW sex determination mechanism. For that reason manipulation of sex for monosex population culture is a case-by-case issue. In very few species, the sex determination mechanism has been studied to the level where it presents opportunities to manipulate sex very early during development, with the initiation of the sex determination cascade. This includes the conserved DM domain genes (*Dmrt*s), which are related to the insect gene *doublesex* (*dsx*) that are located on the sex chromosomes in a handful of species, anti-Müllerian hormone gene located on the Y chromosome (*Amhy*) in the Nile tilapia, *Oreochromis niloticus*, and sex-determining region on the Y chromosome (SRY) in mammals. These factors are evolving rapidly, making it hard to identify, and in many cases they include homologues that are not sex-linked (Sandra and Norma 2009).

### 6.4.2 Sexual Differentiation

Sexual differentiation is the process that leads from an undifferentiated zygote to the differences between males and females. The molecular basis for sexual differentiation is highly variable between species. As an example, sex-lethal (*Sxl*) acts upstream of the sexual differentiation pathway in one of the most studied invertebrate model species *Drosophila melanogaster*. Homologues for *Sxl* were identified in many additional dipteran insect species, in none of which does *Sxl* assume a sex-determining role (Suzuki et al. 2001). *Sxl* differs between males and females. While the male isoform is inactive, the female isoform is an active splice factor which leads to the expression of the female-specific *transformer* (*tra*, a splice factor as well), which then leads to alternatively spliced *doublesex* (*dsx*). While *tra* does not retain its sex-determining role even within the genus (Suzuki et al. 2001), *dsx* retains its sexual differentiation relevance across phyla. For example, the homologue gene in the nematode *Caenorhabditis elegans* (called *mab-3*) encodes a DM domain transcription factor that controls male development. Similarly, the vertebrate *Doublesex* and *mab-3* related transcription factor 1 (*Dmrt1*) is a DM domain-containing transcription factor that governs sexual differentiation in a wide array of species (Raymond et al. 1998). Downstream from these factors (that are

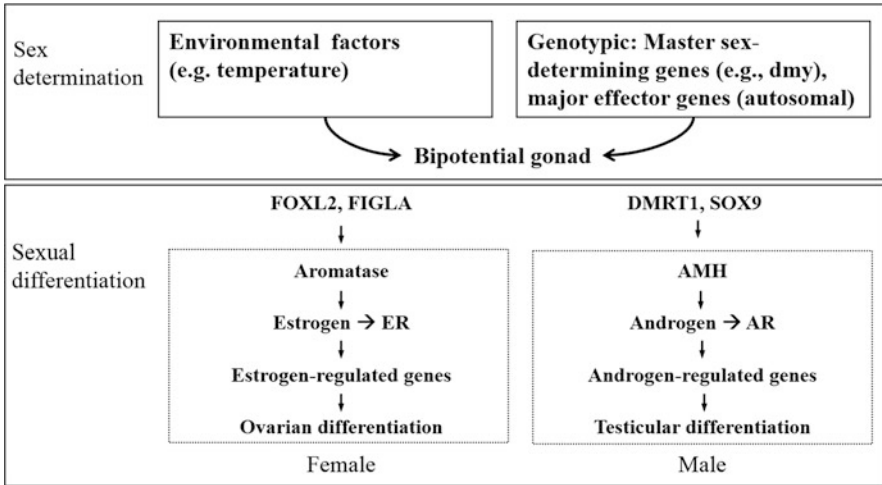
considered sex-determining factors that govern sexual differentiation), the sexual differentiation cascades converge between phyla and include several conserved factors. For example, *amh* and the sex steroid machinery (which includes the enzymes that metabolize the steroids, the steroid secretion system, and their receptors) are conserved in vertebrates (Sandra and Norma 2009).

## 6.5 Hormonal Regulation of Sex Differentiation in Vertebrates

Regulation of sex determination in several species is influenced by environmental factors such as temperature, but in most species it is regulated genotypically. In species where genetic regulation controls the gonad development, a master gene starts a chain of events leading to expression of different genes at different levels and timing, causing the differentiation of the gonad (Pandia 2012). The region where the gonad develops in mammals and cartilaginous fish is called the urogenital ridge as it is a region that gives rise to both the gonads and the urinary system. Initially the gonad is undifferentiated and is “bipotential,” in the sense that given the right cue, it can develop either way. The bipotential gonad has two sets of ducts named the Müllerian ducts that can develop into oviducts and Wolffian ducts that can develop into sperm ducts. At a certain stage, the gonad cells start proliferating and secrete anti-Müllerian hormone (Amh) and testosterone and express *Dmrt1* and the transcription factor *Sox-9* (which promotes Amh expression), leading to testis differentiation. When the signal is not strong enough, an array of other signals will later be expressed following meiosis, leading to the expression of aromatase which converts testosterone to estradiol and the outcome of such event will therefore be differentiation into an ovary (Sandra and Norma 2009; Pandia 2012). In teleost fish, the bipotential gonad does not include two sets of ducts, but one set of ducts (Wolffian) that can develop either way, depending on the presence of the genetic factors described above. Since there are no Müllerian ducts in the developing gonads of teleost fish, it is not clear what the role of Amh is. This is a generalized description of the process (depicted in Fig. 6.1) that has many species-specific variations (Pandia 2012).

The active Amh is a large glycoprotein homodimer of 140 kDa, stabilized by disulfide bridges. While encoded by an autosomal gene in most studied vertebrates, it is encoded by a gene that is located on the Y chromosome in the Nile tilapia (*Amhy*). Amh, also called Müllerian-inhibiting substance (Mis), is a glycoprotein secreted by the Sertoli cells of the fetus (Munsterberg and Lovell-Badge 1991). Amh can be considered as a safe target to use for sex reversal, as it contains species-specific sequences. Testosterone, on the other hand, is produced through steroidogenesis and is generic for many vertebrate species. The use of testosterone or its feminine counterpart estradiol or derivatives thereof is thus environmentally unsafe.

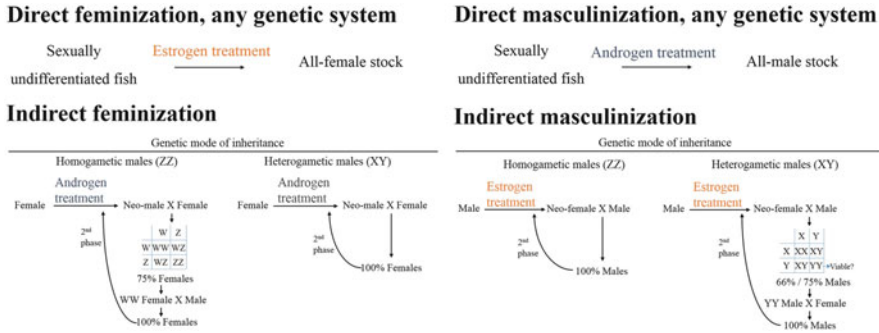




**Fig. 6.1** The key steps in sex determination and sexual differentiation in vertebrates. Sex determination starts with an environmental factor or a genetic factor. In the case of a genetic factor, a master sex-determining gene is defined as the gene that resides on the sex chromosome and initiates the cascade. The major effector gene can be autosomal, and it is the key factor that leads to the translation of the genetic constituent into a sexual differentiation cascade. At the bipotential gonad, different factors are expressed between males and females, leading to sexual differentiation. In females (left), transcription factors like forkhead box protein L2 (FOXL2) and folliculogenesis-specific basic helix-loop-helix protein (FIGLA) express early during ovarian development and are important for its function. In males (right), transcription factors like DMRT1 and SOX9 are important for initiating the male-specific cascade including AMH and biosynthesis and secretion of androgens that bind to their receptors (AR) to initiate a cascade of androgen-regulated genes. In females, aromatase converts the androgens to estrogens which bind to their receptors (ER) and initiate a cascade of estrogen-regulated genes

### 6.5.1 Steroidogenesis

Steroidogenesis is a continuous process of converting the precursor molecule cholesterol into an array of steroid hormones by various enzymes that metabolize it. Steroidogenesis is taking place both in males and females in the gonad as well as additional tissues. Different enzymes convert cholesterol to derivatives known as progestogens followed by a set of enzymatic reactions which convert them to androgens, which is followed by yet another set of enzymes which convert androgens to estrogens. The process is species-specific and includes various androgens and estrogens in different species. Androgens regulate masculinity, while estrogens regulate femininity. It is therefore not surprising that one key enzyme in the sex differentiation process in vertebrates is the aromatase which converts the androgenic hormone testosterone to the estrogenic hormone estradiol (Bao and Garverick 1998). Aromatase is used as a sex marker, since it is expressed in much higher levels in females and can be found in peripheral tissues. Still, it is not as reliable as genetic



**Fig. 6.2** Direct (top) and indirect (bottom) sex reversal from male to female (left) or female to male (right) using androgens and estrogens in vertebrate aquacultured species. While direct manipulation does not require prior knowledge of the genetic mode of inheritance, it affects the indirect approach

DNA markers, and the search for these is still ongoing for many aquaculture important species.

## 6.6 Sex Reversal Induction in Vertebrates

In order to produce monosex populations in vertebrate aquacultured species, usually synthetic estrogens or androgens are administered to induce sex reversal. This method is not environmentally friendly since these compounds end up in waterways (Megbowon and Mojekwu 2014). In several species temperature manipulation proves useful to some extent, although it does not hold for most species. Feminization can be induced directly through administration of estradiol or indirectly through administration of androgens which results in all females through either WW or XX neo-males (functional males that carry a female genetic load). The other way around, masculinization can be induced directly through administration of androgens or indirectly through administration of estrogens which result in either ZZ or YY neo-females (Budd et al. 2015). The options for direct or indirect routes of sexual manipulations, considering the genetic mode of inheritance are depicted in Fig. 6.2. For direct manipulation, prior knowledge of the genetic mode of inheritance is not required, and it is thus simpler. The downside is that this mode is more costly and less ecologically beneficial, since all individuals in the population must be treated, while in the indirect manipulation route, only the brood stock needs to be treated. Indirect feminization is simpler in XX/XY species, as it leads directly to all-female populations, due to eliminating the Y chromosome from the lineage. In ZZ/ZW systems, on the other hand, feminization is more complex as neo-males (ZW) are mated with females (ZW), and only following a second generation, one can isolate the WW females that enable all-female population. Indirect masculinization is simpler in ZZ/ZW species as neo-females are (ZZ), and crossing them with males (ZZ) results in all-male population. In XY/XX species, indirect masculinization is

more complex as neo-females are XY and crossed with XY males. Then the ratio of males in the population through such a cross will be either about 75% or 66.7%, depending on whether YY individuals are viable or not. If they are, the 75% males are expected. Then, these Y males can be isolated and crossed with females to produce all-male populations in the second generation. In order to eliminate the X chromosome from the lineage, one must keep performing indirect feminization on these YY males.

To summarize, the direct route for gaining monosex populations of aquacultured vertebrates is simpler but less eco-friendly. In rainbow trout direct feminization is used since females reach sexual maturity later than males, grow faster, and have superior flesh quality. Yet, there is an issue with incomplete sex reversal that in some cases generates hermaphrodites with ovotestes. Direct masculinization through androgen administration is practiced in Nile tilapia due to faster growth of males in this species. Grass carp is being indirectly feminized as females grow faster in this species. Instead of using feed pellet that includes androgens, the technique used to induce sex reversal in grass carp that does not eat artificial feed is implanting slow release implants with the androgens. Indirect feminization is practiced in salmon due to faster growth rates of the females.

## **6.7 The Case of *Macrobrachium rosenbergii*: The Commercially Most Important Freshwater Prawn**

The global aquaculture trend over the past few decades shows that while capture fisheries are maintained at relatively the same level, aquaculture keeps on growing, dictating the need for better aquaculture techniques to keep up with the growing market demands. In the giant freshwater prawn *Macrobrachium rosenbergii*, this trend is much steeper, necessitating a more immediate solution for improved aquaculture techniques. *M. rosenbergii* males grow faster than females and reach higher weights at harvest. With that, they show a hierarchical population structure and are thus more spread across various sizes as compared with females that grow more uniformly. This species is thus an example for how either all-male or all-female populations are preferable compared with mixed populations, depending on the market demands (Cohen et al. 1988). It was shown that selective harvest of either all-male or all-female population gains higher yields, pointing to the economic advantages of prawn monosex aquaculture. Manual segregation is a common procedure in crustacean aquaculture to separate males and females into separate ponds. Although profitable, this is a labor-intensive, tedious process (Nair et al. 2006). A biotechnological approach has been thus developed, harnessing knowledge of the sex determination and sexual differentiation mechanisms. In crustaceans, sexual differentiation toward maleness is dictated by the circulating androgenic gland hormone, secreted by the male-specific androgenic gland (AG) (Ventura et al. 2011b). This gland governs masculinity development and maintenance. In

*M. rosenbergii* mating normally yields 1:1 sex ratio. Upon AG removal from males at a very early developmental stage, a full and functional sex reversal is achievable from males into neo-females (Sagi and Cohen 1990). Since *M. rosenbergii* males carry two homologous sex chromosomes, designated ZZ, the progeny obtained by crossing neo-females with males is 100% males. This process takes a long time, requires skilled surgeons, and results with very low success rates. Similarly, grafting of AG tissue or cells into females at a very early developmental stage results with neo-males that can generate all-female populations (Malecha et al. 1992). In both cases the success rates were extremely low, up until recently when genetic sex markers were identified (Ventura et al. 2011a).

The significance of the sex markers lies in the fact that for a successful intervention, the manipulation must be done in early developmental stages, prior to the emergence of sexual characteristics (Ventura et al. 2012). This means that 50% of the treated individuals are males and 50% are females. Following manipulation, for example, by AG cells administration, we expect more than half to develop as males. That means that 50% are males and the rest are neo-males. How can we tell them apart? Without sex markers we will have to grow all treated individuals until we can see if they develop into males or females, then discard unsuccessful attempts (in the case of indirect feminization, the females, as we would like to produce neo-males), mate the suspected neo-males with females (a resource demanding process that could take up to a year), grow the progeny of each and every individual cross independently until they show sexual characteristics (up to another 6 months later) and based on the ratio of females to males, decide if the progenitor is indeed a neo-male (bearing ZW sex chromosomes) or a male (ZZ). Using the recently identified sex markers, the need for the tedious validation is alleviated. All that is required is a small piece of tissue from each manipulated individual and within a day all neo-males can be separated with high degree of accuracy.

Using a cDNA library of the AG, an insulin-like encoding gene which is expressed specifically in the AG was identified, termed *Mr-IAG* (Ventura et al. 2009). Like other insulin-like peptides, mature *Mr-IAG* is hypothesized to comprise a B chain linked by two disulfide bridges to an A chain where another disulfide bridge occurs. The time when *Mr-IAG* expression initiates is prior to development of any sexual characteristics, establishing the correct timing for successful sexual manipulation (Ventura et al. 2009). Repetitive injections of dsRNA of *Mr-IAG* into juvenile male prawns resulted with RNA interference (RNAi) and knockdown of *Mr-IAG*, giving rise to a full and functional sex reversal into neo-females (Ventura et al. 2012).

## 6.8 Sex Reversal Induction in Other Crustaceans

Decapoda are the order of ten-legged crustaceans that comprise the aquaculture-relevant species like crabs, crayfishes, lobsters, prawns, and shrimps. Over the past decade, the IAG was identified in more than 20 decapod species, covering

representatives of all major cultured groups (Ventura et al. 2011b, 2014). Attempts to establish all-male or all-female populations in additional crustacean species are ongoing for several commercially important species. The attempts rely on genetic sex markers where available, for a handful of species, and also the IAG, or the AG itself. Additional technologies might include recombinantly produced IAG or a nucleotide vector that will express it. In some cases, the research has led to novel findings, as in the Eastern spiny lobster *Sagmariasus verreauxi*, where novel insulins were identified (Chandler et al. 2015) as well as genetic sex markers that are also the master sex-determining genes.

## 6.9 Concluding Remarks

In summary, IAG is considered to be the key regulator of sexual differentiation in crustaceans. The case study of the giant freshwater prawn serves as a model for sex reversal through AG manipulation or IAG gene silencing. These methodologies are environmentally friendly since the intervention is species-specific, and unlike the practice common in vertebrate species, it does not include hormonal treatment that might affect other organisms. The sexual steroids are not available in crustaceans, a fact that was driving the research to the key sexual differentiating insulin-like hormone, which has led to a safe approach for sexual manipulation that can be translated to similar approaches in vertebrates. As an example, some of the key vertebrate factors highlighted in this chapter, like *amh* and *Dmrt1*, could be utilized in a species-specific way to develop novel techniques for monosex population aquaculture biotechnologies.

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**Part II**  
**Embryonic and Post-embryonic**  
**Development, and the Evolution of the Body**  
**Plan**

## Chapter 7

# Medusa: A Review of an Ancient Cnidarian Body Form



Cheryl Lewis Ames

**Abstract** Medusae (aka jellyfish) have multiphasic life cycles and a propensity to adapt to, and proliferate in, a plethora of aquatic habitats, connecting them to a number of ecological and societal issues. Now, in the midst of the genomics era, affordable next-generation sequencing (NGS) platforms coupled with publically available bioinformatics tools present the much-anticipated opportunity to explore medusa taxa as potential model systems. Genome-wide studies of medusae would provide a remarkable opportunity to address long-standing questions related to the biology, physiology, and nervous system of some of the earliest pelagic animals. Furthermore, medusae have become key targets in the exploration of marine natural products, in the development of marine biomarkers, and for their application to the biomedical and robotics fields. Presented here is a synopsis of the current state of medusa research, highlighting insights provided by multi-omics studies, as well as existing knowledge gaps, calling upon the scientific community to adopt a number of medusa taxa as model systems in forthcoming research endeavors.

## 7.1 Medusozoa: Emergence of the Medusa Body Form

### 7.1.1 *Appeal of the Medusa*

The medusa body form evolved exclusively in Medusozoa (phylum Cnidaria). Taxa bearing this ancient, ingenious body form offer promise as appealing model marine invertebrate systems. The framework behind our current understanding of the medusa (aka jellyfish) is the culmination of morphological and physiological studies conducted over the past two and a half centuries since Linnaeus' *Systema Naturae* (1758). More recently, genetic tools have facilitated improvements in medusozoan

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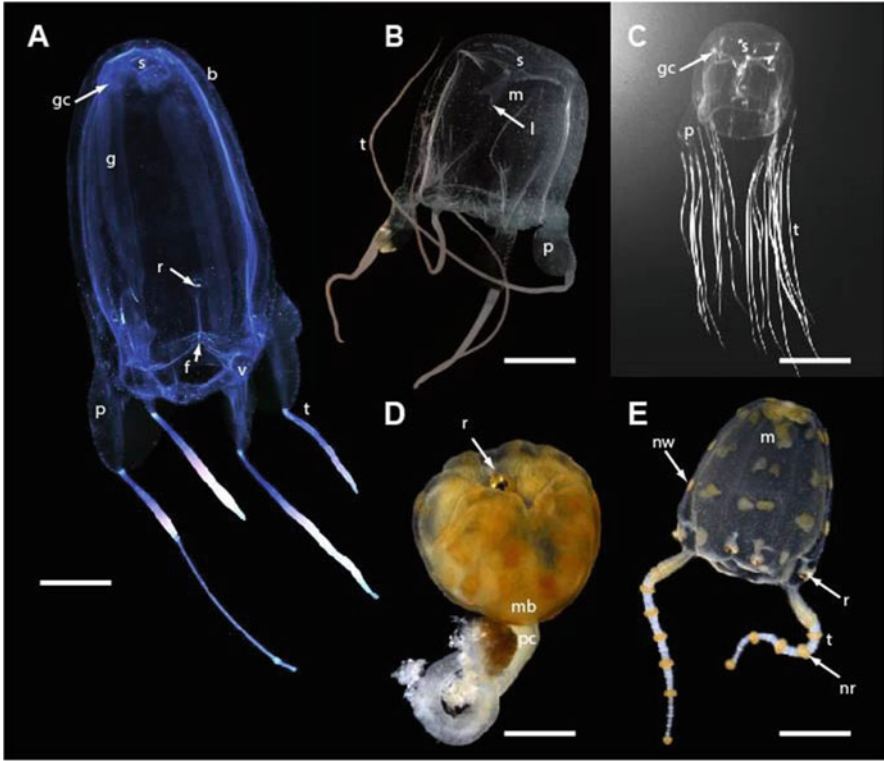
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species delineation and phylogenetic estimates and a better understanding of jellyfish biogeographical distribution. Given their multiphasic life cycles and propensity to adapt to, and proliferate in, a plethora of aquatic habitats, jellyfish are implicated in a number of ecological and societal issues. Now, in the midst of the genomics era, affordable next-generation sequencing (NGS) platforms coupled with publically available bioinformatics tools present the much-anticipated opportunity to explore medusa species as potential model systems. Genome-wide studies of medusae would provide a remarkable opportunity to address long-standing questions related to the biology, physiology, and nervous system of some of the earliest pelagic animals. Furthermore, medusae have become key targets in the exploration of marine natural products, in the development of marine biomarkers, and for their potential applications to the biomedical and robotics fields. Presented here is a synopsis of the current state of medusa research, highlighting insights provided by multi-omics studies, as well as knowledge gaps, calling upon the scientific community to adopt a number of medusa taxa as model systems in forthcoming research projects.

### ***7.1.2 Systematics and Evolutionary Position***

Medusozoa is a monophyletic clade within Cnidaria Verrill 1865, a predominantly marine phylum (~13,000 species rich) with an evolutionary position sister to Bilateria (Ryan et al. 2013; Zapata et al. 2015; Collins 2002, 2009; Marques and Collins 2004; Collins et al. 2006; Daly et al. 2007). Medusozoa contains four major classes Scyphozoa Goette, 1887 (true jellyfish); Cubozoa Werner, 1973 (box jellyfish); Staurozoa Collins and Marques, 2004 (stalked jellyfish); and Hydrozoa Owen, 1843 (hydroids, hydromedusae, and siphonophores) (Collins 2009) (but see Straehler-Pohl 2017) organized into two monophyletic clades: Hydrozoa (Trachylina plus Hydroidolina) and Acraspeda (Staurozoa plus Cubozoa and Scyphozoa) (see Gegenbaur 1856; Haeckel 1880). Molecular analyses, including recent phylogenomic reconstructions (albeit with a limited number of medusa species), have recovered strong support for a single origin of the medusa body form in Medusozoa (Zapata et al. 2015; Kraus et al. 2015), with a complete loss of medusa occurring in Staurozoa (see below) and multiple losses within Hydrozoa (Cartwright and Nawrocki 2010). All species of Cubozoa (~50 species in two major lineages Carybdeida and Chirodropida) (Fig. 7.1) and Scyphozoa (~250 species in two major lineages Coronatae and Discomedusae (i.e., Semaestomeae plus Rhizostomeae) (Fig. 7.2) display a life history that involves the metamorphosis of a microscopic planula into a sexual medusa, after going through a sessile polyp stage in most taxa (Collins 2009; Zapata et al. 2015; Ceh et al. 2015; Yamamori et al. 2017). Conversely, Staurozoa (~50 species) taxa remain as “stalked jellyfish,” never becoming motile medusae. Hydrozoa (Fig. 7.3) is composed of two major monophyletic clades: Trachylina and Hydroidolina (Cartwright and Nawrocki 2010; Zapata et al. 2015; Bouillon et al. 2006). As the most species-rich (~3700 species) and diverse medusozoan class, Hydrozoa has traditionally been split into four main lineages—Anthomedusae, Leptomedusae, Linnomedusae, Narcomedusae, and

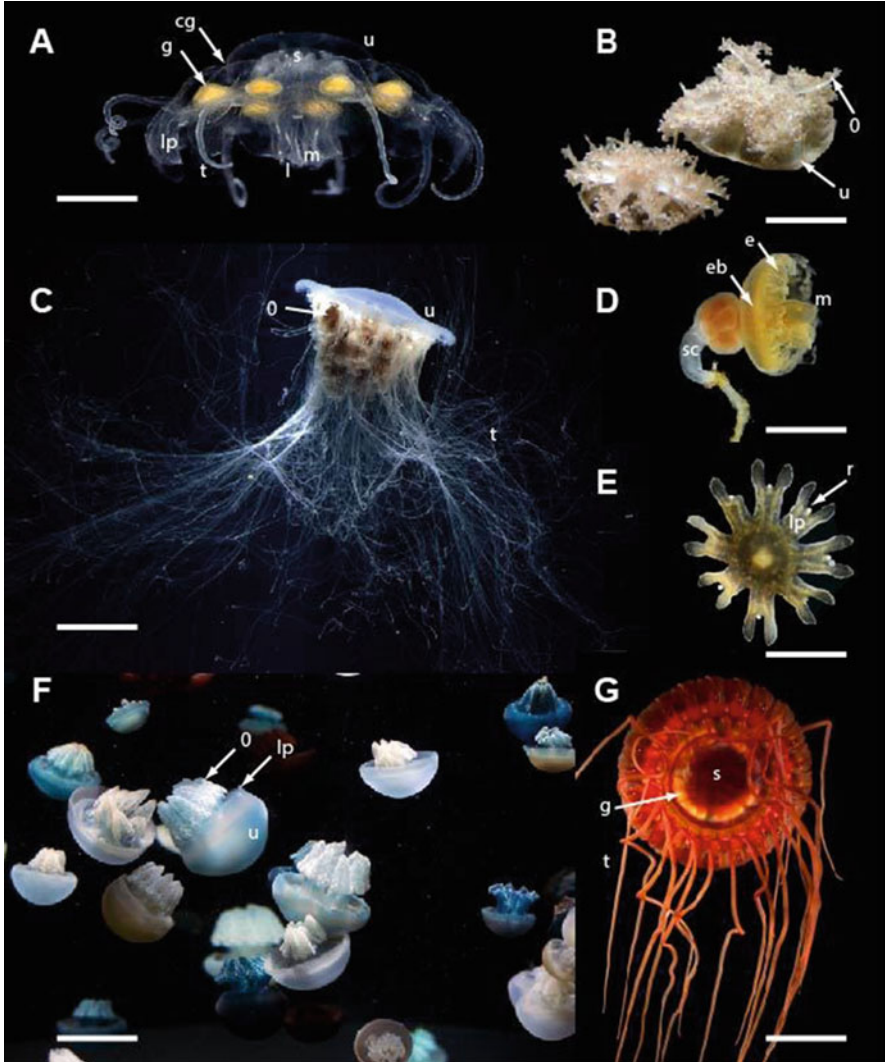


**Fig. 7.1** Cubomedusae. (A) *Alatina alata*. (B) *Carybdea brevipedalia*. (C) *Chironex fleckeri*. (D). Cubopolyp (unidentified) releasing a medusa bud apically. (E) Newly liberated cubomedusae (from C) Abbreviations: *b* bell, *f* frenalium, *g* gonads, *l* lips, *m* manubrium, *mb* medusa bud, *nr* nematocyst ring, *nw* nematocyst wart, *p* pedalium, *pc* polyp column, *r* rhopalium, *s* stomach, *t* tentacle, *v* velarium. Scale bars: 2.5 cm (A and B), 7 cm (C), 1 mm (D and E)

Trachymedusae—but not all are monophyletic (Collins et al. 2008; Collins 2009; Zapata et al. 2015). However, recent phylogenomic reconstruction resolves Trachylina as sister to Hydroidolina which consists of Leptothecata, Filifera (paraphyletic), Aplanulata, Capitata and Siphonophora (Zapata et al. 2015). Despite this extensive biodiversity seen in Hydrozoa, only about 850 species are estimated to produce a medusa stage (Gibbons et al. 2010). The exact number of hydrozoan species with a medusa is not clearly defined, as it is currently infeasible to rear all taxa under laboratory conditions to validate the presence or absence of a medusa in the life cycle.

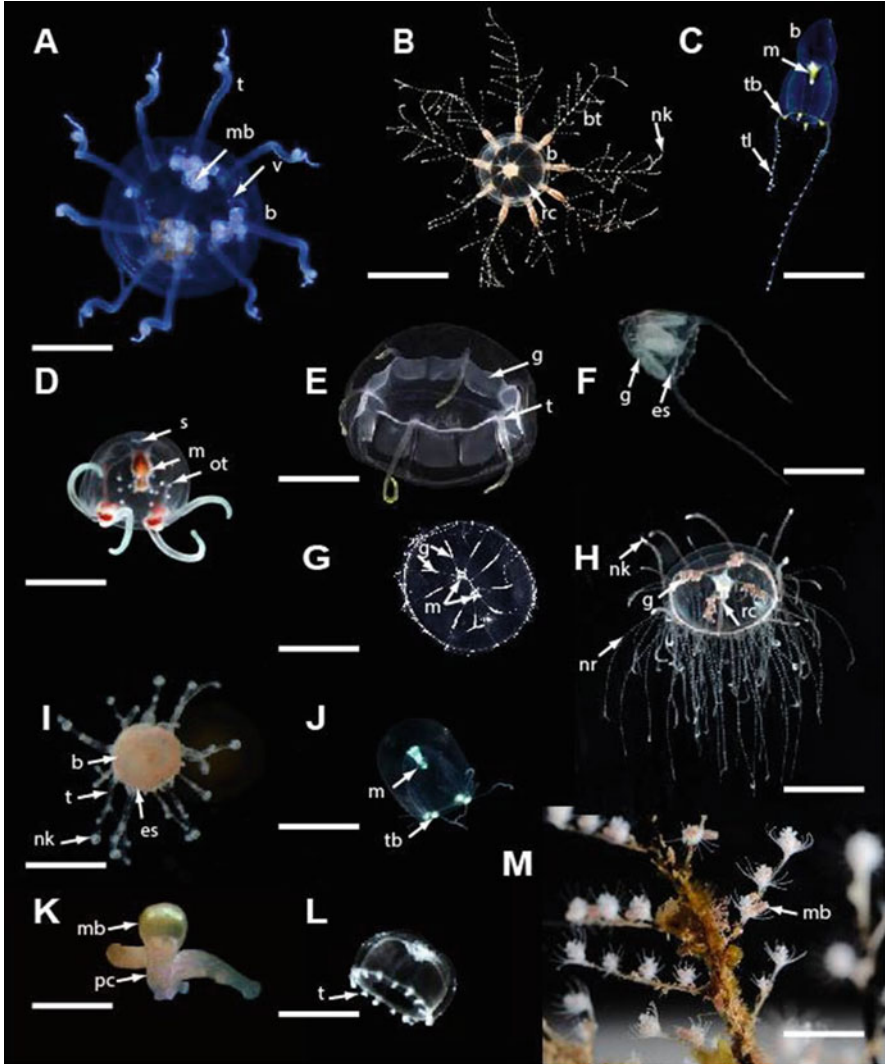
### 7.1.3 Defining Medusa

In this work, the term medusa (plural medusae) designates the body form or development stage corresponding solely to the free-swimming juvenile or sexual



**Fig. 7.2** Scyphomedusae. (A) *Nausithoe* sp. (B) *Cassiopea andromeda*. (C) *Cyanea capillata*. (D) *Cassiopea andromeda*, strobila releasing an ephyra bud apically. (E) *Cephea* sp. ephyra. (F) *Catostylus mosaicus*. (G) *Atolla wyvillei* (oral view). Abbreviations: cg coronal groove, e ephyra, eb ephyra bud, g gonads, l lips, lp lappets, m manubrium, o oral arms, r rhopalium, s stomach, sc strobila column, t tentacle, u umbrella. Scale bars: 1 mm (A), 5 cm (B and C), 3 mm (D and E), 8 cm (F), 3 cm (G)

stage present in some medusozoan taxa. Taxa excluded from the definition of medusa herein are stauromedusae (stalked jellyfish); hydrozoans of the orders Siphonophorae (including *Physalia*) and pelagic members of Capitata (*Vellela* and *Porpita*) which are colonies made up of specialized polypoid and medusoid forms functioning as an individual unit floating or hydrostatically propelling in the water



**Fig. 7.3** Hydromedusae. (A) *Lizzia blondina* (oral view) with medusae budding from radial canals. (B) *Cladonema radiatum* (aboral view) with branching tentacles and terminal nematocyst knobs. (C) *Zancleopsis* sp. with fluorescent manubrium and two tentacles bearing tentilia. (D) *Podocoryna* sp. with pigmented tentacle bulbs and manubrium, bearing oral tentacles. (E) *Pseudaegina rhodina* with scalloped bell and gonads and tentacles originating on the exumbrella. (F) *Amphinema* sp. with fluorescent gonads and two tentacles. (G) *Sugiura* sp. (oral view) exhibiting manubrium and radial canal supernumerary. (H) *Vallentinia gabriellae* with two types of marginal tentacles ending in adhesive nematocyst knobs. (I) *Eleutheria* sp. (aboral view) crawling on the substrate, branched tentacles ending in adhesive nematocyst knobs. (J) *Bougainvillia* sp. with fluorescent tentacle bulbs and manubrium, bearing oral tentacles. (K) *Craspedacusta sowerbii* polyp with lateral medusa bud. (L) *Craspedacusta sowerbii* newly released medusa. (M) *Pennaria* sp. hydroid releasing medusa buds. Abbreviations: *bt* branching tentacle, *es* eye spot (ocelli), *g* gonads, *lp* lappets, *m* manubrium, *mb* medusa bud, *nr* nematocyst ring, *nk* nematocyst knob, *ot* oral tentacles, *pc* polyp column, *rc* radial canal, *t* tentacle, *tb* tentacle bulb, *tl* tentilia, *u* umbrella, *v* velum. Scale bars: 2 mm (A, B, D, E, G, K, L), 5 mm (C, F, H, I, J, M)

column; Ceriantharia (Anthozoa) taxa exhibiting a medusoid form during early development (and sometimes into maturity) that is analogous to a true medusa (Stampar et al. 2015); and any other gelatinous zooplankton.

## 7.2 Evolution of an Innovative Bauplan

The first medusa experts believed jellyfish lacked nerves, an anus, and a vascular system and were incapable of independent muscle contraction, respiration, or internal digestion. This section provides an overview of key morphological attributes of medusae observed in different medusozoan lineages stemming from more than 260 years of well-documented research on these remarkable gelatinous animals (Collins 2009; Bentlage et al. 2010; Daly et al. 2007; Zapata et al. 2015; Cartwright and Nawrocki 2010; Boero et al. 2008; Arai 1997; Lamarck 1801; Garcia Rodriguez et al. 2018; Cuvier 1830; Lesson 1843; Agassiz 1862; Linnaeus 1758; Wrobel and Mills 1998; Schiariti et al. 2012; Thiel 1936; Gibbons et al. 2010; Bouillon et al. 2006).

The discussion below highlights the diversity and complexity of the medusa bauplan, despite its diploblastic arrangement and lack of brain. The list below is not meant to be exhaustive, given the extensive diversity of form and function that remains to be described in jellyfish and because exceptions to the rule are often the norm when working with medusae.

Currently, the underlying molecular and cellular interactions governing the diversity of medusa morphological and physiological traits are not well known. However, whole genome studies on sessile cnidarians (i.e., *Hydra* and *Nematostella*) indicate that their molecular makeup consists of many elements shared with bilaterians, such as transcription factors regulating mesoderm and nervous system development, homeobox genes controlling the formation of body parts during early embryonic development, the ability to control developmental regulatory genes via distal enhancer elements, and epigenetic modification of DNA and chromatin (Technau and Schwaiger 2015). Future genomics studies on medusa taxa will provide an understanding of how these conserved molecular components are involved in structuring the medusa bauplan and how they regulate complex and coordinated behavior in the motile medusa form.

### 7.2.1 *Medusa Locomotion*

#### **Umbrella/Bell**

A medusa is a solitary, free-swimming life stage consisting of a radially symmetric convex gelatinous **umbrella** or **bell** (the latter term used mainly, but not exclusively, for cubomedusae), composed of 98% water and salt encased in a colloidal, matrix containing very little carbon (Acuña et al. 2011) that contracts and dilates

facilitating locomotion (Knight 1866). The medusa body is diploblastic—consisting of endoderm and ectoderm—separated by mesoglea, a variably thick layer that reinforces medusa structure. All cubomedusae have a cuboid bell (Fig. 7.1), the width of which varies in mature medusae from 4 mm (*Copula sivickisi*) to 30 cm (*Chironex fleckeri*); body parts (e.g., tentacles, gonads) are present in fours (i.e., tetramerous organization). Locomotion in cubomedusae is achieved by contraction of the **velarium** (marginal bell turnover) (Fig. 7.1A), propelling the medusa during swimming (Colin et al. 2013). The velarium is secured by four buttresses called **frenula** (singular frenulum) (Fig. 7.1A) positioned at right angles with the subumbrella wall and containing a series of **velarial canals**. Scyphomedusae (Fig. 7.2) and hydromedusae (Fig. 7.3) vary widely in bell shape and number of serial body parts; synapomorphies have traditionally served as useful classification clues. In hydromedusae (except *Obelia*) propulsion is achieved by the **velum** (Fig. 7.3A)—a thin muscular flap of tissue on the umbrella margin that projects inward, partially occluding the subumbrellar opening. Scyphomedusae have neither a velarium nor a velum but facilitate locomotion by other structures—coronal groove (Fig. 7.2A), scalloped margin (Fig. 7.2A, C, G), or mushroom-shaped umbrella (Fig. 7.2F). Scyphomedusae are among the largest medusae, with adults varying in **umbrella** width from 25 mm (*Linuche unguiculata*) to 2 m (*Cyanea capillata*) (Fig. 7.2C), while hydromedusae are often microscopic but vary in bell width from less than 2 mm (*Cladonema radiatum*) (Fig. 7.3B) to 25 cm (*Aequorea macrodactyla*).

### Medusa Muscle

Medusae display a broad range of muscle patterning and organization, permitting specialized functions at different life stages vital to locomotion, defense, and feeding and digestion (for a review of cnidarian muscles, see Leclère and Röttinger 2017; Seipel and Schmid 2005; Satterlie et al. 2005). As cnidarians, medusozoan muscles are mainly composed of specialized epithelial cells with smooth myofilaments (epitheliomuscular cells), constituting the principal building block of the endoderm and ectoderm layers. Muscle patterning and mechanisms of contraction differ across medusozoan clades (Seipel and Schmid 2005; Satterlie et al. 2005; Azuma et al. 1986). An interesting innovation is the ability for certain deep-sea coronate scyphomedusae (e.g., *Periphylla periphylla*), which inhabit extreme oxygen minimum layer ecosystems, to scale metabolic enzyme activities in the swimming muscle and tentacle tissue based on oxygen availability (Thuesen et al. 2005). The phylogenetic position of Cnidaria and the novel muscular pattern of the motile medusa umbrella make jellyfish a nice model for studying muscle evolution (for discussion on cnidarian smooth and striated muscles, see Seipel and Schmid 2005; Sanders et al. 2014; Leclère and Röttinger 2017).

### Symmetrization, Regeneration, and Transdifferentiation

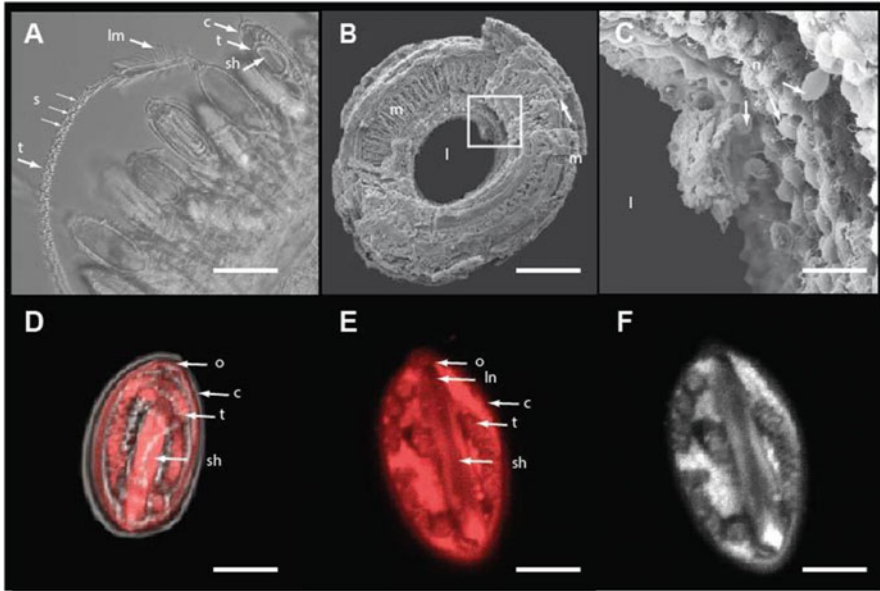
Regeneration (i.e., the ability to heal a wound or regain a lost body part) is an important trait for animal survivorship. Cnidarian polyps have amazing regenerative ability, but true regeneration of amputated body parts has yet to be definitively documented in medusae. In fact, what was previously perceived as body part regeneration in scyphomedusae is now properly attributed to the unique self-repair

process called “symmetrization”: coordination of the medusa musculature to reorganize body parts following an amputation event to restore essential global radial symmetry (see Abrams and Goentoro 2016). Probably the most famous case of so-called medusa regeneration is in the hydromedusa *Turritopsis dohrnii* which reverts back and forth between medusa and polyp stage, earning it the common name “immortal jellyfish” and the focus of anti-aging research (Miglietta et al. 2006). Until recently, medusa life cycle reversal was considered rare, but the scyphomedusa *Aurelia* can also revert back to its polyp stage, triggered by particular cellular processes (e.g., apoptosis and/or interstitial cell proliferation), suggesting medusa transdifferentiation may be more common than previously thought (Piraino et al. 2004; He et al. 2015). Ontogeny reversal may benefit the jellyfish under extreme environmental conditions, facilitating broad dispersal (e.g., in ballast water) to habitats outside the native geographic range (Pia Miglietta and Harilaos 2009). Recently, in vivo techniques were used to characterize epithelial wound healing in medusae for the first time by imaging tiny live *Clytia hemisphaerica* hydromedusae (Kamran et al. 2017). Findings revealed that both cell crawling (over the wound) and purse string (actin cable contraction) mechanisms were involved, suggesting an ancient evolutionary origin (>600 mya) of wound healing, prior to the divergence of Cnidaria. Given that many structural and regulatory genes are shared between cnidarians and vertebrates (Kraus et al. 2015; Technau and Steele 2011; Technau and Schwaiger 2015), continued efforts to uncover fundamental cellular interactions and molecular signaling pathways in medusa repair should prove relevant to studying wound healing and regeneration in other animal systems (for a detailed discussion, see Kamran et al. 2017).

## 7.2.2 Medusa Feeding and Digestion

### Lures and Filaments

**Tentacles** are long thin, usually high contractile, structures primarily used in prey capture, feeding, and defense (and sometimes copulation (Lewis and Long 2005)). Medusa tentacles are composed of both circular and longitudinal muscle fibers, bearing rings of **nematocyst** (Figs. 7.1E and 7.4) arranged around a longitudinal canal that runs the length of the tentacle lumen (Fig. 7.4B, C) or **nematocyst clusters**. The number of tentacles varies broadly among scyphomedusae (though some lack tentacles (Fig. 7.2B, F)) and hydromedusan taxa but is more conserved in cubomedusae which bear tentacles exclusively at each of the four corners of the bell extending from a wing-like structure called a **pedalium** (Fig. 7.1A–C). Carybdeida cubomedusae (Fig. 7.1A, B) possess a single tentacle per pedalium (with variation seen in *Tripedalia binata* and *T. cystophora*), while Chirodropida cubomedusae can possess up to 15 tentacles per pedalium (*Chironex* spp.) (Fig. 7.1C). In contrast, some hydromedusae have swollen tentacle bases at the bell margin called **tentacle bulbs** (*Bougainvillia* sp.) (Fig. 7.3C, D, J). Recent gene expression studies suggest that nematocyst development occurs within cubomedusae (*Alatina alata*) pedalia (Fig. 7.1A) (Lewis Ames et al. 2016; Lewis Ames and Macrander 2016) and



**Fig. 7.4** Cubomedusae tentacle nematocysts. (A) Intact and discharged rod-shaped, microbasic p-mastigophores in partially homogenized tentacle tissue of *Malo filipina* (light microscopy). (B) *Copula sivickisi* tentacle cross section showing nematocysts lining lumen wall (environmental SEM). (C) Magnification of boxed section in B showing nematocysts lining lumen wall. (D and E) Intact heterotrichous microbasic eurytele isolated from tentacle of *Alatina alata* (confocal microscopy, fluorescent rhodamine g staining), (F) *A. alata* (digitally desaturated version of E). Abbreviations: *c* capsule, *l* lumen, *lm* lamellae, *ln* lancet, *m* muscle, *n* nematocyst, *o* operculum, *s* spine, *sh* tubule shaft, *t* tubule. Scale bars: 30  $\mu\text{m}$  (A), 200  $\mu\text{m}$  (B), 20  $\mu\text{m}$  (C), 10  $\mu\text{m}$  (D–F)

hydromedusa tentacle bulbs (Houlston et al. 2010) and that mature nematocysts populate the tentacles via migration into the tentacle lumen (Fig. 7.4A–C).

### Digestive Tract

The medusa **gastrovascular cavity** (or gastric cavity) is a sac (often lined with cilia) into which food is passed after ingestion via the **manubrium** (feeding tube) (Figs. 7.1B, 7.2A, D, and 7.3C, D, G, H, J) and primarily where digestion occurs. It consists of the **gastric peduncle**—a gelatinous bulge protruding into the roof of the subumbrella that bears the stomach and/or manubrium. In cubomedusae and scyphomedusae, the **stomach** (Figs. 7.1A–C and 7.2A, G) is further divided into subcomponents forming four **gastric pockets (ostia)** or multiple **stomach pouches**, respectively, which extend into the sides of the bell. In contrast, the gastrovascular cavity of hydromedusae consists of a simple sac, lacking divisions (Fig. 7.3D). The **manubrium** is a pendant prolongation of the gastrovascular cavity that extends into the subumbrella (Figs. 7.1B, 7.2A, D, and 7.3C, D, G, H, J), found in most medusae, except rhizostome (Fig. 7.2B, F) and some semaestome scyphomedusae (Fig. 7.2C). The manubrium ends with a **mouth**, often bearing lips (Figs. 7.1B and 7.2A), that opens within the subumbrella to ingest prey items present in the water



column or that are passed to the mouth by the marginal tentacles. Alternatively, many semaestome medusae have four or more frilly **oral arms** (Fig. 7.2C) and a central mouth for gathering and ingesting large prey items, while rhizostome medusae have a system of eight branched, lobed oral arms (Fig. 7.2F), the edges of which bear many tiny openings called **mouth lobes** for feeding on tiny organisms.

**Gastric cirri** (Fig. 7.1A, C) are found in the stomach or stomach pouches of most cubomedusae (except Carukiidae cubomedusae) and scyphomedusae; they are often laden with nematocysts (refer to Sect. 7.3) to help subdue prey and may also aid in digestion. In scyphomedusae that lack gastric cirri (e.g., *Drymonema*), oral arms completely envelop the prey, and external enzymatic digestion takes place. Digestion has not been broadly studied in medusae, but evidence suggests that intracellular digestion occurs in the endodermal cells following endocytosis of food particles; phagocytosis has been observed in jellyfish species with food vacuoles (e.g., *Aurelia*) (Arai 1997). Following digestion in the stomach, food particles are passed along the **radial canals** (Fig. 7.3B, H), which are circulatory tubes (varying in number by species) connecting the gastrovascular cavity with the canal system (e.g., marginal ring canal, tentacle canal, or velarial canals), which plays an important role in distributing food and oxygen throughout the umbrella and in eliminating waste. Digestion rates in jellyfish are highly temperature dependent and vary by medusa species and types of prey items. Proteolytic and lipolytic enzymes known to play a vital role in digestion have been documented in the stomach and oral arms of several jellyfish species (for a review see Arai 1997). Additionally, recent RNA-Seq studies of genes expressed in the gastric cirri of the cubomedusae *A. alata* (Fig. 7.1A) revealed an abundance of toxins and toxin-like enzymes in the gut, in the absence of food items (Lewis Ames and Macrander 2016; Lewis Ames et al. 2016), suggesting these bioactive proteins are perpetually produced and may serve a dual role (e.g., digestion and venom composition).

### **Autotrophic Feeding Through Symbionts**

Medusozoans are among the most important aquatic carnivores. As major consumers of planktonic and benthic organisms (e.g., fish, crustaceans, worms) (Boero et al. 2008; Doyle et al. 2014; Wrobel and Mills 1998), medusae utilize their tentacles extensively for prey capture and defense; a lack of available prey items results in starvation, shrinkage, and death. However, some rhizostome medusa, such as *Cassiopea* spp. (Fig. 7.2B, D) that host symbiotic photosynthesizing dinoflagellates (*Symbiodinium* spp. zooxanthellae), can survive longer periods of starvation when light situations are amenable to photosynthesis, suggesting zooxanthellae may produce and transfer nutrients (e.g., fatty acids) that are vital for regular metabolic function (Mortillaro et al. 2009).

### 7.3 Nematocysts: Novel Stinging Structures

Medusozoans are united by the presence of **nematocysts** (Fig. 7.4), which are specialized intracellular venom-filled organelles (within the broader category of cnidocysts) used in prey capture and defense against predation (Mariscal 1974; Kass-Simon and Scappaticci Jr. 2002; Östman 2000). For a review of jellyfish venom and bioactive toxins, see Starcevic and Long 2013; Cegolon et al. 2013; Mariottini 2014; Jouiaei et al. 2015a. Nematogenesis involves the development of a nematocyst (a secretory product of the Golgi apparatus) within the cnidarian-specific cell called a **nematocyte**. The double-wall nematocyst capsule (Fig. 7.4A, D–F) is reinforced by proteins orthologous to the bacterial enzyme subunit poly-gamma-glutamate capA (pgsA)—a natural polymer that helps maintain high osmotic pressure of up to 150 bar in the nematocyst until a trigger induces the explosive release of a tightly coiled microscopic hypodermic tubule in the fastest known biological process (<3 ms) (David et al. 2008; Hwang et al. 2010; Kass-Simon and Scappaticci Jr. 2002). The innovative structure and biomechanics of nematocysts, and their ability to penetrate human skin with an ultrafast injection system, make them an excellent choice for expanding biotechnology applications that either inhibit nematocyst discharge, thereby preventing human envenomation, or replicate nematocyst structural efficiency in micro-devices for active drug delivery (Lotan 2016).

#### 7.3.1 *Medusa Cnidome*

The cnidome (nematocyst inventory) varies broadly among medusa taxa, and during ontogeny, providing systematic value that aids in taxon-level identification in medusozoans. Broadly, nematocysts are categorized as penetrant or adherent (Östman 2000); they are primarily used in prey capture and defense and sometimes in locomotion (Anderson and Bouchard 2009). More than 30 known types of nematocysts have been classified based on morphology alone (Östman 2000; Mariscal 1974; Gershwin 2006; Weill 1934). Although no exclusively medusozoan nematocyst category has been identified, medusozoan nematocysts differ from those of anthozoans by the presence of an apical hinged operculum (Fig. 7.4D–F) (rather than an apical flap or cap) (Reft and Daly 2012; Östman 2000; Rachamim et al. 2014). More similarities in cnidome are seen among different species of medusozoans than between medusozoans and anthozoans (Östman 2000). Nematocyst types common to all medusozoans include euryteles (Fig. 7.4D–F), birhopaloids, and isorhizas, while microbasic b-mastigophores occur in some hydromedusae and cubomedusae, but not in scyphomedusae. Among medusozoans, only cubomedusae have p-mastigophores (Fig. 7.4A), and although desmonemes and stenoteles are generally associated with hydromedusae, the latter are reportedly present in at least one cubozoan polyp (see Arneson and Cutress 1976).

Nematocysts are abundant in medusa tentacles, but they are also found in the gastrovascular cavity of some cubomedusae and scyphomedusae where they are thought to aide in digestion (Lewis Ames and Macrander 2016; Conant 1898; Arai 1997). A unique case involves the discovery of penetrant and adherent nematocysts in the female ovaries and male testes, respectively, of the cubomedusae *Copula sivickisi* and associated with spawned reproductive material, where they are thought to provide protection for the embryo strand and an adherent benefit for spermatophores when passed to females during copulation (Lewis and Long 2005; Garm et al. 2015; Toshino 2014; Hartwick 1991; Garcia Rodriguez et al. 2018). The most unique documented case of extra-tentacular nematocysts in a cubomedusa is the recent finding of nematocyst nests in *C. sivickisi* within subgastric sacs, formerly thought to function as sperm storage structures (Garcia Rodriguez et al. 2018).

### 7.3.2 Cellular and Molecular Classification of Nematocysts

Nematocytes are among the most complicated eukaryote cell types known. Overall, nematocytes contain pre- and postsynaptic elements and interact via synapses with neurons, sensory cells, and possibly other nematocytes. Nematocyst discharge is custom-regulated via endogenous pathways and a choreographed integration of mechanosensory and chemosensory information exchange by both inter- and intracellular signaling molecules (Anderson and Bouchard 2009). Nematocyst firing also involves light activation of a receptor on the apical structure (operculum) initiating voltage- and  $\text{Ca}^{2+}$ -dependent exocytosis (Anderson and Bouchard 2009; Gitter et al. 1994; Kass-Simon and Scappaticci Jr. 2002; Plachetzki et al. 2012). In the past decade, the rising trend in characterizing nematocyst molecular components using multi-omics techniques (proteomics and transcriptomics) has revealed nematocyst-specific structural proteins (e.g., minicollagens, outer wall antigen), novel bioactive proteins (e.g., CaTx toxin family), and conserved toxins and toxic-like enzymes (e.g., metalloproteases) (for discussion on medusozoan nematocyst structural and bioactive proteins, see Ponce et al. 2015; Jouiaei et al. 2015b; Lewis Ames and Macrander 2016; Weston et al. 2013; Junior et al. 2014).

## 7.4 Neurogenesis

Basic features of animal nervous systems were present in several clades of early diverging metazoans. Investigating the minimalistic circuits of early diverging pre-bilaterian animals has potential to reveal fundamental principles about the basic design of neural circuits and potential evolutionary trajectories related to neurogenesis in early animals (for reviews of the cnidarian nervous system, see Bosch et al. 2016; Rentzsch et al. 2016; Watanabe et al. 2009; Galliot et al. 2009).

### 7.4.1 *Neural Circuits*

Advanced neural integration and fast neuronal communication are essential for medusae, many of which perform intricate and specialized behaviors (Albert 2011; Stewart 1996; Garm et al. 2007a, 2016; Lewis and Long 2005; Bentlage et al. 2010; Gibbons et al. 2010), display sensitivity to sound (Solé et al. 2016), or exhibit a sleep-like state (Nath et al. 2017). Functionally sophisticated neurophysiological specialization in medusozoans includes bidirectional chemical synapsis (Anderson 1985), diverse peptide-gated channels for signaling (Durrmagel et al. 2010), axons with more than a single impulse propagation (Horridge et al. 1962), chemical and small molecule neurotransmitters (e.g., catecholamines, serotonin, acetylcholine) and neuropeptides (Kass-Simon and Pierobon 2007), ion channel receptors, nematocysts (Kass-Simon and Scappaticci Jr. 2002), statocysts (i.e., gravity sensing organs) (Horridge 1969), and image-forming lens eyes (Nilsson 2013). However, medusozoan nervous systems are often dismissed as being structurally simple, functioning as diffuse nerve nets with varying degrees of localized grouping and specialization. This generalization about medusozoan nervous systems is extrapolated from findings of cellular and molecular studies on sessile cnidarian models (e.g., *Hydra*) and just a handful of medusa species (for a review of jellyfish nervous systems, see Katsuki and Greenspan 2013). Studies indicate that some fundamental genetic components of vertebrate nervous systems that have long existed in cnidarians may have been co-opted for neuronal communication in animals with complex nervous systems (for a list of neurogenesis orthologues, see Bosch et al. 2016; You et al. 2008). Advances in genetic editing and engineering methods (e.g., CRISPR/Cas9) (Rentzsch et al. 2016) and adoption of transgenic techniques and cloning (Technau and Schwaiger 2015), coupled with calcium imaging capabilities of entire neural circuits, are paving the way for scientists to study medusozoans as alternates to more complex vertebrates nervous systems (for a review of neurogenesis, see Galliot et al. 2009; Bosch et al. 2016).

### 7.4.2 *Sensory Perception and Balance: The Medusa Pacemaker*

Jellyfish sense organs (**rhopalia**) occur near the bell margin or at the base of the tentacle in medusae bearing individual (or multiple) photoreceptors called **ocelli** that are sometimes lensed. Rhopalia (Figs. 7.1A, D, E and 7.2E) are part of the medusa pacemakers, as they have associated statocysts (or statoliths)—acellular crystalline structures that function as balance organs (Horridge 1969). Information from these balance and sensory organs is transferred to an associated marginal center of the nervous center and then to the nerve nets, playing a vital neurological role in facilitating scyphomedusae and cubomedusae behaviors (Garm and Mori 2009). Scyphomedusan rhopalia (variable in number, but usually eight or more) (Fig. 7.2E)

have a statocyst, a sensory epithelia, and an ocellus. Among the set of these marginal pacemakers, there is a constant shifting of control during sensory uptake; rhopalia removal renders the medusa incapable of spontaneous umbrella contractions (Satterlie 2002). The conduction system linking the rhopalia pacemakers, known as the motor nerve net, is responsible for spreading excitation via a single pulse generated throughout the entire subumbrellar muscle sheet (known as “diffuse conduction”) in a nonpolarized manner (for a detail explanation of the conduction system initiating swim contractions, see Satterlie 2002).

Cubomedusae **rhopalia** (Fig. 7.1A, D, E) differ significantly from other rhopalia: always four in number, they are suspended by a peduncle within an indented rhopaliar niche on each side of exumbrella (above the bell margin). The rhopaliar niche is partially or entirely covered by a flap (or flaps) of tissue forming the rhopaliar niche ostium. Each cubomedusan rhopalium (Fig. 7.1A, D, E) invariably possesses a **statocyst** and two central **complex lens eyes** flanked by two sets of simple **slit and pit eyes** (Conant 1898; Nilsson et al. 2005; Bielecki et al. 2014; Lewis et al. 2013), facilitating the evolution of vision-guided behavior in cubozoans (Garm et al. 2007b, 2008, 2016; Bielecki et al. 2014; Coates et al. 2006). Gene expression studies of several cubomedusae revealed that proteins involved in vision and the phototransduction pathway (e.g., opsins and J-crystallins) are abundant in rhopalia (Piatigorsky et al. 2001; Kozmik et al. 2003, 2008a, b; Suga et al. 2010; Lewis Ames et al. 2016; Feuda et al. 2014; Plachetzki et al. 2010; Plachetzki et al. 2005, 2007). Some phototransduction proteins are also expressed in cubomedusan extraocular tissues (Bielecki et al. 2014; Liegertová et al. 2015; Desmond Ramirez et al. 2011; Lewis Ames et al. 2016), and in planulae (e.g., *Alatina alata*) which possess eye spots (i.e., rudimentary photoreceptors) (Lewis Ames et al. 2016), suggesting paralogous opsins evolved subsequent functional diversification in cubozoans. Physical and molecular findings support the presence of a cubomedusan “central nervous system” that interacts with diffuse nerve networks (for a review see Satterlie 2011; Albert 2011). Cubomedusan pacemaker characteristics depend on the marginal nerve ring connecting all four rhopalia to one another and to junctions at the base of each of the four tentacles (Satterlie et al. 2005; Satterlie 2002, 2011). Elaborate rhopalia are integrated with the rest of the nervous system via the marginal nerve network, and visual output modulates the pacemaker system (thought to be located in the upper region of the rhopaliar peduncle) controlling bell contractions (Satterlie 2002). Unlike the other medusozoan classes discussed herein, hydromedusae lack rhopalia (Satterlie 2002), and only certain species possess statocysts (Horrige 1969); the swim-control system is found in the inner marginal nerve ring. In hydromedusae with ocelli (photosensitive eyespots along the bell margin) (Fig. 7.3I) phototropism is observed. However, hydromedusae that lack ocelli are also capable of light-sensitive behavior, evidenced by their diel vertical migration capabilities (Mills 1983), a behavior common in deep-sea coronate scyphomedusae (e.g., *Atolla* spp.) (Fig. 7.2G).

## 7.5 Medusa Life Cycles

### 7.5.1 Sexual Reproduction

Generally, medusa **gonads** are gonochoristic (separate sexes), although hermaphroditism has been documented in the scyphomedusa *Chrysaora hysoscella* (Morandini and Marques 2010; Gershwin and Collins 2002). Scyphomedusan gonads (variable by taxon) are typically located in the floor of the stomach (Fig. 7.2A, G), peripheral to gastric cirri (Arai 1997). Fertilization in scyphomedusae can occur internally in the gastrovascular cavity of the female, following which time zygotes may be released externally into the water column, or brooded in pouches (*Aurelia*) or within the ovaries (*Cyanea*) (Fig. 7.2C). In cubomedusae, gonads (Fig. 7.1A) are located within subcomponents of the gastrovascular cavity called gastric ostia. Of the eight cubozoan species for which sexual reproduction has been documented, most exhibit internal fertilization in the gastrovascular cavity, with external fertilization documented for just two species (Toshino et al. 2015b). All cubomedusae have two pairs of gonads at each of the four interradial (eight hemigonads in total) that are generally leaf-like in shape (Fig. 7.1A). Cubomedusae exhibit the most elaborate sexual reproduction strategies with at least one species forming monthly spermcasting aggregations (*Alatina alata*) (Fig. 7.1A) (Lawley et al. 2016; Lewis et al. 2013). Possibly more remarkable is the fact that tripedaliid cubomedusae *Tripedalia cystophora* and *C. sivickisi* engage in copulation—males pass spermatozoa to females for internal fertilization by way of sperm storage structures in the female's velarial canals (in *C. sivickisi*) and subsequent release of planula larvae (Lewis and Long 2005; Lewis et al. 2008; Marques et al. 2015; Stewart 1996; Garcia Rodriguez et al. 2018). *Copula sivickisi* is the only known truly sexually dimorphic medusa, with males and females exhibiting conspicuous discrepancies in gonad shape and function (Lewis and Long 2005; Garcia Rodriguez et al. 2018). In hydromedusae, sexes are also generally separate, but in contrast to other classes of jellyfish, hydromedusan gonads are ectodermal in origin (with few exceptions) (Fig. 7.3E–H). Following gametogenesis in the gonad ectoderm, hydromedusae gametes are shed directly into the water column (rather than passing through the gastric cavity). Gonad organization is highly variable in hydromedusae, with disparity in the number and location of gonads on the medusa body (e.g., manubrium (*Bougainvillia*)) (Fig. 7.3J), stomach (*Proboscoidactyla*), or radial canals (*Vallentinia gabriellae*)) (Fig. 7.3H), which serves as an important taxonomic feature for species identification.

### 7.5.2 Metagenetic Life Cycles

Medusa life histories are typically characterized by metagenesis—alternation of generations that starts with a motile planula settling as a benthic polyp and then metamorphosing into a pelagic medusa life stage (refer below in Sect. 7.5.2 for

exceptions). In medusa taxa with metagenetic life cycles, the liberated planula attaches to a substrate where it becomes a sessile life form capable of feeding and reproducing asexually as buds that settle adjacent the primary polyp as secondary polyps. Polyps can be solitary or colonial and with or without tentacles. The most elaborate polyps are seen in hydrozoan taxa as interconnected structures composed of dendritic or stolon-like arrangements (Houliston et al. 2010) called hydroids (Fig. 7.3M) that are sometimes capable of sexual reproduction (Cartwright and Nawrocki 2010; Bouillon et al. 2006). Additionally, taxon-specific motile asexual buds are released that move away from the vicinity of the source polyp before settling as new polyps, thereby augmenting possibilities for colonization, e.g., nonciliated frustules (e.g., *Craspedacusta sowerbii* (Jankowski et al. 2008)), ciliated planuloid buds (e.g., *Cassiopea* spp. (Hofmann et al. 1996)), creeping polyps (*Copula sivickisi* (as *Carybdea sivickisi*) (Lewis and Long 2005)), and swimming polyps (*Morbakka virulenta* (Toshino et al. 2013)). Under adverse ecological conditions, jellyfish species are also known to exhibit several encysted diapause stages called podocysts (Jankowski et al. 2008; Lewis et al. 2012; Ikeda et al. 2011; Toshino et al. 2013; Arai 2009), blastocysts (Lawley et al. 2016), or planulocysts (Dong et al. 2008). In these cases, polyps or planulae, respectively, are reduced to a desiccated form with low metabolic activity that persists until suitable environmental conditions are restored (Ikeda et al. 2011). It has been suggested that these cysts provide an advantage for facilitating broad geographic dispersal (Lawley et al. 2016) and potential jellyfish blooms (Arai 2009). The diversity and plasticity of these life forms and life history modes across Medusozoa means that medusae can colonize a plethora of ecosystems (Condon et al. 2013; Lucas et al. 2014; Dawson and Hamner 2009; Ceh et al. 2015; Gibbons et al. 2010).

### Medusa Buds

Metagenesis may be the ancestral developmental mechanism in Medusozoa (Antonio Carlos Marques and Collins 2004). This adaptation presents a type of buffer zone accounting for seasonal variation in environmental conditions, with the asexual polyp form dominating when resources are scarce (winter), and then when conditions and nutrients are favorable for growth (spring), medusae are released for maturation and subsequent sexual reproduction (Mills 2001; Chiaverano et al. 2013). The modularity of the polyp facilitates exponential growth of medusa populations from a solitary embryo (Aglieri et al. 2014).

The life cycle of most scyphomedusa species starts with a single ephyra (juvenile medusa) being released (via transverse fission) from the apical portion of a strobila polyp in a process called strobilation (Fig. 7.2D, E) (but see Helm et al. 2015). For a review of the evolution and development of scyphozoan jellyfish, see Helm (2018). Depending on the species, medusae can be released either in multiples via polydisc strobilation (*Aurelia* spp.) (Brekhman et al. 2015; Purcell et al. 2009; Fuchs et al. 2014) or as singletons via monodisc strobilation (*Cassiopea* spp.) (Hofmann et al. 1978, 1996) (Fig. 7.2B, D). To date, the life history of eight cubozoan species has been described, and in all cases cubozoan medusae are generated from cubopolyps by apical metamorphosis of the polyp into a juvenile

medusa (Fig. 7.1D, E) (Toshino et al. 2015a). Cubomedusae metamorphosis is dubbed “complete” when the entire cubopolyp is transformed into a juvenile medusa and “partial” (also called modified strobilation (Straehler-Pohl and Jarms 2005)) when only the apical portion of the cubopolyp is transformed, leaving a remnant of the stalk to regenerate into a functional polyp after the release of the cubomedusa. Most metagenetic hydrozoan species that make medusa buds (~733 species estimated by Gibbons et al. (2010)) are thought to employ lateral budding to generate medusae from solitary (Fig. 7.3K, L) or colonial polyp structures (hydroids) (Fig. 7.3M) (Marques and Collins 2004; Boero et al. 2008; Cartwright and Nawrocki 2010; Gibbons et al. 2010; Bouillon et al. 2006). Distinctively, lateral budding involves the generation of a third layer of tissue (entocodon) in between the endoderm and ectoderm, which some have suggested is homologous to bilaterian mesoderm (Seipel and Schmid 2005; Boero et al. 1998). Additionally, species of the hydrozoan genus *Millepora* (fire coral) have calcareous skeletons (similar to anthozoan corals) that produce medusae from a swollen receptacle known as an ampulla (Soong and Cho 1998; Hickson 1890). *Millepora* medusae are short-lived, releasing gametes into the water via the manubrium within an hour and subsequently dying several hours later.

### 7.5.3 Exceptions to Metagenesis

#### Holoplanktonic Development

Although the medusa is the most conspicuous life stage, most jellyfish species are considered “meroplanktonic” because the presence in the water column of the pelagic life stages (planulae or medusa) is short-lived. In contrast, “holoplanktonic” animals remain in the plankton for the full duration of their life, metamorphosing into medusae directly from planulae or actinula larval forms (Boero et al. 2008; Marques and Collins 2004; Collins et al. 2006; Daly et al. 2007; Collins 2009; Gibbons et al. 2010). Approximately 93 species of holoplanktonic medusa species are estimated within Hydrozoa (traditionally within Trachymedusae and Narcomedusae) (Gibbons et al. 2010), while only a few holoplanktonic species have been reported in Scyphozoa families: Pelagiidae (Aglieri et al. 2014) and Periphyllidae (Jarms et al. 2002). The evolution of a holoplanktonic life cycle in some medusae may provide the advantage of feeding and undergoing complex developmental changes as part of the nutrient-rich plankton and of broader dispersal potential over greater distances as compared to metagenetic taxa, which depend on the availability of suitable substrates (Gibbons et al. 2010; Dong et al. 2008).

#### Medusa Bell Fission

Several species belonging to hydromedusan families (Aequoridae, Dipleurosomatidae, Campanulariidae, and Sugiuridae) can generate daughter medusae in a process known as medusa bell fission (Minemizu et al. 2015). Several variations of the process exists, including splitting via direct fission to generate two daughter medusae (*Aequorea macrodactyla*) (Stretch and King 1980) or via



longitudinal fission (schizogony) (*Gastroblasta raffaelei*) (Gravili et al. 2007; Russell 1953). The presence of body part supernumerary (Fig. 7.3G) in taxa with bell fission is considered to be a display of morphological traits seen in the colonial polyps, also exhibited in the solitary medusa stage that is likely attributed to differential expression of regulatory genes in the single shared genome.

### **More Life Cycle Anomalies**

Some notable examples of the medusa representing a mosaic of polypoid and medusoid characters include *Clytia mccradyi* whose gonads are replaced with gonothecae containing developing medusae, and several hydromedusae whose manubrium (*Zanclaea medusopolypata*) or radial canals (*Lizzia blondina*) (Fig. 7.3A) bear hydranths with medusa buds (for a review, see Gravili et al. 2007; Boero et al. 2000). Additional enigmatic life histories include *Halammohydra*, which exhibits a pelago-benthic life cycle occupying sand on the sea bottom (Collins et al. 2008; Clausen 1967), and “crawling medusae” of the genera *Staurocladia* and *Eleutheria* (Fig. 7.3I) which, following release from their hydroids, crawl around in tide pools (sometimes swimming) or adhering to rocks and seaweed (Hirano et al. 2000).

## **7.6 Medusa Biogeography**

The ingenuity of the medusa body form provides osmotic and biomechanical versatility, permitting adaptation of jellyfish to a plethora of marine ecological niches (Hosia et al. 2008; Lewis et al. 2013; Mortillaro et al. 2009; Bentlage et al. 2009), brackish water environments (Lucas 1996; Lasley et al. 2016; Calder and Burrell 1969), and even freshwater habitats (Jankowski et al. 2008; Lewis et al. 2012), over the past 600 million years (Cartwright and Collins 2007; Daly et al. 2007). The recent advancement of molecular tools for studying population genetics has proven important in delineating jellyfish taxa previously thought to be cosmopolitan species (Dawson 2005; Schroth et al. 2002; Bayha et al. 2010), and in corroborating hypotheses of global distribution (Lawley et al. 2016). Future genomics-level biogeographic studies on marine and freshwater jellyfish should improve our understanding of the mechanisms underlying broad geographical distribution of medusae across oceans.

### **7.6.1 Jellyfish Proliferations**

The wide range of biphasic (or multiphasic) life cycles exhibited by medusozoans requires dramatic metamorphosis on a physiological, behavioral, ecological, and molecular scale, the basis of which may constitute an evolutionary advantage in the oldest animal lineage with a complex life history. The incredible plasticity of jellyfish life cycle adaptation to highly variable environments gives way to large

temporal and spatial fluctuations in the abundances of certain taxa (Condon et al. 2012). The potential for a jellyfish species to cause a bloom (sudden unpredictable proliferation) is integrally linked to the abundance of medusae liberated from polyps (i.e., strobilation) (Purcell et al. 2009).

Molecular components of the conserved retinoic acid signaling pathway are known to be involved in strobilation in several medusa species (for a list of metamorphosis genes, see Fuchs et al. 2014; Holstein and Laudet 2014; Brekhman et al. 2015; Kostrouch et al. 1998). The discovery of molecular cues that potentially drive complex morphological and physiological changes during medusa metamorphosis has fueled many studies aimed at determining potential environmental drivers that might activate or deactivate these pathways, such as temperature (You et al. 2008), light (Mortillaro et al. 2009), iodine (Prieto et al. 2010), indole compounds (Kuniyoshi et al. 2012; Yamamori et al. 2017), UVB exposure, global oscillations (Condon et al. 2013; Lucas et al. 2014), xenobiotics (Schroth et al. 2005), hormone/pheromones (Berking et al. 2005), current drift (Fossette et al. 2015), and symbiosis with dinoflagellates (Hofmann et al. 1996). For a review of the known natural cues and molecular mechanisms of strobilation induction, see Helm (2018). A medusa reference genome would permit further investigation of the genetic pathways underlying induction of metamorphosis in a broad range of medusa taxa, with the purpose of clarifying the relationship between metagenesis and jellyfish proliferations.

### **7.6.2 Jellyfish Blooms: A Bad Rap**

Jellyfish blooms are increasingly recognized as a dire ecological and societal concern. Blooms have been associated with human-instigated environmental perturbations, construction of underwater structures, and artificial coastal installations that provide increased recruitment potential for jellyfish polyps and subsequently interfere with human recreational, commercial, and economic activities (Vodopivec et al. 2017; Purcell et al. 2007; Purcell 2012). Some noteworthy examples in the news and literature include jellyfish clogging fishing nets, devouring commercial catch, destroying aquaculture facilities, clogging power plant cooling systems, and causing sea bather's eruption and lethal human envenomation, in extreme cases (Boero et al. 2008; Graham and Bayha 2007; Leone et al. 2013; Purcell et al. 2007; Brinkman et al. 2015; Rossetto et al. 2009). Hence the concerted effort within the scientific community to understand the science of blooms, and to address the question of the effects on ocean health, if jellyfish blooms are indeed on the rise (Lucas et al. 2014; Condon et al. 2013; Kingsford and Mooney 2014; Dawson et al. 2014; Bayha and Graham 2013; Condon et al. 2012; Doyle et al. 2014).

### 7.6.3 *Ecological and Societal Benefits of Medusae*

Jellyfish are important components of the gelatinous zooplankton community, particularly because of their position in the food web as prey for many different taxa and predators of fish larvae and other planktonic organisms (Lucas et al. 2014; Doyle et al. 2014; Condon et al. 2012). The oscillating nature of gelatinous zooplankton plays an important role in shaping marine ecosystems (Boero et al. 2008), but too often the ecological and societal benefits of jellyfish (Doyle et al. 2014) are overshadowed by the negative focus on jellyfish blooms.

Medusozoans are a vital part of the aquatic food web, representing the primary food source for many large marine vertebrates (e.g., sea turtles, sunfish and penguins) (Thiebot et al. 2017), as well as many other pelagic and benthic consumers (Arai 2005). Many marine organisms are known to digest medusozoans and utilize either a portion of the body or the entire animal in defense (e.g., octopus) (Hoving and Haddock 2017) or via a process known as kleptocnidae (sequestration of nematocysts from cnidarian prey) (Goodheart and Bely 2017; Greenwood 2009). Humans also consume medusae because of their high nutritive value (for a complete review of worldwide medusa consumption, see Brotz et al. 2017; Brotz 2016).

Large biomass offered by proliferating jellyfish could provide a source of bioactive compounds including peptides, collagens, gelatin, water-soluble minerals, and biopolymers, making medusae a valuable resource for health-, food-, cosmetic-, and biomedical-related industries (Leone et al. 2013). In particular, rhizostome scyphomedusae have been targeted for potential marine-derived compounds with nutraceutical potential (Khong et al. 2016) and pharmacological properties (for a complete review of medusae as potential therapeutic agents and drug candidates, see Leone et al. 2013). In particular, jellyfish collagen has found a special niche in the biomedical field as effective biomaterial for improved cell culture protocols, wound care, collagen regeneration, tissue engineering, and hemostatic applications (Hoyer et al. 2014; Cheng et al. 2017; Sang et al. 2011; Pustlauk et al. 2016; Krishnan and Perumal 2013). Furthermore, hydromedusae aequorin and green fluorescent proteins (GFPs) are employed, respectively, to measure intracellular  $\text{Ca}^{2+}$  levels and as fluorescent reporters for labeling cells or controlling gene expression in synthetic biology (Shimomura 2005).

### 7.6.4 *Medusa-Inspired Robotics*

Medusa motility has inspired the development of three-dimensional computational fluid dynamics models simulating swimming hydrodynamics (Katija et al. 2015; Katija and Jiang 2013; Colin et al. 2013) with potential to advance underwater propulsion and flight dynamics-related applications for both military and commercial agencies. Because of the aerodynamic body form and great maneuverability and stability, flight dynamics modeled after the “flying” motion of jellyfish jet propulsion

have been the basis of the construction of microaerial vehicles (MAVs)—biologically inspired “bird-like” alternatives to helicopters or drones (Fang et al. 2017; Ristroph and Childress 2014). Furthermore, the United States Naval Undersea Warfare Center and the Office of Naval Research have collaborated to build self-powered robotic surveillance jellyfish that can potentially navigate the sea (or sky) utilizing self-harvested energy for surveillance missions of unlimited duration (University of Texas at Dallas & Festo 2009; Villanueva et al. 2011). With the artificial muscles’ efficient use of energy, these robotic jellyfish could potentially serve as points of contact in covert networks of widely distributed environmental sensors.

## 7.7 Emerging Medusa Model Systems: Insights to Be Gained

Currently, the only medusozoan with a reference genome is *Hydra*—a freshwater solitary polyp. The release of the first *Hydra* genome (Chapman et al. 2010) sparked a wave of additional multi-omics studies on *Hydra* (Juliano et al. 2014; Wenger and Galliot 2013; Galliot 2012), resulting in a well-annotated genomic resource that continues to broaden our understanding of cnidarian cellular and molecular complexities (Bosch et al. 2016; Quirin et al. 2016). However, in the absence of an annotated reference genome for any medusa species, major gaps remain in our understanding of the molecular components underlying the evolution of biological processes mediating complex behaviors in free-living medusozoans (i.e., jellyfish).

Based on reports of jellyfish genomics or transcriptomics studies in the scientific literature and NGS data sets deposited into the NCBI Short Read Archive (SRA), approximately 16 medusa candidates have been identified as potential emerging medusa model systems: hydromedusan *Hydractinia*, *Podocoryna*, and *Clytia hemisphaerica* (SRA and reviewed in Houliston et al. 2010; Sanders et al. 2014; Frank et al. 2009; Leclère and Röttinger 2017) and *Millepora* spp., *Turritopsis* sp., and *Craspedacusta* sp. (SRA); scyphomedusan *Aurelia* (SRA and reviewed in Brekhman et al. 2015; Nakanishi et al. 2015; Fuchs et al. 2014; Berking et al. 2005; He et al. 2015; Kuniyoshi et al. 2012; Albert 2011), *Rhopilema esculentum*, *Nemopilema nomurai*, *Chrysaora* spp., *Cyanea* spp., *Stomolophus meleagris*, and *Cassiopea* sp. (SRA, and for a review of the latter, see Ohdera et al. 2018); and cubomedusan *Alatina alata* (SRA and reviewed in Lewis Ames and Macrander 2016; Lewis Ames et al. 2016; Lawley et al. 2016; Lewis et al. 2013), *Tripedalia cystophora* (Bielecki et al. 2014; Liegertová et al. 2015), *Chironex fleckeri* (SRA) and *Chiropsalmus quadrumanus* (SRA). Undoubtedly, many of the knowledge gaps presented in this review regarding medusa biology, physiology, evolution, and proliferation can be addressed upon the release of medusa reference genome(s).

Furthering the vision to advance medusa genomics research will require effective participation by jellyfish scientists in academia, public aquariums, and other research facilities, toward establishing effective protocols for maintaining and manipulating

medusa cultures, thereby permitting reproducibility of findings by the broader scientific community. Finally, continued interdisciplinary collaborations will ensure selection of appropriate target species whose genomes can best help inform evolutionary relationships among early animals, facilitate the development of bio-monitoring tools for early detection of jellyfish proliferations, and have potential applications to aquaculture and to biomedical and pharmaceutical industries.

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# Chapter 8

## Sea Urchin Larvae as a Model for Postembryonic Development



Andreas Heyland, Nicholas Schuh, and Jonathan Rast

**Abstract** Larvae are a diverse set of postembryonic life forms distinct from juveniles or adults that have evolved in many animal phyla. Echinoids (sea urchins and sand dollars) generate rapidly developing, morphologically simple, and optically transparent larvae and are a well-established model system supported by a broad array of genomic resources, experimental approaches, and imaging techniques. As such, they provide a unique opportunity to study postembryonic processes such as endocrine signaling, immunity, host–microbe interactions, and regeneration. Here we review a broad array of literature focusing on these important processes in sea urchin larvae, providing support for the claim that they represent excellent experimental study systems. Specifically, there is strong evidence emerging that endocrine signaling, immunity, and host–microbe interactions play major roles in larval development and physiology. Future research should take advantage of sea urchin larvae as a model to study these processes in more detail.

**Keywords** Thyroid hormones · Nitric oxide · Histamine · Microbiota · IL-17 · Signaling · Phagocytosis · Innate immunity

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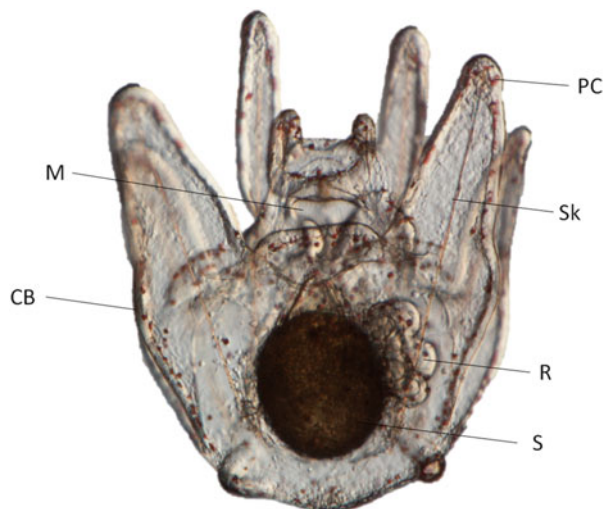
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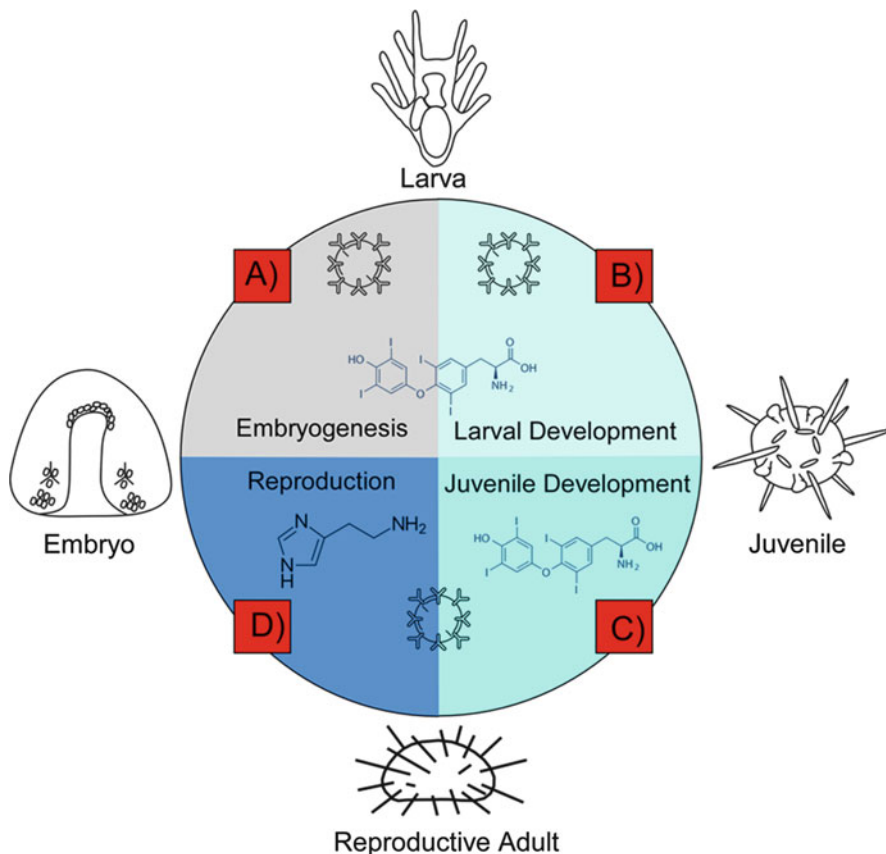


## 8.1 Larval Forms as Experimental Models in Physiology and Development

Larval forms are widespread among animal phyla, and as such they represent an important part of postembryonic development (Carrier et al. 2017). While discussions about what constitutes a larva can be found elsewhere (Bishop et al. 2006a; Carrier et al. 2017; Hodin et al. 2010; McEdward 2000; McEdward and Janies 1993), they encompass several unique characteristics, which are further discussed in this chapter (Figs. 8.1 and 8.2): (1) larvae are morphologically, physiologically, and developmentally separate entities from embryos, juveniles, and reproductive adults within the life cycle of an organism; (2) larval life cycles can be contrasted with direct life cycles, where embryos transition directly into a juvenile form; (3) metamorphosis is an intricate part of the larval life cycle during which the larva is transformed into the juvenile; and (4) the transition from larva to juvenile is frequently accompanied by an ecological transition (settlement), as juveniles inhabit different ecosystems from the larvae. Here we argue that larvae present a unique opportunity to study postembryonic development, especially among marine invertebrate phyla, which feature an impressive diversification of form and function. Technically, larvae have the advantage over adult or juvenile forms in that their morphologies are relatively simple and therefore amenable to rigorous experimentation. In this review, we provide a summary of research focusing on hallmarks of postembryonic development. Research on echinoderm larvae, especially sea urchin larvae, has contributed to our understanding of the evolution of endocrine and neuroendocrine signaling systems, immunity, asexual reproduction, and regeneration.

**Fig. 8.1** Larva of the sea urchin *Strongylocentrotus purpuratus* after 3 weeks of development in the lab. Specimen features eight larval arms with skeletal rods (Sk) and a developing juvenile rudiment (R). Also visible are pigment cells (PC) dispersed over the surface of the larva. The ciliated band (CB) surrounds the entire larval body and is involved in transporting food particles to the larval mouth (M). Food particles are digested in the larval stomach (S)

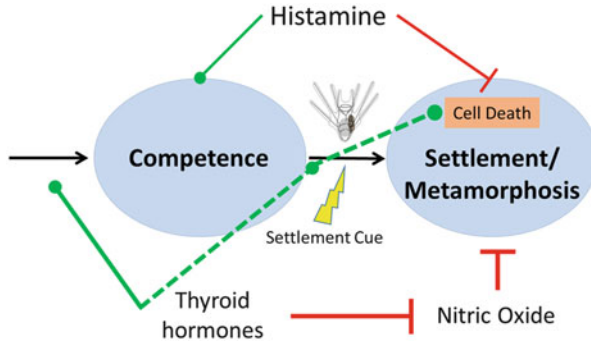




**Fig. 8.2** Schematic of an indirect sea urchin life cycle with major life history stages—reproductive adult, embryo, larva, and prereproductive juvenile. The postembryonic period is indicated in blue tones and consists of larval development (the focus of this chapter), juvenile development, and reproduction. Embryogenesis (a) initially results in a larval form, which metamorphoses, (b) after a sometimes extensive period of swimming and feeding in the plankton. Settlement defines the transition from the plankton to the benthos. Juveniles require months to years, depending on the species, to reach reproductive maturity (c). Reproduction occurs by free-spawning gametes (sperm and eggs) into the water column (d). This chapter focuses on the role of endocrine signaling, immunity, and regeneration in postembryonic development. The molecules indicated in the figure are histamine (HA) and thyroxine, a thyroid hormone (TH), respectively. Circles with receptors represent immune system function

## 8.2 A Framework for Hormonal Action in Larval Development and Metamorphosis of the Sea Urchin

Research on the involvement of hormones in echinoderm life histories has led to the emergence of a conceptual framework of their functions and interactions in larval development and metamorphosis (Fig. 8.3). While their detailed molecular



**Fig. 8.3** Conceptual diagram of hypothesized endocrine regulatory network underlying larval development and metamorphosis in the purple sea urchin *S. purpuratus*. Histamine (HA), thyroxine (T<sub>4</sub>), and nitric oxide (NO) all have been shown to regulate larval development and metamorphosis. The function of these signaling molecules may converge on metamorphic competence, the stage immediately preceding settlement, via the regulation of programmed cell death (apoptosis). Round ends indicate activation while line ends indicates inhibition. For references see text. Regular lines indicate mechanisms that have been extensively tested. Broken lines indicate hypothesized mechanism for which only preliminary or no evidence exists to date

mechanisms remain elusive in many instances, their involvement in critical larval functions, such as skeletogenesis and programmed cell death (PCD), makes them attractive candidates for functional physiological studies and offers insight into the evolution of endocrine signaling. Here we will briefly discuss the current state of knowledge on the hypothesized endocrine and neuroendocrine regulatory network underlying larval development and metamorphosis (Fig. 8.3).

### 8.2.1 *The Hypothesized Endocrine and Neuroendocrine Network of Sea Urchin Larval Development*

Thyroid hormones (THs) are regulators of animal development and physiology. For example, these hormones are both necessary and sufficient for the metamorphic transition from a tadpole to an adult frog (for review see Tata 1996). Similarly, THs have been shown to regulate metamorphosis and other life history transitions in echinoderms, urochordates, cephalochordates, and mollusks (for review see Taylor and Heyland 2017). The function of THs (specifically T<sub>4</sub>) in echinoids (sea urchins and sand dollars) include, but is not limited to the acceleration of development, specifically juvenile skeletogenesis, and reduction of larval arm length (Flatt et al. 2006; Heyland and Hodin 2004; Heyland et al. 2005, 2009; Heyland and Moroz 2006; Heyland et al. 2001, 2004) (Fig. 8.2). THs are endogenously synthesized from incorporated iodine in the purple sea urchin *S. purpuratus* (Miller and Heyland 2010, 2013), and this endogenous synthesis can be complemented by the exogenous hormone sources such as from the consumed phytoplankton (Heyland et al. 2006,

2009; Taylor and Heyland 2017). In fact, iodine incorporation into sea urchin epithelial cells occurs via conserved mechanisms, shared between plants and animals, which are distinct from TH synthesis mechanisms in mammals and other vertebrates (Miller and Heyland 2013). Finally, it appears that THs activate programmed cell death (PCD) in the arm tips of competent sea urchin larvae, promoting the transition from larva to juvenile (Taylor et al. [in review](#)). Because of this compelling evidence for TH function in invertebrates, sea urchin larvae provide an excellent model to study underlying mechanisms.

Histamine (HA) is an essential signaling molecule across prokaryotes and eukaryotes (Kyriakidis et al. 2012), with widespread functions ranging from neuronal signaling to immunity in animals (Nassel 1999; Roeder 2003; Tabarean 2016). In sea urchins, it is functionally involved in the formation of the fertilization envelope (Leguia and Wessel 2006) and has been identified as a natural settlement cue in several Australian echinoid species (Swanson et al. 2004, 2012). Recent evidence also suggests that it functions as a regulator of metamorphic competence (Lutek et al. 2018; Sutherby et al. 2012). Specifically, these studies show that HA levels and sea urchin histamine receptor 1 (suH1R) expression increase during larval development, peaking at metamorphic competence. Furthermore, the sea urchin genome contains two putative sequences for histamine receptors, one of which we recently functionally characterized (Lutek et al. 2018). This work suggests that suH1R is expressed in neuronal clusters in the mouth region of the larva and inhibits PCD in competent larvae.

In addition to TH and HA, nitric oxide (NO) has been shown to signal in metamorphically competent sea urchin larvae (Fig. 8.2). Specifically, endogenously released NO functions as an inhibitor of settlement (Bishop and Brandhorst 2001a, b). Furthermore, this pathway appears to interact with TH signaling in TH treatment of competent sea urchin larvae results in the reduction of NOS (nitric oxide synthase—NO synthesis enzyme)-positive neurons (Bishop et al. 2006b). These results suggest that TH may function antagonistically to NO signaling in competent larvae. Still, the functional relevance of this interaction requires further studies, along with a detailed analysis of the interactions between NO, TH, and HA.

## 8.2.2 Putative Function of TH Signaling in Skeletogenesis

The formation of a skeleton in postembryonic development is common to many animal groups. Sea urchin larvae prominently and beautifully feature a calcareous endoskeleton (Fig. 8.1), which provides a structure for the larval arms (for review see Decker and Lennarz 1988). The formation of the larval skeleton from a subset of primary mesenchyme cells (PMCs) in the ventrolateral cluster is the result of a complex gene regulatory network (GRN) involving transcription factors and signaling systems, which have been studied in detail in *S. purpuratus* and related species (reviewed in McIntyre et al. 2014). Importantly, the PMC GRN is a target of activated (phosphorylated) mitogen-activated protein kinase (MAPK, ERK 1/2)

signaling, and the transcription factors Ets1 and Alx1 are key mediators of MAPK inputs (McIntyre et al. 2014). Intriguingly, genes involved in this mechanism are present in several other echinoderm groups lacking skeletons, suggesting that the evolution of larval skeletons involved the activation of the entire module by specific signaling factors. One such candidate is vascular endothelial growth factor (VEGF), which is essential for proper formation of larval spicules in the sea urchin embryo (Koga et al. 2016).

The function of the larval skeletons is intimately linked to swimming and feeding performance and therefore dependent on environmental input. For example, sea urchin larval arms grow longer when food availability is limited (Heyland and Hodin 2004; Miner 2005; Miner and Vonesh 2004; Strathmann et al. 1992). This response effectively increases the length of the ciliated band and allows the larva to capture more food particles, hence compensating for the lack of food in the environment (Hart and Strathmann 1994). In addition to the transcription factors and signaling molecules involved in larval skeleton formation, several genes with specific functions in skeletal elongation have been identified in the sea urchins *S. purpuratus* and *Lytechinus variegatus*, including the transmembrane protein P16, SM50, MSP130, advillin, and carbonic anhydrase. These genes are important candidates for analyzing the molecular basis of arm elongation in sea urchins under low food conditions (McAlister and Miner 2018).

Neurosecretory mechanisms have been shown to be involved in the regulation of arm length. For example, Adams et al. (2011) demonstrated that dopamine is involved in the process that induces reduced feeding structures under abundant food conditions. Furthermore, Heyland and Hodin (2004) have shown that increased levels of TH, which potentially originate from algae, can produce a phenotype similar to the high food phenotype (i.e., shorter arms in high food conditions in comparison to low food conditions). These findings suggest that receptors detecting these signals can directly or indirectly modulate the GRN underlying skeletogenesis and apoptosis (see below). However, the link between these signaling events and skeletogenesis remains largely unknown. Note that recent findings also suggest a link between this plastic response and changes in the larval microbiome (further discussed below).

### **8.2.3 *Function of Hormonal Signaling in Programmed Cell Death (PCD)***

PCD plays a central role in both normal development and disease. In animals with larval stages, PCD is linked to postembryonic development and metamorphosis (Tata 1996). Specifically, hormones such as TH in vertebrates and ecdysteroids in insects are major regulators of this process. For example, THs regulate PCD in frog tail resorption (Ishizuya-Oka et al. 2010), and ecdysteroid action is a major regulator of PCD in insect metamorphosis (Buszczak and Segraves 2000). The complement of

PCD-related proteins (i.e., caspases, CARD, Apaf, BIR, Bcl domain proteins, and others) in basal animal groups and sea urchins is more similar to vertebrates (in comparison to model systems such as fruit flies and nematodes where PCD has been historically studied), indicating that these cell death mechanisms may have evolved early in animal evolution. Hence, we can gain important insights into the mechanisms of PCD by studying it in larval development of the sea urchin (Robertson et al. 2006).

Recent research on TH and HA signaling strongly suggests a link between endocrine signals and PCD in sea urchin larvae. In addition to the work outlined above on HA signaling regulating PCD in competent larvae, our data also suggest that T4 signals upstream of the skeletogenesis GRN in PMCs (Taylor and Heyland 2017; Taylor et al. *in review*). Part or all of this interaction may be responsible for the observed shortening of skeletal elements in response to T4 treatment. This hypothesis requires more detailed investigations into the regulation of various proteases critical for PCD, such as caspases and matrix metalloproteinases (MPPs), by THs. Cell death is closely linked to changes in the extracellular matrix (ECM). Specifically, caspases as well as MPPs have been shown to affect cell–cell interactions in PCD (Herren et al. 1998). More importantly, MMPs have been identified as the main players in PCD during the metamorphic transition of frogs and insects, and their function is regulated by metamorphic hormones (THs and ecdysteroids, respectively) (Mathew et al. 2010; Page-McCaw et al. 2007). While the functional role of these important PCD regulators in sea urchins remains largely unknown, previous work has identified MMPs as regulators of sea urchin skeletogenesis (Ingersoll and Wilt 1998), and several forms of MMPs are expressed during embryogenesis (Ingersoll and Pendharkar 2005). Furthermore, these proteases may play a functional role in mutable collagenous tissues, a type of tissue, which has received considerable attention in echinoderms due to its ability to undergo reversible changes in mechanical properties (Ribeiro et al. 2012).

### **8.3 Sea Urchin Larvae as an Experimental Model for Bacterial Colonization and Host-Microbe Interactions in Postembryonic Development**

Developmental biology has traditionally carried the view that multicellular organisms are discrete, individual entities. Advances in microbiology and sequencing technology have led to the emergence of a “holobiont” view: multicellular eukaryotes are hosts to vast microbial ecosystems continuous with the surrounding environment, forming indivisible biological systems (Gilbert et al. 2012; McFall-Ngai et al. 2013). These microbial residents participate in most (if not all) physiological processes, but much remains to be understood about interactions between microbes and developing hosts. The life history and habitat diversity found among echinoderms (e.g., tropical, temperate, arctic; benthic, planktonic, or brooded) could be

useful in delineating how the acquisition of distinct microbiota interacts with environment, feeding, development, and more, in the context of similar and dissimilar host genomes (Williams and Carrier 2018). This section will review the small but promising literature on bacterial microbiology in sea urchin larvae, as well as future directions for the field.

Previous research has isolated bacteria from adult echinoids using traditional culture techniques (Table 8.1). Unkles (1977) demonstrated that *Echinus esculentus* guts and cavity fluid were associated with *Gammaproteobacteria* (*Pseudomonas*, *Aeromonas*, and *Vibrio*) and some *Bacteroidetes* (specifically *Flavobacteria*), while the peristomial membrane had a distribution intermediate between those tissues and the surrounding environment. More recently, Hakim et al. (2015, 2016) used next-generation 16S sequencing to characterize the gut microbiome of adult *L. variegatus*, revealing not only distinct bacteria in urchin tissue and the environment, but also distinct communities associated with different parts of the gut. Pharynx and gut were dominated by *Epsilonproteobacteria* relative to the digesta, which were primarily colonized by *Gammaproteobacteria*, other *Proteobacteria*, and *Bacteroidetes* (including *Flavobacteria*). The surrounding seagrass and ocean water were relatively enriched for *Cyanobacteria* and *Alphaproteobacteria*, respectively. *Strongylocentrotus intermedius* and *Strongylocentrotus nudus* gonads were reported to be exclusively colonized by *Bacteroidetes*, although this may be a result of the *Bacteroidetes* (specifically *Tenacibaculum*)-focused 16S PCR primers used (Balakirev et al. 2008). Regardless, distinct relative abundances of *Flavobacteria* and *Sphingobacteria* were associated with the “usual” and “gray” *S. intermedius* phenotypes, but not with their depth below sea level, which may indicate a functional role associated with these phenotypes (Balakirev et al. 2008). Kiselev et al. (2013) isolated 22 strains (predominantly *Gammaproteobacteria* but also *Bacteroidetes* and *Firmicutes*) of 9 genera (*Aliivibrio*, *Bizionia*, *Colwellia*, *Olleya*, *Paenibacillus*, *Photobacterium*, *Pseudoalteromonas*, *Shewanella*, *Vibrio*) from adult *Strongylocentrotus pallidus* cavity fluid and exposed them to congeneric *S. intermedius* embryos. *Aliivibrio*, *Vibrio*, *Colwellia*, *Shewanella*, and *Photobacterium* spp. slowed or arrested growth by 3 days postfertilization; the remaining strains had no noticeable effect (Kiselev et al. 2013). Conversely, some of these strains induced apparent increases in pigment cell numbers (also see below) and viability, which may imply a degree of bacteria-immune synergy (Kiselev et al. 2013). These studies illustrate several recurring themes: adult echinoids in diverse habitats (Clyde Sea for *E. esculentus*, Gulf of Mexico for *L. variegatus*, Sea of Japan for *S. intermedius* and *S. nudus*, Sea of Okhotsk for *S. pallidus*) are similarly colonized by culturable *Proteobacteria* and *Bacteroidetes* relative to their diets and surrounding environments. Guts and coelomic fluid are associated with *Gammaproteobacteria* (particularly the genera *Colwellia*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, and *Vibrio*) and *Bacteroidetes* (particularly class *Flavobacteria*). These taxa are similarly overrepresented using culture-independent 16S sequencing approaches (Hakim et al. 2015, 2016) and may be associated with an array of functions (De Ridder and Foret 2006; Williams and Carrier 2018).

**Table 8.1** Bacterial phyla associated with adult and larval sea urchins

Stage	Tissue(s)	Sea urchin species	Method	Bacterial phyla	Reference
Adult	Gut, cavity fluid, peristomial membrane	<i>Echinus esculentus</i>	Culture, morphology, staining	<i>Bacteroidetes</i> <i>Proteobacteria</i> ( $\gamma$ ) Other	Unkles (1977)
Adult	Gonad	<i>Strongylocentrotus intermedius</i>	Culture, 16S	<i>Bacteroidetes</i>	Balakirev et al. (2008)
Adult	Gonad	<i>Strongylocentrotus nudus</i>	Culture, 16S	<i>Bacteroidetes</i>	
Adult	Cavity fluid	<i>Strongylocentrotus pallidus</i>	Culture, 16S	<i>Bacteroidetes</i> <i>Firmicutes</i> <i>Proteobacteria</i> ( $\gamma$ )	Kiselev et al. (2013)
Adult	Pharynx, gut tissue, gut digesta, feces	<i>Lytechinus variegatus</i>	16S	<i>Bacteroidetes</i> <i>Proteobacteria</i> ( $\alpha$ , $\beta$ , $\gamma$ , $\delta$ , $\epsilon$ ) Other	Hakim et al. (2015) and Hakim et al. (2016)
4-arm larva	–	<i>Strongylocentrotus purpuratus</i>	Culture, 16S	<i>Actinobacteria</i> <i>Bacteroidetes</i> <i>Cyanobacteria</i> <i>Proteobacteria</i> ( $\alpha$ , $\beta$ , $\gamma$ ) Other	Ho et al. (2017)
4-, 6-, 8-arm larvae	–	<i>Strongylocentrotus purpuratus</i>	16S	<i>Bacteroidetes</i> <i>Cyanobacteria</i> <i>Planctomycetes</i> <i>Proteobacteria</i> ( $\alpha$ , $\beta$ , $\gamma$ ) <i>Verrucomicrobia</i> Other	Carrier and Reitzel (2018)
4-, 6-, 8-arm larvae	–	<i>Mesocentrotus franciscanus</i>	16S	<i>Bacteroidetes</i> <i>Cyanobacteria</i> <i>Planctomycetes</i> <i>Proteobacteria</i> ( $\alpha$ , $\beta$ , $\gamma$ ) <i>Verrucomicrobia</i> Other	
4-, 6-, 8-arm larvae	–	<i>Strongylocentrotus droebachiensis</i>	16S	<i>Bacteroidetes</i> <i>Cyanobacteria</i> <i>Planctomycetes</i> <i>Proteobacteria</i> ( $\alpha$ , $\beta$ , $\gamma$ ) <i>Verrucomicrobia</i> Other	

Among echinoderms, fewer studies have focused on endogenous microbiota of sea urchin larvae (Table 8.1). Ho et al. (2017) characterized 89 bacterial strains associated with 4-arm feeding *S. purpuratus* larvae; the vast majority were *Gammaproteobacteria*, notably genera *Alteromonas*, *Colwellia*,



*Pseudoalteromonas*, *Pseudomonas*, and *Vibrio*. Representatives of the *Actinobacteria*, *Bacteroidetes*, and *Cyanobacteria* were also present. Repeated isolation experiments and comprehensive 16S sequencing corroborate these results: larvae are associated primarily with *Proteobacteria*, *Bacteroidetes*, and *Cyanobacteria*, distinct from the communities present in their surrounding environment (Ho et al. 2017). Culturable diversity turned out to be lower than that revealed with 16S sequencing, and associated diversity was lower when larvae were raised in the laboratory than in the field (unpublished data). *Cyanobacteria* and some types of *Proteobacteria* may be especially difficult to culture, at least using traditional methods. However, much of the microbiota appears to be relatively stable, even when larvae are raised in artificial seawater, suggesting that the host–microbe interactions underlying these associations could be studied in the lab.

Microbial transmission can occur in several dimensions, (vertical vs. horizontal, parental vs. exogenous, larva to adult, etc.), and many larvae-associated taxa overlap with taxa previously identified in adults, raising questions about the dynamics of the host–microbiota relationship in echinoid development and life history. Likely compartments for bacterial colonization are the digestive tract and the ectoderm, either its external surface or the subcuticular space (Cameron and Holland 1983, 1985). Subcuticular bacteria have been observed in adult echinoids (De Ridder and Foret 2006; Holland and Neilson 1978) and some echinoderm larvae (Cameron and Holland 1983, 1985; Holland and Neilson 1978; Cerra et al. 1997). Still, to our knowledge, this phenomenon has not been observed in sea urchin larvae. 16S fluorescent *in situ* hybridization reveals that larval bacterial load is miniscule until about 3–4 days of development, coinciding loosely with the onset of feeding, after which the vast majority of associated bacteria are present in the mouth and gut lumen (unpublished data) (Smith et al. 2008). Small numbers of bacteria are sometimes associated with the external ectoderm, but are rarely or never seen in the cuticle. Blastocoelar bacteria are generally only seen in injured or dead larvae, or under experimental conditions, where they are rapidly cleared by phagocytes (Ho et al. 2017).

In the face of environmental variation, sea urchin larvae acclimate physiologically by differentially expressing genes as well as adjusting phenotypic traits (see also discussion of phenotypic plasticity above). For many other animal taxa, acclimation is a combined physiological as well as microbial response, whereas in the latter, the community composition and their relative proportions of the host-associated microbiome are restructured. In a recent study, Carrier and Reitzel (2018) tested the hypothesis that feeding-induced plasticity in urchin larvae (a host response to environmental variation) correlates with a phenotype-specific microbiome, a proxy for hologenomic acclimation. In their study, they find support that across a phenotypic continuum, urchin larvae associate with phenotype-specific microbiome, of which was independent of dietary state (i.e., food quantity), developmental stage, and ecological drift. This pattern was convergent between *S. purpuratus*, *S. droebachiensis*, and *Mesocentrotus franciscanus* larvae, even though they associated with species-specific microbiomes. Furthermore, the authors find support that the magnitude that phenotypic plasticity is expressed strongly

correlates with the degree to which the larval host differentially associates with the microbiome. Collectively, these data could represent an additional layer of adaptation to changing environmental feeding conditions, reinforcing the view that echinoid larval microbiota are associated with feeding.

Several bacterial taxa found in sea urchin larvae are consistently associated with settlement and metamorphosis and have been hypothesized to be functionally involved in the induction of settlement (Table 8.2). For example, Huggett et al. (2006) isolated culturable bacteria from coralline algae and demonstrated settlement of the sea urchin *Heliocidaris erythrogramma* in response to biofilms produced by *Pseudoalteromonas*, *Vibrio*, *Shewanella*, *Photobacterium*, and *Pseudomonas* strains. Settlement is significantly reduced or blocked in *H. erythrogramma* and *Tripneustes gratilla* sea urchin larvae, as well as *Acanthaster planci* sea star larvae, when inducing macroalgae or rocks are cleared of their associated bacteria (Dworjanyan and Pirozzi 2008; Huggett et al. 2006; Johnson et al. 1991). *A. planci* larvae were observed by electron microscopy to settle and metamorphose only on regions colonized by bacteria, which included *Alteromonas* or *Pseudomonas*, and *Vibrio* (Johnson et al. 1991). Mos et al. (2011) tested a broad set of potential settlement cues and concluded that seaweeds with their associated bacteria were the most effective settlement cue for *T. gratilla*. Furthermore, *H. erythrogramma* settlement was found to be strongly correlated with the diversity of *Amphiroa anceps* and *Corallina officinalis* macroalgae-associated bacterial communities, but no significant relationship was observed for *Holopneustes purpurascens*, which settled consistently in response to algae-derived HA (Nielsen et al. 2015). Together, these results suggest that competent larvae may respond to specific bacterial strains or at least algal–bacterial consortia, depending on the echinoid and bacterial species involved. Further study is required to elucidate the molecular cues associated with bacteria and algae that regulate this process and the pathways of bacterial transfer and colonization between food sources, adults, larvae, and juveniles.

## 8.4 Sea Urchin Immunity in Larval Development

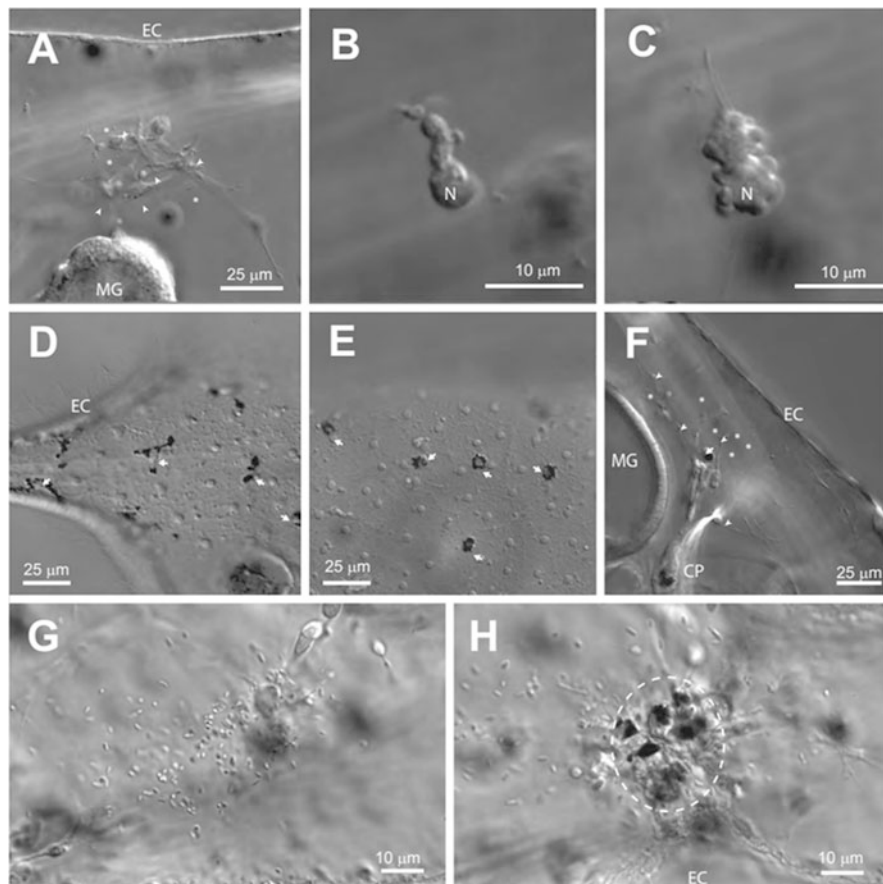
Echinoderms have had an important place in the history of immunology ever since Ilya Metchnikoff first described phagocytosis in asteroid and echinoid larvae (Metchnikoff 1893; Tauber 2003). Though the field of immunology has been primarily focused on vertebrate systems, with strong contributions from insects, there is every reason to believe that sea urchins have need of a complex immune system, and much research has confirmed that this is the case. Sea urchins are relatively long-lived invertebrates, and some species are among the animals with the longest lifespans (Ebert and Southon 2013). Marine environments are dense microbial ecosystems full of potential pathogens. Prolonged life expectancy in such environments implies broad and robust antimicrobial immunity (Bodnar and Coffman 2016; Buckley and Rast 2012). Embryos and larvae must survive to metamorphic competence in similar environments as their adults, suggesting a

**Table 8.2** Bacterial genera associated with induction of larval settlement

Echinoderm species	Bacterial phyla	Bacterial genera	Induction cue	Method	Reference
<i>Acanthaster planci</i>	<i>Proteobacteria</i> ( $\gamma$ )	<i>Alteromonas/Pseudomonas</i> <i>Vibrio</i>	Live bacteria associated with the macroalgae <i>Lithothamnium pseudosorum</i>	Culture, morphology, biochemistry	Johnson et al. (1991)
<i>Holopneustes purpurascens</i>	<i>Proteobacteria</i> ( $\gamma$ )	<i>Photobacterium</i> <i>Thalassomonas</i> <i>Vibrio</i>	Monoculture biofilms	Culture, 16S	Swanson et al. (2006)
<i>Heliocidaris erythrogramma</i>	<i>Actinobacteria</i> , <i>Bacteroidetes</i> , <i>Proteobacteria</i> ( $\alpha, \gamma$ )	<i>Alteromonas</i> <i>Aestuuriibacter</i> <i>Cytophaga</i> <i>Micrococcus</i> <i>Thalassomonas</i> <i>Photobacterium</i> <i>Pseudalteromonas</i> <i>Pseudomonas</i> <i>Shewanella</i> <i>Vibrio</i>	Monoculture biofilms	Culture, 16S	Huggett et al. (2006)
<i>Triploneustes gratilla</i>	<i>Proteobacteria</i> ( $\gamma$ )	<i>Pseudalteromonas</i> <i>Thalassomonas</i> <i>Vibrio</i>	Live bacteria, bacterial supernatants, mixed biofilms	Culture, 16S	Mos et al. (2011)

need for broad immune competence in these stages as well. Today, there is much work describing the immune systems of adult echinoids, and immunity in other life history stages are beginning to receive renewed attention (reviewed in Smith et al. 2018). This work has been greatly aided by access to genome sequences from the purple sea urchin and several other echinoderms, which reveal the presence of a broad suite of genes encoding innate immune receptors, signals, and effectors (Buckley and Rast 2017; Hibino et al. 2006; Sea Urchin Genome Sequencing et al. 2006). Although sea urchins do not have the same adaptive immune system that characterizes vertebrate immunity, they share developmental and regulatory systems with vertebrates that are also important in the larva. This section will review literature and emerging perspectives on sea urchin larval immunity and address potential future directions for the field.

Several mesenchymal cell types in sea urchin embryos and larvae are capable of rapid recognition and phagocytosis of foreign particles in the blastocoel as demonstrated in both *L. variegatus* (Silva 2000) and in *S. purpuratus* (Ho et al. 2017). Embryos and larvae also express humoral immune effectors such as complement factors (Shah et al. 2003) and antibacterial peptides (Li et al. 2014). The development and morphology of several larval mesenchymal cell lineages have been well described (Gibson and Burke 1987; Ruffins and Ettensohn 1993; Tamboline and Burke 1992), and a subset of these was recently shown to have specific immune functions (Buckley et al. 2017; Buckley and Rast 2017; Ho et al. 2017). In the purple sea urchin embryo, two populations of secondary mesenchymal cells are specified at the blastula stage, which give rise to several types of immune cells in the larva. These include five cell categories (Fig. 8.4): (1) *pigment cells* contain red granules and are motile with several dendritic cell-like pseudopodia (Gibson and Burke 1987; Ho et al. 2017). In unchallenged larvae, these cells are closely associated with the ectoderm and form clusters near the apex and arm tips. Upon bacterial challenge, they become rounded and hypermotile and often enter the blastocoelar space (Ho et al. 2017). (2) *Globular cells* are large cells filled with round vesicles that impart an unusual, popcorn-like appearance. Expression of a lineage-specific membrane attack complex/perforin family (MACPF)-like factor suggests possible effector function for these cells (MACPFA2 Ho et al. 2017). Several motile globular cells typically patrol the blastocoel, consistent with an immune surveillance function. (3) *Filopodial cells* include the primary phagocytes of echinoid larvae. This heterogeneous cell category possesses small, rounded cell bodies and extended long (10–50  $\mu\text{m}$ ) cytoplasmic projections, forming a dynamic intercellular network connecting the basal surfaces of the gut and ectodermal epithelia throughout the blastocoelar space (Ho et al. 2017). Upon bacterial challenge, a subpopulation of filopodial cells activates the echinoid-specific antimicrobial effector SpTransformer (185/333). (4) *Ovoid cells* are elliptical phagocytes that appear when bacteria are introduced into the blastocoel. These cells are not observed in unchallenged larvae. The developmental origin of these cells is not known but they may be an activated form of filopodial cell (Ho et al. 2017). (5) *Amoeboid cells* are small, highly motile comma-shaped cells (Gibson and Burke 1987; Ho et al. 2017). Their role in



**Fig. 8.4** Cellular immune response in the sea urchin larva. **(a)** A complex of several filopodial cells phagocytosing injected *Vibrio diazotrophicus* (*V.d.*). **(b)** An amoeboid cell in the blastocoel. **(c)** A globular cell with several filopodia in the blastocoel. **(d)** Pigment cells apposed to the ectoderm just after injection of *V.d.* showing inactivated extended morphology. **(e)** Pigment cells several hours after injection showing rounded activated morphology. **(f)** A group of interacting filopodial cells and pigment cells at site of injected *V.d.* **(g)** Site of injection 5 min after introduction of *Vibrio lentus*. Some cells have already begun to accumulate in area of bacteria. **(h)** Same region after 30 min. A large complex of several cell types has accumulated including filopodial cells, globular cells, pigment cells, and other uncharacterized cell types (surrounded by dotted line). *CP* coelomic pouch, *EC* ectoderm, *MG* midgut, *N* nucleus; arrow, pigment cell nucleus; arrowhead, filopodial cells; asterisk, selected bacterial cells

immunity is unclear, but they migrate rapidly to bacterial infection sites and carry out complex interactions with epithelia and other immunocytes (Ho et al. 2017).

In four-arm purple sea urchin larvae, exposure to laboratory *Escherichia coli*, either through blastocoelar microinjection or from the surrounding seawater, does not induce a strong response (Ho et al. 2017). However, *Vibrio diazotrophicus*, a marine bacterium isolated from adult *Strongylocentrotus droebachiensis* (Guerinot

et al. 1982), in culture seawater induces reproducible and synchronous changes in larval immunocyte behavior. Specifically, the midgut epithelium becomes dramatically thickened, and pigment cells retract their projections and migrate to the blastocoelar space and basal surfaces of the mouth, esophagus, and gut. Increased interactions between pigment cells and the various classes of blastocoelar cells are also observed (Ho et al. 2017). Pigment cell migration by 24 h is reduced considerably when larvae are exposed to killed vs. live *Vibrio* (Ho et al. 2017), so the response likely depends on activity of the living bacteria. In addition, a rapid and vigorous phagocytic response is induced when *Vibrio* are injected into the larval blastocoel (Ho et al. 2017).

#### 8.4.1 *The Larval Immune Gene Response and IL-17*

As mentioned above, the purple sea urchin genome encodes a broad panel of immune factors, including orthologs of transcription factors involved in vertebrate immunity (e.g., SCL/TAL2, E2A/HEB/ITF2, GATA1/2/3, PU.1), cytokines and receptors (e.g., IL-1, IL-17, TNF), complement factors (e.g., C3/4/5, Bf), antimicrobial peptides (e.g., Strongylocins), and an expansive repertoire of innate immune receptors (e.g., TLRs, NLRs, SRCRs) (for a comprehensive list, see Buckley and Rast 2017; Hibino et al. 2006). However, the degree to which this immune toolkit is utilized in the embryo and larval stages was initially unclear. This question was addressed by sequencing whole transcriptomes from four-arm *Strongylocentrotus purpuratus* larvae exposed to *Vibrio diazotrophicus* in a time series over a 24-hour period (Buckley et al. 2017). Coincident with the changes in immune cell behavior described above, expression of homologs of genes involved in every layer of vertebrate immune response also is evident, including transcription factors (Atf2, CEBP $\alpha$ , and  $\gamma$ , NF- $\kappa$ B), signaling adaptors (NF $\kappa$ B $\zeta$ , TNFAIP3, Traf6), cytokines (IL-17, TNF), and effectors (SpSoul1 and the echinoid-specific SpTransformer (185/333) family) (Buckley et al. 2017). Genomic analysis finds 35 *Strongylocentrotus purpuratus* IL-17 homologs separated into 10 subfamilies; a small group of IL-17 ligands belonging to two subfamilies (SpIL17-1 and SpIL17-4) are strongly upregulated early in response to bacterial exposure. Whole-mount in situ hybridization (WMISH) localizes expression of both families to the midgut and hindgut epithelia. Since these events happen very early in response to exposure and the gut lumen is a primary site of bacterial exposure and recognition, these results implicate the gut epithelium as a primary regulator of immune response. Another group, SpIL17-9, is expressed in a similarly rapid fashion in adult coelomocytes following *Vibrio diazotrophicus* injection.

Two IL-17 receptors (IL17-R1 and IL17-R2) have been identified in the purple sea urchin genome via the presence of SEF/interleukin-1 receptor (SEFIR) domains (Buckley et al. 2017; Buckley and Rast 2017; Hibino et al. 2006). WMISH experiments localize expression of both receptors at low levels to multiple tissues and cells throughout the larva (Buckley and Rast 2017). Larvae raised from zygotes

microinjected with antisense morpholinos targeting the IL17-R1 exhibit reduced expression of an array of downstream genes, including IL17-4, after *Vibrio diazotrophicus* exposure, suggesting a feedback mechanism (Buckley et al. 2017). Further study is required to elaborate other potential immune pathways, but these studies demonstrate that the IL17/IL17-R system is a major regulator of immune response in sea urchin larvae.

#### **8.4.2 Partitioning the Immune System Between Embryo, Larva, and Adult**

Although immune genes are expressed from the beginning of development, analysis of embryonic RNAseq profiles (Tu et al. 2014) indicates a sharp increase in the complexity of immune gene expression at the onset of feeding. This coincides with the differentiation of several immune cell types (Ho et al. 2017; Solek et al. 2013), the establishment of robust immune signaling systems (Buckley et al. 2017), and increasing complexity of microbial associations (see above). The immune system is further elaborated in terms of immune cell number and efficiency of response as the larva grows. The initial wave of immune cells is derived from embryonic mesoderm that is subdivided by a GRN involving transcription factors that have important homologs in vertebrate hematopoietic systems (Schrankel et al. 2016; Solek et al. 2013). The source of later larval immune cells remains unknown. In larvae, expression of the transcription factors necessary for early immune cell specification is maintained in the coelomic pouches and in a small group of mesenchymal cells at the aboral larval apex (Solek et al. 2013). These areas are candidate sources for the expansion of larval immune cells as the larva grows.

The immune cells of larvae and adults have similarities as well as differences (Ho et al. 2017; Solek et al. 2013). The red spherule cells of adults share expression of transcriptional regulators and enzymes with larval pigment cells, as do adult and larval phagocytic cells. Larval amoeboid cells share morphology and behavior with the amoeboid forms of adult colorless spherule cells. In contrast, there are no obvious morphological counterparts of vibratile cells and several other adult immune cell types in the larva. In terms of recognition and effector gene expression, there appear to be distinct differences between larvae and adults. Although some of these may fall away as different immune activation mechanisms are explored, it seems that the larva expresses a set of immune genes that overlap, but also differ, from those of the adult and that to some extent the genome encodes two immune systems that operate in these distinct life stages (Ho et al. 2017). How the immune system is elaborated during the course of larval development, how it may function at metamorphosis, and how the larval immune system contributes to that of the adult are questions that remain to be explored.

## 8.5 Asexual Reproduction and Regeneration

### 8.5.1 *Regeneration in Echinoderm Larvae*

Regeneration is an essential component of postembryonic development in animals, as it provides the option to rebuild vital organs and tissues. This process is widespread among invertebrates and has been extensively studied in adult echinoderms for over a century (Candia and Paolo 2010). Still, it has been known for some time that the capability of regeneration is not restricted to adults and that larvae can regenerate body parts as well. Generally, regeneration proceeds through a series of processes, which include wound healing, cell fate changes, cell migration and proliferation, as well as cell–cell signaling. In some animals, such as cnidarians, regeneration originates from highly proliferative stem cells (Holstein et al. 2003). The generality of this mechanism among animals remains unclear however.

Bipinnaria larvae of sea stars can regenerate most larval structures after bisection (i.e., cutting the animal into an anterior and posterior half), including the stomach, mouth, intestine, and anal opening (Vickery et al. 1999a, b, 2001a, b; Vickery and McClintock 1998). A recent study on cell proliferation during regeneration in the bat star *Patiria miniata* shows a marked reduction immediately following bisection, prior to the onset of wound-proximal proliferation (Hinman and Cary 2017). Transcriptome analysis of bisected larvae also suggests conservation of gene expression changes associated with wound healing and proliferation with those found in other animals with regenerative capabilities. Still, localization of specific target genes (e.g., SRAP—sea start regeneration-associated protease, vasa, dysferlin, and vitellogenin) and a detailed analysis of cellular changes occurring after bisection during the regeneration process (Oulhen et al. 2016) suggest that larval regeneration in *P. miniata* also involves broad cellular changes instead of just proliferation by pre-specified stem cell populations. These findings suggest that regeneration follows a distinct differentiation pattern. Future research will have to clarify these regenerative mechanisms in more detail, and sea urchin larvae provide an ideal model organism for such investigations.

### 8.5.2 *Asexual Reproduction Via Budding in Sea Urchins*

In contrast to vertebrates, where sexual reproduction is the predominant reproductive strategy, other animal taxa have evolved asexual life cycles involving the generation of larval or adult forms without a preceding embryogenesis. Larval budding has been described in four of the five echinoderm classes, i.e., ophiuroids (Balser 1998), asteroids (Rao et al. 1993), and more recently, echinoids (Eaves and Palmer 2003; Vaughn and Strathmann 2008) and holothuroids (Eaves and Palmer 2003). While the mechanisms underlying this process remain largely unknown, its widespread distribution warrants a more detailed analysis. A variety of abiotic factors such as



pH, salinity, temperature, and turbulence have been shown to induce budding (Allen et al. 2018). Additionally, it has been shown that conspecific larvae in the environment as well as predators may elicit a budding response in sea urchin larvae (Allen et al. 2018). Still, the mechanisms underlying these events, as well as their adaptive significance within the life cycle of an organism, remain elusive. Presumably, some mechanisms involved in regeneration are also important for budding as the larval body needs to be rebuilt. Other processes however may be quite different as regeneration likely involves a stronger response of the immune system during wound healing. Moreover, bisected larvae can draw from a pool of differentiated cells, while buds, which typically consist of only one or two cell types, need to transdifferentiate these cells into a variety of other cell types. It will be interesting to investigate the similarities and differences of molecular and cellular mechanisms between budding and regeneration among sea urchin larvae.

## 8.6 Synthesis

- Larval forms are morphologically distinct from the juvenile. Functional evidence on endocrine and immunity functions during larval development indicates the existence of similarities and differences between the larva and the adult.
- While hormones fulfill critical functions in the internal regulation of metamorphosis and settlement, the ecological transition from the planktonic to the benthic environment appears to involve interactions with bacteria, through biofilms or secreted factors.
- While cell death plays a key role in postembryonic development, its function is strongly linked to endocrine signaling and potentially the immune response.
- The complexity of immune gene expression and of the cellular immune system increases over the course of larval development.
- The acquisition of complex microbiota and the elaboration of cellular immunity and immune gene expression are temporally and functionally associated with the onset of feeding in planktotrophic larvae.

### Box 1

*Adaptive immunity:* Also known as anticipatory immunity, adaptive immunity is found in all vertebrates where immune receptors are somatically assembled from incomplete genomic elements and selected for binding to nonself in the individual. Immunoglobulins and T-cell receptor systems in jawed vertebrates and variable lymphocyte receptor (VLR) systems in jawless vertebrates are thus far the only well-described examples of this type of receptor system.

*Budding:* Form of asexual reproduction, which has been observed in a diversity of marine invertebrate larvae. It results in the formation of a new

(continued)

**Box 1** (continued)

individual by cleaving off ectoderm and endodermal cells from the larva, which can then form a completely new larval body.

*Holobiont*: A multicellular eukaryote host and its associated microbial symbionts (or microbiota). The combined genomes of the host and its microbiota (host genome + microbiome) are the hologenome.

*Innate immunity*: Protective systems based on nonself-recognition receptors that are directly encoded in the genome like any other gene. Receptors that mediate this type of immunity generally target highly conserved microbial molecules and associated infection signals. These systems are found throughout animals.

*Larva*: Developmental stage in animals with indirect life histories, preceding juvenile and/or adult stages and following embryogenesis. Larvae are dispersed through the water column, transition into a juvenile via a sometimes drastic metamorphosis, and frequently occupy a different habitat than the juvenile and/or adult. Bipinnaria and pluteus larvae are example of sea star and sea urchin larval stages, respectively.

*Larval skeletogenesis*: The formation of an endoskeleton in sea urchin and other larval forms. Sea urchin larvae require the skeleton to swim and feed. Note that in sea urchin larvae, skeletogenesis occurs in both, the larval and juvenile compartment.

*Metamorphosis*: Developmental transition from a larva to a juvenile, which typically involves the development of juvenile structures inside the larva as either a rudiment or imaginal disks. Once juvenile structures matured, larval structures will be destroyed as part of the metamorphic transition.

*Programmed cell death (apoptosis)*: A form of cell death, which is mediated by specific intracellular programs.

*Settlement*: Transition from the planktonic to the benthic habitat upon induction by specific environmental cues. In marine invertebrate larvae, settlement links the planktonic with the benthic part of the live cycle.

*16S*: A gene encoding the RNA component of the prokaryotic 30S small ribosomal subunit. Useful in both universal and specific identification of microbes due to the presence of both highly conserved regions, present in all prokaryotes, and hypervariable regions, which evolve more rapidly, and can distinguish closely related taxa to genus- or species-level resolution.

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# Chapter 9

## The *Ciona* Notochord Gene Regulatory Network



Michael Veeman

**Abstract** Complex gene regulatory networks are at the heart of cell fate specification and differentiation. The simple chordate *Ciona* has remarkable advantages for the dissection of these regulatory networks, including a stereotypically chordate but extremely small and simple embryo, a streamlined and compact genome, and highly efficient transgenesis by electroporation. Here we use the *Ciona* notochord as an example of how these characteristics can be exploited to understand both the early network controlling cell fate as well as the tissue-specific network controlling notochord differentiation and morphogenesis.

### 9.1 *Ciona* as a Model for Chordate GRN Analysis

Ascidians such as *Ciona* are among the closest invertebrate relatives of the vertebrates and are long-standing model organisms for studying chordate development (Passamaneck and Di Gregorio 2005; Satoh and Levine 2005; Munro et al. 2006; Lemaire 2009; Stolfi and Christiaen 2012; Satoh 2014). Adult *Ciona* are sessile, filter-feeding hermaphrodites. Their embryos, however, share a conserved chordate embryonic body plan with the vertebrates, including a hollow dorsal neural tube and notochord. Early development is extremely rapid, giving rise to a swimming chordate tadpole larva in only ~18 h. The tadpole consists of ~2000 cells comprising only 6 main tissues: epidermis, neural, muscle, notochord, mesenchyme, and endoderm. The egg is ~130–140  $\mu\text{m}$  in diameter, giving rise to a particularly small and compact embryo that can be imaged in its entirety and with fine subcellular detail in a single confocal field of view. Early development is highly stereotyped and proceeds along fixed and well-characterized cell lineages.

After the nematode *C. elegans*, *Ciona intestinalis* was the second animal genome to be sequenced (Dehal et al. 2002). It is similarly compact, with a heterochromatic

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genome of only ~150 Mb. It contains ~15,000 predicted genes, with a typical gene-gene spacing of ~10 kb. There is relatively little repetitive DNA. Phylogenies based on genomic information indicate that it is the tunicates, including *Ciona*, and not the cephalochordates that are the closest invertebrate relatives of the vertebrates (Delsuc et al. 2006). They diverged, however, before the two genome duplication events at the base of the vertebrate lineage and consequently have simpler sets of developmentally important genes such as signaling molecules and transcription factors (Panopoulou et al. 2003; Dehal and Boore 2005). Ascidian genomes are extremely polymorphic, likely reflecting both a high mutation rate and a large effective population size (Small et al. 2007; Tsagkogeorga et al. 2012).

*Ciona* are prolific broadcast spawners and produce large amounts of sperm and eggs. They can be induced to spawn naturally or else sperm and eggs can be easily removed by dissection. It is straightforward to obtain thousands of synchronously developing embryos that require only natural or artificial seawater as a culture medium to reach the swimming larval stage. The eggs or early embryos can be microinjected, but one can also introduce plasmid DNAs into fertilized eggs by a straightforward and very efficient electroporation procedure (Corbo et al. 1997b). This allows for fast-paced research involving transgenesis, including dominant-negative constructs, tagged protein fusions, and, in particular, reporter constructs to map candidate *cis*-regulatory regions.

*Ciona intestinalis* is the ascidian most widely studied as a model organism. It is highly invasive (Zhan et al. 2010) and currently has a broad global distribution in temperate saltwater harbors and marinas that makes it readily available to investigators in North America, Europe, Japan, and elsewhere. There are two cryptic types of *Ciona intestinalis* that differ genetically and in subtle aspects of both larval and adult morphology (Caputi et al. 2007; Nydam and Harrison 2011; Pennati et al. 2015). The Type A (thought to be originally from the Pacific) and Type B (originally Atlantic) populations are relatively cross-fertile in the lab (Sato et al. 2014) but only overlap in a small part of the English Channel. Population genetic analysis shows there has been little or no gene flow in the sympatric zone for several thousand years (Bouchemousse et al. 2016). It has thus been suggested that Type A and Type B represent distinct species, with Type A best known as *Ciona robusta* and Type B as the true *Ciona intestinalis* (Pennati et al. 2015). The original genome sequence and the majority of experimental work have been on Type A, and the ascidian community is currently in a state of flux as to the best terminology.

Outside the *Ciona intestinalis/robusta* species complex, several other ascidians are also used as model organisms, including *Ciona savignyi*, *Halocynthia roretzi*, *Boltenia villosa*, *Phallusia mammillata*, *Botryllus schlosseri*, and various species of *Molgula*. *Phallusia* has a particularly transparent egg and embryo and is becoming increasingly popular for the direct imaging of living embryos. *Halocynthia* has an egg more than twice the size of *Ciona*, making approaches based on microsurgery and single-blastomere microinjection more straightforward. The early fate specification of ascidian cell types including notochord has been studied in parallel in *Halocynthia* and *Ciona*, and I apologize to my *Halocynthia* colleagues for the *Ciona*-centric focus of this review.

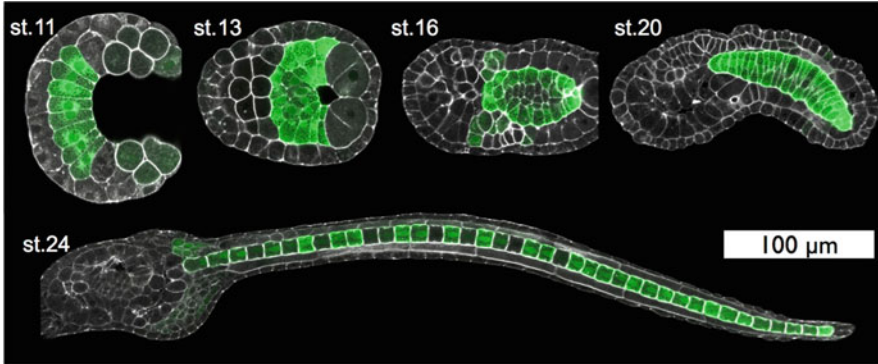
Excellent foundations are in place for the systematic analysis of *Ciona* gene regulatory networks (GRNs). There are published in situ hybridization expression patterns for most of the predicted transcription factors and signaling molecules and many other genes (Satou et al. 2001b; Kusakabe et al. 2002; Fujiwara et al. 2002; Imai et al. 2004; Azumi et al. 2007). A provisional GRN for the early embryo has been constructed by knocking down 104 different transcription factors and signaling genes and assessing the transcriptional effects on the other components (Imai et al. 2006). ChIP-chip data has been published for several of the most important early transcription factors (Kubo et al. 2010). Morpholino-based gene knockdown is widely used (Satou et al. 2001a), and TALEN and CRISPR approaches are becoming common as well (Yoshida et al. 2014; Sasaki et al. 2014; Stolfi et al. 2014; Sasakura et al. 2017; Gandhi et al. 2017). Strong community databases help to integrate genomic, gene expression and functional data (Sierra et al. 2006; Endo et al. 2011; Brozovic et al. 2016, 2017). For a broader discussion of cis-regulatory analysis in *Ciona*, there are several excellent reviews (Kusakabe 2005; Kubo et al. 2009; Wang and Christiaen 2012; Stolfi and Christiaen 2012).

## 9.2 The *Ciona* Notochord as a Model for Understanding Morphogenesis and Differentiation

Many aspects of *Ciona* development have been studied in the context of transcriptional regulatory networks, including the early embryo as a whole (Imai et al. 2006), the neural lineages (Esposito et al. 2017; Imai et al. 2009), and the presumptive cardiopharyngeal mesoderm (Tolkin and Christiaen 2012). In this review we focus on the developing notochord. It expresses a rich and dynamic set of morphogenetic cell behaviors (Jiang and Smith 2007), making it an excellent model for studying the transcriptional regulation of morphogenesis.

The ascidian notochord consists of only 40 cells that intercalate into a tapered single-file column. The anterior 32 “primary” notochord cells are derived from blastomeres A7.3 and A7.7, which are anterior vegetal cells located at the equatorial margin of the embryo in contact with anterior animal cells (Nishida 1987). The early embryo is bilaterally symmetric, so there are four primary notochord cells when first induced at the 64-cell stage. Near the onset of gastrulation, these divide once mediolaterally to form an arc of eight cells. The final two divisions are oriented parallel to the AP axis and transform the arc into a flat plate of 32 cells (Carlson et al. 2015). The posterior eight notochord cells are derived from the posterior vegetal B8.6 cell pair, which divide twice to give eight cells at the posterior of the notochord plate. Figure 9.1 shows an overview of key stages in notochord morphogenesis.

The 40 postmitotic cells in the notochord primordium become mediolaterally elongated and aligned as they intercalate with one another to form a single-file column (Munro and Odell 2002). As in vertebrates, this process of convergent extension involves mediolaterally biased cell protrusions and is dependent on the



**Fig. 9.1** *Ciona* notochord morphogenesis. Confocal images of *Ciona* embryos electroporated with a notochord-specific *Bra*>GFP transgene (green). Cell cortices are labeled with phalloidin (white). Not every notochord cell is labeled because of transgene mosaicism, and there is also fainter ectopic expression in mesenchyme cells in some image. The stages indicated are from the series of Hotta (Hotta et al. 2007a), and all the embryos are oriented with anterior to the left

noncanonical Wnt/planar cell polarity pathway (Jiang et al. 2005). An FGF ligand expressed in the overlying medial neural plate has been proposed to act as a guidance cue for intercalating notochord cells (Shi et al. 2009), but, as in vertebrates, the cues controlling mediolateral intercalation remain mysterious. Ephrin signaling has also been implicated in notochord intercalation, possibly by influencing apical-basal polarity independent of PCP-dependent planar polarity (Oda-Ishii et al. 2010).

Notochord cells from the left and right halves of the embryo do not alternate perfectly but instead show considerable variation in their precise patterns of intercalation (Nishida 1987). This is one of the few developmental processes in ascidians that is not highly stereotyped. It is not, however, completely random, and anterior-posterior position in the notochord primordium is highly correlated with anterior-posterior position in the intercalated notochord (Carlson et al. 2015). Intercalation in the secondary notochord lineage is considerably more stereotyped than in the primary lineage.

As in other chordates, *Ciona* notochord morphogenesis is intimately associated with the formation of a perinotochordal basement membrane. Mutation of *Laminin- $\alpha$ 3/4/5* leads to severe defects in notochord development (Veeman et al. 2008). The early intercalation of notochord cells is relatively normal, but there is a failure of “boundary capture” behaviors at the notochord periphery. When intercalating notochord cells reach across to make contact with muscle and other adjacent cell types, they normally flatten out and make a smooth boundary. In the *Laminin- $\alpha$ 3/4/5* mutant, they instead show persistent protrusive behaviors and will intercalate between adjacent non-notochord cells and migrate chaotically within the tail. Fibronectin has also been shown to be important for notochord morphogenesis but with a distinct CRISPR phenotype in which intercalation begins but fails to complete (Segade et al. 2016).

When the notochord cells complete intercalation to form a single-file column of 40 cells, they are initially shaped like highly oblate cylinders. Over the next several hours, they change from disk-shaped to drum-shaped. This cell-shaped change drives extensive elongation of the tail and is dependent on actomyosin-based cell contractility (Dong et al. 2011). Late in this process, the cells develop a distinctive equatorial groove as a result of flows of cortical actomyosin that form a contractile belt at the equator (Sehring et al. 2014).

As in many chordates, the post-intercalation *Ciona* notochord tapers from a thicker middle to finer anterior and posterior ends. This involves both unequal cleavages in the notochord lineage as well as regional differences in the timing of when cells complete intercalation (Veeman and Smith 2013). Cells nearer the ends complete intercalation first, giving them a head start on the disk-to-drum shape change that makes them narrower.

After the disk-to-drum shape change, distinctive pockets start to form at the center of the round contact surfaces between adjacent notochord cells. Unlike the intracellular vacuoles that form in many vertebrate notochords, these pockets are extracellular (Dong et al. 2009). The pockets increase in size until they eventually connect to form a continuous lumen running the length of the notochord. The connection between adjacent pockets does not involve a membrane fusion event but instead a partial reversal of intercalation that allows adjacent pockets to connect through cell-cell junctional rearrangements. The initial formation of the extracellular lumen pockets involves the acquisition of an unusual form of epithelial polarity in which apical polarity markers become localized to the notochord-notochord contact surfaces at *both* the anterior and posterior side of each cell (Denker et al. 2013). Polarity markers become further localized such that apical markers are localized to the walls of the extracellular lumen pockets and basolateral markers to the periphery of the cell. At these stages, the notochord can be thought of as a bizarre-stacked epithelium in which each cell in the column has two separate apical surfaces.

There are other manifestations of cell polarity in the *Ciona* notochord that are likely independent of apical-basal polarity. Myosin light chain becomes enriched on the anterior notochord cell surfaces during the disk-to-drum shape change (Newman-Smith et al. 2015), and the cell nuclei become localized to the posterior (Jiang et al. 2005). These phenomena are at least partially dependent on the planar cell polarity pathway and present an unusually simple “one-dimensional” system to study planar polarity (Kourakis et al. 2014).

Despite the notochord lumen being extracellular, the ascidian notochord is similar to vertebrates in that secretory function leads to a volume increase that is resisted by the perinotochordal ECM. In *Ciona* this requires an SLC26 family member, which is a putative electroneutral anion transporter (Deng et al. 2013). Although turgor pressure within the notochord has never been directly measured, it is inferred to exist and to contribute to the notochord’s mechanical properties as a flexible but incompressible hydrostatic skeleton that helps to convert alternating contractions of the left and right blocks of tail muscle cells into the whiplike motion of the tail in larval swimming.

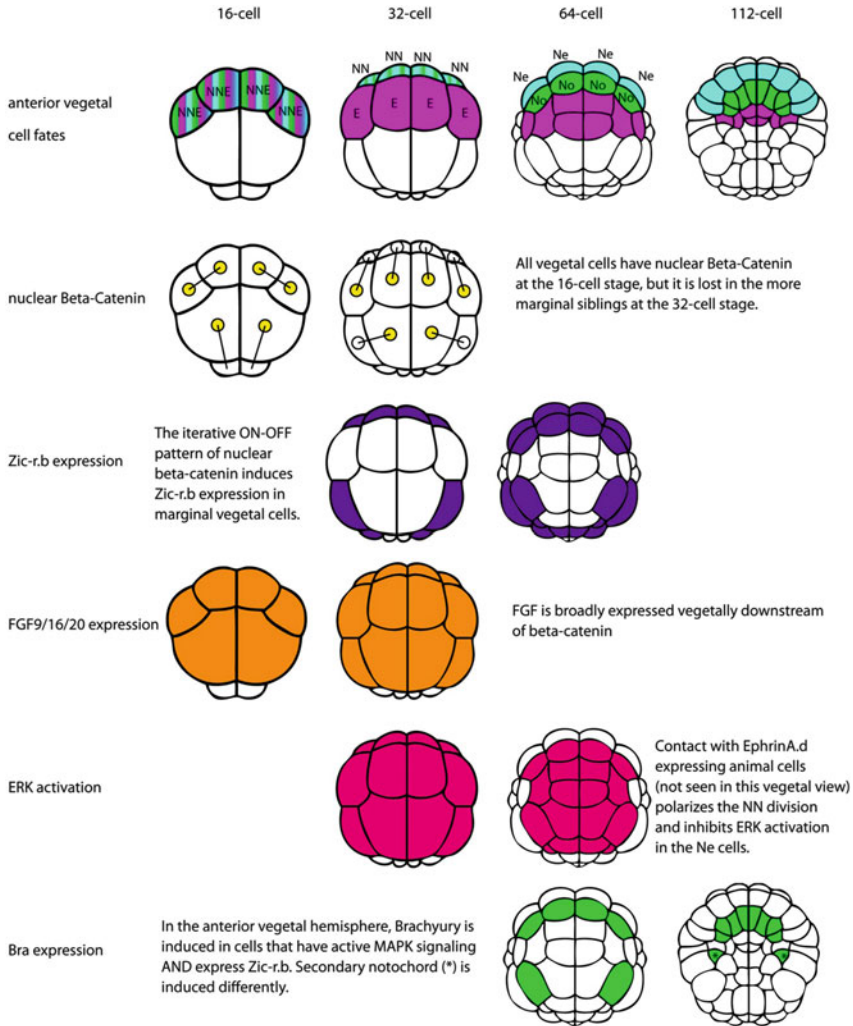
### 9.3 Establishing Primary Notochord Fate

Cell fate specification in primary (A-line) notochord has been intensively studied, and a causal chain of events is now understood that links symmetry-breaking events in the fertilized egg all the way to the tissue-specific expression of the key notochord transcription factor Brachyury (Bra) at the 64-cell stage. Two consecutive cytoplasmic rearrangements in the first cell cycle establish orthogonal patterning systems (Nishida 2005). The first ooplasmic segregation is required to establish distinct germ layers along the animal-vegetal axis, with ectodermal fates largely derived from the animal hemisphere, endodermal fates from the vegetal pole, and mesodermal fates from the vegetal hemisphere margin. The second ooplasmic segregation establishes “posterior” versus “anterior” fates though, as noted by Lemaire (2009), these commonly used labels are not perfectly aligned with the true AP axis.

The *Ciona* embryo is bilaterally symmetric, and its first three cleavages are all nearly orthogonal. The first division separates the future left and right halves, the second division separates anterior and posterior fates, and the third division establishes distinct animal and vegetal hemispheres. The anterior animal (a-line), posterior animal (b-line), anterior vegetal (A-line), and posterior vegetal (B-line) quadrants all exhibit distinct signatures of zygotically expressed genes as early as the 16-cell stage (Imai et al. 2006). This includes the signaling molecules EphrinA.d, which is broadly expressed in the animal hemisphere (Picco et al. 2007), and Fgf9/16/20, which is expressed in most of the vegetal hemisphere (Imai et al. 2002a; Bertrand et al. 2003). The primary notochord becomes fate-restricted at the 64-cell stage from anterior vegetal cells near the animal-vegetal margin of the embryo.

The first ooplasmic segregation leads, through an uncertain mechanism, to the nuclear localization of maternal beta-catenin on the vegetal side of the embryo and the specification of mesendodermal cell fates (Hudson et al. 2013; Imai et al. 2000, 2016). At the 16-cell stage, there are two anterior vegetal cell pairs, A5.1 and A5.2, which have been referred to as the “NNE” cells as they largely give rise to notochord, posterior neural, and endodermal fates. Both of these cell pairs have nuclear beta-catenin, which is required for NNE fate (Hudson et al. 2013). A5.1 and A5.2 each divide to give a more vegetal daughter (“E”: A6.1 and A6.3) that is restricted to endoderm and a more animal daughter that contributes to notochord and posterior neural fates (“NN”: A6.2 and A6.4). This lineage decision again involves beta-catenin, which is nuclear at the 32-cell stage in the E cells but not the NN cells (Hudson et al. 2013). Figure 9.2 shows an overview of key expression patterns and interactions in primary notochord fate specification.

The NN cells A6.2 and A6.4 each divide to give one daughter that contributes to posterior neural plate (Ne: A7.4 and A7.8) and one that gives rise to primary notochord (No: A7.3 and A7.7). The Ne cells are in direct contact with cells from the anterior animal quadrant, whereas the No cells are not. This segregation of notochord and neural fates is dependent on FGF signaling (Yasuo and Hudson 2007). Several FGFs are expressed in the vegetal hemisphere, but the FGF signal transducer ERK only becomes activated by phosphorylation in the No cells and not



**Fig. 9.2** Primary notochord induction. Key gene expression patterns and interactions are shown at the indicated stages. All cartoon views are of the vegetal side of the embryo with anterior to the top. Blastomeres labeled as NNE are fated to predominantly form neural tissue, notochord, and endoderm. The E cells are largely fate-restricted to endoderm, whereas the NN cells still have mixed neural/notochord fate. The No cells are fate-restricted to notochord, whereas the Ne cells contribute mostly to posterior neural fates

the Ne cells. This differential competence involves Ephrin signaling from the animal hemisphere (Picco et al. 2007). The entire animal hemisphere expresses EphrinA.d, which polarizes the NN cells such that they divide to give a more animal daughter in which FGF signaling is inhibited and a more vegetal daughter that is competent to respond to broadly expressed FGFs by becoming notochord. Both Fgf9/16/20 and

Fgf8/17/18 are important, with FGF9/16/20 most critical in the early Ne/No binary fate switch, while both are involved in later aspects of establishing/maintaining notochord fate (Yasuo and Hudson 2007).

As in all chordates, the T-box transcription factor Bra is a key marker of notochord fate (Yasuo and Satoh 1993; Showell et al. 2004). In vertebrates and cephalochordates, Bra is initially expressed broadly throughout the presumptive mesoderm but becomes restricted to only the axial mesoderm (presumptive notochord) over time. In *Ciona*, Bra is expressed only in cells fate-restricted to notochord (with the exception of transient expression in the parent of the secondary lineage founder cell B7.3) (Corbo et al. 1997b) and does not have broader roles in mesoderm induction.

Bra induction is dependent on the transcription factors FoxA.a (formerly known as Fkh/HNF3 $\beta$ ), FoxD, and Zic-r.b (formerly ZicL) (Imai et al. 2002b, c, 2006), as well as FGF signals (Imai et al. 2002a; Yasuo and Hudson 2007) likely transduced by ETS class transcription factors (Miya and Nishida 2003; Hudson et al. 2016). FoxA.a, FoxD, Zic-r.b, and Fgf9/16/20 are all downstream of beta-catenin signaling, and both FoxD and Fgf9/16/20 are direct transcriptional targets (Imai et al. 2002b; Oda-Ishii et al. 2016).

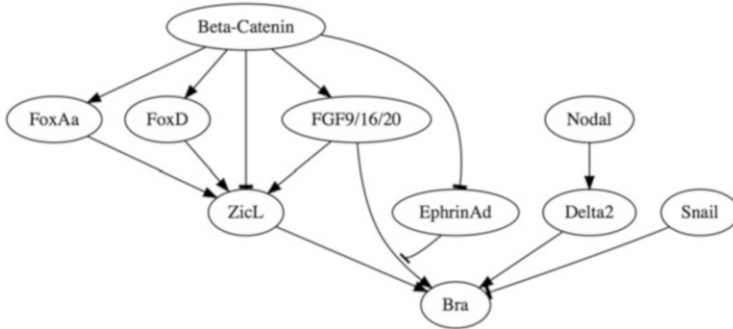
Zic-r.b acts downstream of FoxA.a and FoxD (Imai et al. 2002c, 2006) and is ultimately restricted to marginal cells through a differential role for beta-catenin signaling in consecutive cell cycles (Hudson et al. 2013, 2016). FoxA.a, FoxD, and Fgf9/16/20 are all induced in the NNE cells at the 16-cell stage and are required for NNE cell fates. At the 32-cell stage, the NN and E cells both continue to express FoxA.a, FoxD, and FGF9/16/20 but differ in that nuclear localization of beta-catenin persists in E but is lost in NN. In the E cells, nuclear beta-catenin together with these other mesendodermal determinants induces the endoderm-specific transcription factor Lhx3. In the absence of nuclear beta-catenin in the NN cells, FoxA.a, FoxD, and Fgf9/16/20 induce Zic-r.b expression (Hudson et al. 2016). Zic-r.b is also under negative regulation by the transcriptional repressors Prdm1-r.a and Prdm1-r.b which restrict its expression in the anterior animal hemisphere (Ikeda and Satou 2017).

At the 64-cell stage, the E cells express Lhx3 and phosphoERK, the No cells express Zic-r.b and phosphoERK, and the Ne cells express Zic-r.b but lack ERK activation because it is repressed by Ephrin signaling from the animal hemisphere. For A-line notochord, it is likely this overlap of Zic-r.b expression and FGF-dependent ERK activation that induces Bra expression. Figure 9.3 shows a network diagram of transcriptional and signaling interactions controlling Bra expression.

## 9.4 Secondary Notochord Induction

The mechanisms of Bra induction are somewhat different in the B-line (posterior vegetal)-derived secondary notochord lineage, which requires Delta/Notch signaling but not Zic-r.b (Imai et al. 2002c, 2006; Hudson and Yasuo 2006). Figure 9.4 shows an overview of key expression patterns and interactions. The earliest B-line



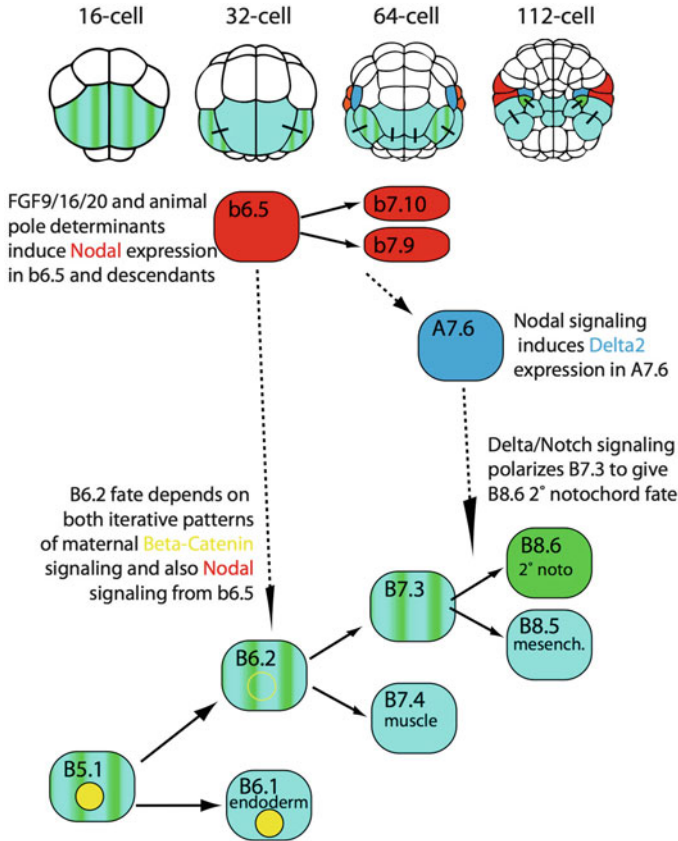


**Fig. 9.3** Gene regulatory networks upstream of Brachyury. Composite view of the best characterized regulatory interactions upstream of Bra. Positive interactions are noted with an arrowhead and negative interactions with a bar. Most but not all of these interactions have been shown to be direct. Not all of these interactions would be active in any particular cell type/stage

blastomere to be fate restricted to notochord is B8.6 at the 112-cell stage. Bra, however, is first expressed in B8.6's parent B7.3 at the 64-cell stage and is rapidly downregulated in the sibling cell B8.5 which instead forms mesenchyme.

A two-step relay mechanism involving both Nodal and Delta/Notch signaling is required for secondary but not primary notochord induction (Hudson and Yasuo 2006). This involves a lateral signaling center in the b6.5 cell pair that is also required for mediolateral patterning in the A-line (posterior) neural plate (Hudson and Yasuo 2005). The b6.5 cell pair is located at the sides of the 32-cell embryo near both the animal/vegetal boundary and the anterior/posterior boundary. It expresses Nodal, and Nodal expression persists in its descendants until gastrulation. The mechanisms controlling Nodal expression in b6.5 are not well understood, but it is downstream of Fgf9/16/20.

Nodal inhibition blocks secondary but not primary notochord induction (Hudson and Yasuo 2006). Single-blastomere injections of antimorphic Nodal receptor at the 8-cell stage show that Nodal signaling is required for secondary notochord induction in the B-line cells from which the secondary notochord arises, but blocking Nodal signaling in the A-line (anterior vegetal) cells unexpectedly blocked secondary notochord induction as well. Nodal thus acts in at least two different ways in secondary notochord induction. It acts in the B-line, most likely through signaling from b6.5 to the secondary notochord precursor cell B6.2; but it also acts more indirectly via effects on A-line-derived cells. This latter interaction has been shown to involve a relay mechanism using Delta2, which is expressed in A7.6 downstream of Nodal expression in its neighbor b6.5. A7.6 is in contact with B7.3 and is required for inducing secondary fate via canonical, gamma-secretase-dependent, Su(H)-dependent Notch signaling. This induction is likely direct, as there are functionally important Su(H) sites in the *Bra* enhancer (Corbo et al. 1998). As in the primary notochord, secondary notochord induction also requires FoxA.a, FoxD, and Fgf9/16/20, though not Zic-r.b.



**Fig. 9.4** Secondary notochord induction. Key gene expression patterns and interactions are shown at the indicated stages. All cartoon views are of the vegetal side of the embryo with anterior to the top

## 9.5 The Notochord Transcriptome

Until recently, most of the known notochord-enriched genes came from a subtractive hybridization screen for genes upregulated in response to misexpression of *Bra* in endoderm and other tissues under the control of the *FoxA.a* enhancer (Takahashi et al. 1999a; Hotta et al. 2000). This identified ~40 *Bra*-inducible genes with varying degrees of enrichment in the notochord. These have become known as “Brachyury-downstream” genes, as they are likely to be direct or indirect targets of *Bra*. This initial Brachyury-downstream gene set has been tremendously valuable and formed the foundation for functional studies of these genes’ roles in notochord morphogenesis (Hotta et al. 2007b, 2008) as well as the regulation of notochord-specific

expression (Di Gregorio and Levine 1999; Dunn and Di Gregorio 2009; Takahashi et al. 2010).

Another strategy for identifying *Ciona* notochord genes is by orthology to known notochord genes in other chordates. Kugler and collaborators examined the expression of 73 *Ciona* orthologs of vertebrate genes known to be expressed in the notochord and found that 20 were expressed in the *Ciona* notochord (Kugler et al. 2008). Interestingly, they found that six of these were not upregulated in response to *FoxA.a*>Bra, suggesting that there is a class of notochord gene that would be missed by screening for transcriptional targets of Bra misexpression.

More recently, two groups have taken an alternate strategy based on transcriptional profiling of notochord cells purified by flow cytometry from dissociated embryos expressing a notochord-specific fluorescent transgene (Jose-Edwards et al. 2011; Reeves et al. 2017). Jose-Edwards and colleagues used microarray-based profiling on sorted notochord cells to identify seven new transcription factors expressed in the notochord: Spalt-like A (*sallA*), Lmx-like, Stat5/6-b, Fos-a, NFAT5, AFF, and Klf5 (Jose-Edwards et al. 2011).

Reeves and colleagues performed RNAseq-based transcriptional profiling on sorted notochord and not-notochord cells from stage 16 (early intercalation) and stage 19.5 (mid-intercalation) and identified 1364 genes with enriched expression in the notochord (Reeves et al. 2017). Extensive validation by in situ hybridization showed that ~90% of the tested genes were expressed in the notochord at the predicted stages. This notochord-enriched gene set was enriched for gene ontology terms related to the extracellular matrix, cell adhesion, and cytoskeleton, and orthologs of 112 of the genes had previously characterized notochord expression in either mice or zebrafish.

Previous analysis of gene expression in the notochord had drawn a distinction between “notochord-specific” genes expressed only in the notochord and “notochord-predominant” genes expressed in the notochord as well as in distinct other cell types but not ubiquitously (Hotta et al. 1999). The notochord RNAseq data, however, suggests that these are not meaningful distinctions as there is a smooth and continuous distribution of notochord enrichment scores.

Reeves and collaborators revisited the *FoxA.a*>Bra overexpression experiment but used RNAseq instead of subtractive hybridization as a sensitive, genome-wide readout. They identified 925 genes upregulated by Bra misexpression but found surprisingly little overlap, only ~20%, between these and the FACSseq notochord-enriched gene set. Many notochord-enriched genes are not induced in response to Bra misexpression, indicating that the “Brachyury-downstream” genes may not be a representative subset of notochord genes.

## 9.6 Cis-regulatory Control of *Brachyury* Expression

The transcriptional regulation of notochord-specific expression has received considerable attention. The first gene to be analyzed was *Brachyury* itself in the paper that first established electroporation as a viable method for *Ciona* transgenesis (Corbo et al. 1997b). Corbo and colleagues identified a 434 bp region upstream of the *Bra* transcriptional start site that drove relatively notochord-specific expression in reporter assays. This enhancer region contains two predicted suppressor of hairless-binding sites that have been shown to indeed function downstream of Su (H) (Corbo et al. 1998) and likely act in vivo as part of the Notch/Delta relay for secondary notochord induction. The enhancer region also contains Snail sites that are essential for repressing *Bra* expression in muscle cells (Fujiwara et al. 1998). Zic-r.b is a direct transcriptional activator of *Bra* and binds to two distinct binding sites in the minimal enhancer (Yagi et al. 2004). FGF signaling acts directly on the *Halocynthia Bra* enhancer via ETS-binding sites (Miya and Nishida 2003), but this has not yet been experimentally confirmed for the main *Bra* enhancer in *Ciona*.

*Bra* also has an upstream “shadow” enhancer that can drive notochord-specific expression independent of the proximal 434 bp region discussed above (Farley et al. 2016). This CRM depends on both a Zic-r.b site and two ETS sites, consistent with the essential role of Zic-r.b and FGF in primary notochord induction. Interestingly, all of the Zic-r.b and ETS sites in the shadow enhancer are predicted to have relatively low affinity for their cognate TFs but are optimally spaced and oriented for strong, notochord-specific expression. This fits with a model in which transcription factor binding sites might be tuned by evolution and frequently “suboptimized” to balance both the strength and the tissue specificity of expression (Farley et al. 2015, 2016).

Tissue-specific transcriptional regulators are often involved in positive feedback loops that help to mediate stable and irreversible cell fate decisions (Crews and Pearson 2009). Morpholino knockdown of *Bra*, however, actually leads to an increase in *Bra* expression, indicating that it may be a negative regulator of its own expression (Imai et al. 2006). There is evidence for positive autoregulation in the *Halocynthia Bra* enhancer (Takahashi et al. 1999b), but there also appear to be considerable differences in notochord induction between these species (Darras and Nishida 2001; Lemaire 2009; Takatori et al. 2010; Hudson et al. 2016). *Bra* autoregulation remains underexplored given its potential functional importance.

## 9.7 Notochord Effector Gene Regulation

Because of the ease of cis-regulatory analysis by the electroporation of reporter constructs (Corbo et al. 1997b) coupled with the early identification of a large number of notochord-specific genes (Takahashi et al. 1999a; Hotta et al. 2000), the cis-regulatory basis of tissue-specific expression in the *Ciona* notochord has

received considerable attention. Many notochord-specific genes are direct transcriptional targets of Brachyury and/or FoxA.a, but others are expressed without direct input from these trans-acting factors (Di Gregorio and Levine 1999; Passamanek et al. 2009; Katikala et al. 2013; Jose-Edwards et al. 2015). Unlike many of the other transcription factors upstream of *Bra* expression, *FoxA.a* is highly expressed in the differentiating notochord, although it is also expressed in other midline structures including the ventral neural tube and endodermal strand (Corbo et al. 1997a).

*Tropomyosin-like* was the first Brachyury-downstream gene shown to be a direct Bra target (Di Gregorio and Levine 1999). Notochord-specific expression was mapped to a 114 bp enhancer upstream of the transcriptional start site containing two predicted Bra sites that bound Bra protein in gel-shift assays. Another Brachyury-downstream gene, *Leprecan*, is also a direct Bra target (Dunn and Di Gregorio 2009). A 581 bp CRM driving notochord-specific expression was identified roughly aligned with *Leprecan*'s eighth intron. The 3' end of this minimal region contains several predicted Bra-binding sites that bind Bra in vitro in gel-shift assays and are required for notochord expression in reporter assays.

Direct binding of FoxA.a was first demonstrated for a cis-regulatory module (CRM) associated with *tune*, a novel gene named for its expression in tunicate notochord and endoderm (Passamanek et al. 2009). An upstream region of only 155 bp recapitulates its normal expression pattern in reporter assays and contains two predicted Bra sites and one FoxA.a site. Both the FoxA.a site and one of the Bra sites are essential for reporter activity and both bind their cognate proteins in gel-shift assays.

Takahashi and colleagues systematically tested the upstream 3 kb regions of 20 Brachyury-downstream genes in reporter assays and found that 8/20 gave the expected expression pattern (Takahashi et al. 2010). They mapped these eight more finely and found that they could typically be truncated down to 0.5–1 kb regions while maintaining the normal expression pattern.

Katikala and colleagues tested candidate regions from 17 notochord genes and identified regions driving notochord expression for 10/17, though several of these were also expressed ectopically (Katikala et al. 2013). They mapped these more finely to 65–300 bp minimal regions that could still drive notochord expression, and then identified and mutated all the potential Bra-binding sites. They found four genes where a single Bra site was required for notochord expression. They found another four genes where two Bra sites acted cooperatively, in the sense that mutating either site alone reduced but did not eliminate notochord expression but mutating both sites together did eliminate it. They also found two genes where the minimal CRM contained no predicted Bra-binding sites. Remarkably, there was a correlation between these three classes of minimal CRM and the onset of expression of the endogenous gene. The genes with two cooperative Bra sites tended to be first expressed earliest, during early-mid gastrulation. The genes with a single Bra site were first expressed during mid-late gastrulation. The genes with no Bra sites did not initiate expression until late in neurulation.

This correlation suggested a model in which genes with multiple cooperative Bra sites are induced first at low levels of Bra expression. The target genes with single

Bra sites are induced somewhat later as Bra expression rises, and the later genes are activated indirectly by intermediary transcription factors. It is a compelling model, but it remains to be determined whether it can fully explain the temporal dynamics of direct and indirect Bra target genes.

Jose-Edwards and collaborators dissected 14 *Ciona* CRMs driving notochord expression by systematically mutating predicted transcription factor binding sites (Jose-Edwards et al. 2015). They found that 9 of the 14 required Bra and/or FoxA.a sites for notochord expression but that 6 required other motifs including putative bHLH, KLF, and Myb sites. For the genes with essential Bra/FoxA.a sites, there was little evidence for any consistent pattern or arrangement of these sites. It appears that notochord enhancers are extremely diverse, both in terms of the transcription factors involved and the arrangement of their binding sites.

Despite extensive analysis of the cis-regulatory control of numerous notochord genes, the structure of the GRN acting downstream of Bra remains poorly understood. Bra ChIP-seq identified more than 2000 *Ciona* genes with evidence for Bra occupancy, suggesting that a very large number of genes could potentially be regulated directly (Kubo et al. 2010). Arguing against a shallow regulatory model, however, are the observations that there are many other notochord-enriched transcription factors that first become expressed over a broad range of stages (Jose-Edwards et al. 2011; Reeves et al. 2017), that many notochord genes are not upregulated by Bra misexpression (Capellini et al. 2008; Reeves et al. 2017), and that several CRMs have been dissected that drive notochord expression independent of Bra sites (Jose-Edwards et al. 2015). Most of these other notochord TFs have not yet been studied for a role in notochord differentiation, but Tbx2/3 has been shown to be required for normal notochord morphogenesis and to directly or indirectly regulate at least 20 notochord genes (Jose-Edwards et al. 2013).

In addition to the likely complexity of the GRN acting within the differentiating notochord, there is also some evidence that posttranscriptional regulation may be important in controlling notochord-specific gene expression. The microRNA *miR-124* is enriched in the developing nervous system, and many of the genes it is predicted to silence are notochord-enriched genes downstream of Bra (Chen et al. 2011).

## 9.8 Fine Spatial Patterning

Most of the known notochord genes are expressed uniformly within the notochord, but several genes have been identified that are differentially expressed in notochord subregions (Reeves et al. 2014). These include genes that are differentially expressed between the primary and secondary notochord lineages, genes that are upregulated at the anterior and posterior notochord tips, and a TGF- $\beta$  ortholog that is expressed in a posterior to anterior gradient. There are also multiple genes that are expressed in a stochastic subset of notochord cells that vary from embryo to embryo (Oda-Ishii and Di Gregorio 2007; Reeves et al. 2014). The functional significance of these

expression patterns is not known, and the mechanisms controlling them are not understood. It is clear, however, that the notochord is more finely spatially patterned than previously believed.

## 9.9 Is *Brachyury* a True Master Regulator Gene?

The master regulator concept holds that many cell types (or tissues, structures, organs, etc.) are defined by the expression of a single cell type-specific transcription factor that not only controls the transcriptional program within that cell type but can also reprogram other cell types to that fate (Chan and Kyba 2013). MyoD, for example, has been described as a master regulator of muscle fate as it can reprogram fibroblasts and other cell types to become a muscle (Weintraub et al. 1989). It is an appealing concept, both in terms of understanding fate specification in early embryos as well as understanding how animal body plans might evolve.

Bra has been widely described as a master regulator of ascidian notochord fate (Yasuo and Satoh 1998; Takahashi et al. 1999a). It is the earliest known marker of the presumptive notochord cells (Yasuo and Satoh 1993; Corbo et al. 1997b), and it is required for notochord fate (Chiba et al. 2009). Misexpressing Bra outside the notochord induces expression of a large number of genes normally expressed in the notochord (Takahashi et al. 1999a). There is a degree of circularity to that argument, however, as until recently most of the known notochord genes were specifically identified by virtue of being upregulated in response to *FoxA.a*>Bra (Takahashi et al. 1999a). RNAseq analysis of both flow-sorted notochord cells as well as *FoxA.a*>Bra misexpressing embryos, however, shows that ~80% of notochord-enriched genes are not upregulated in response to Bra misexpression (Reeves et al. 2017). This suggests that Bra might not be a true master regulator gene as formally defined, as misexpressing it outside the notochord only partially reprograms other cells to a notochord-like transcriptional profile.

One possible explanation is that Bra indeed has a unique position at the top of the transcriptional cascade for notochord differentiation but is unable to completely reprogram other cell types to notochord fate because of the cell-type-specific regulatory networks already at work in those cells. A non-exclusive alternative explanation is that Bra does not act alone at the top of the notochord-specific transcriptional hierarchy but instead acts in parallel with other yet to be identified factors. Jose-Edwards and colleagues found that the notochord transcription factor Lmx-like is expressed normally in Bra mutants, giving initial support to the idea that there may be notochord-specific transcription independent of Bra (Jose-Edwards et al. 2011). Distinguishing between these hypotheses will require both RNAseq analysis of Bra mutant embryos as well as a more thorough dissection of the notochord GRN.

## 9.10 Current Questions in *Ciona* Notochord Gene Regulation

Beyond the question of whether Bra is a true master regulator or instead acts in a more combinatorial fashion, it is now clear that the *Ciona* notochord GRN is deep rather than shallow. Numerous transcription factors are expressed in the notochord (Jose-Edwards et al. 2011; Reeves et al. 2017), and several notochord CRMs have been found to be independent of direct Bra binding (Katikala et al. 2013; Jose-Edwards et al. 2015). The network relationships between the transcription factors acting to control notochord-specific gene expression have yet to be elucidated. Are there multiple layers of hierarchy downstream of Bra or just one? Is it a cascade or are there positive or negative feedback loops?

A comprehensive dissection of the notochord GRN would be an essential step toward understanding how the structure of the GRN relates to the function of the effector genes it controls. In the sea urchin mesendoderm GRN, distinct transcription factors are thought to act in a highly modular way to control different morphogenetic behaviors (Wu and McClay 2007; Wu et al. 2008; Saunders and McClay 2014). Do distinct transcription factors control distinct notochord cell behaviors such as mediolateral intercalation, elongation and lumen formation, or is there some other regulatory logic?

Dozens of notochord genes have been subjected to relatively detailed cis-regulatory dissection, but it appears that there is no straightforward enhancer code controlling notochord-specific expression. FoxA.a and Bra sites are commonly involved, but no clear pattern has emerged for how these sites are arranged (Jose-Edwards et al. 2015). Despite this diversity of cis-regulatory architecture, one would hope that there must be aspects of underlying logic to be gleaned. A highly parallelized approach for simultaneously testing thousands of CRM variants was essential in developing the recent enhancer suboptimization hypothesis (Farley et al. 2015) and will likely be powerful for many types of cis-regulatory analysis.

Other interesting questions for the future involve the evolution of tissue-specific regulatory networks. Aspects of notochord induction appear to be quite different between *Halocynthia* and *Ciona* despite remarkably similar embryonic lineages and morphology (Darras and Nishida 2001; Lemaire 2009; Takatori et al. 2010; Hudson et al. 2016). Notochord-specific enhancers are often highly divergent even between *Ciona intestinalis* and *Ciona savignyi* (Jose-Edwards et al. 2015). How and why has the ascidian notochord GRN evolved, and what are the constraints upon its evolution? How well is it conserved with cephalochordates and vertebrates? These broader questions will be increasingly addressable as the *Ciona* GRN becomes increasingly well understood.



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# Chapter 10

## Model Systems for Exploring the Evolutionary Origins of the Nervous System



**Karri M. Haen Whitmer**

**Abstract** The development of nervous systems can be seen as one of the key transitions in animal evolution, allowing the efficient integration of sensory input and motor output and the expedient transmission of impulses over relatively long distances inside an organism. With the increased availability of genome sequences for animals at the base of the metazoan phylogenetic tree, two alternative hypotheses have been proposed regarding nervous system evolutionary origins, ultimately prompting a debate whether an enormously complicated system like the nervous system could have evolved more than once. This review summarizes what is currently known about nervous system origins, concentrating on the evolution of synapse components, with respect to phylogenetic knowledge of early diverging animal groups, comprising members of the Porifera, Ctenophora, Placozoa, and Cnidaria.

### 10.1 Introduction

Neural processing requires an enormous diversity of genes that allow the organism to detect and respond to stimulus. These processes are normally accomplished by propagation of action potentials in neurons by voltage-gated ion channels embedded in axonal membranes and synthesis of specialized chemical messengers, neurotransmitters or neuropeptides, for release into the synaptic cleft, and a variety of pre- and postsynaptic receptors, channels, enzymes, and structural proteins. The immensely complex networks required for the function and regulation of the metazoan nervous system have specialized and adapted over time, with precludes to these likely as early as the deep Cryogenian in the ancestors of animals (Erwin et al. 2011). Due to the relative inability to study soft tissues like nerves and muscles through analysis of the fossil record, research on the origins of the nervous system rely upon the study of

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genomes, transcriptomes, and proteomes of living animals that may have features similar to ancestors long lost to the mode and tempo of animal evolution.

## 10.2 A Minimal Nervous System

Although upon initial inspection, the idea of reconstructing nervous system origins by studying differences in the anatomy and physiology of extant animals might seem relatively uncomplicated, progress has been hampered by a lack of definition for what a minimal nervous system might look like. That is, when examining species for the presence of nervous systems, what nervous system characters should one look for? Since it may be difficult to simply define nervous systems based upon their cellular structure (see Kristan 2016), we must turn to the genes that are expressed in neural cells. Are there neuron-specific trans-phylum, pan-neuronal genes that can help us to definitively classify the presence/absence of nervous systems in animals? Could these help us to characterize a minimal nervous system or tell us whether an animal's ancestors did or did not have a nervous system? Certainly there are many well-defined modules of proteins that are required for neural transmission, including, but not limited to, proteins that encode voltage-gated ion channels and rectifier channels required for the transmission of impulses along membranes; biosynthetic machinery for synthesis of neurotransmitters and neuropeptides; presynaptic protein modules for vesicle packaging and calcium-dependent trafficking and docking; structural and functional postsynaptic proteins, including an array of ligand-gated channels; and other receptors for detection and integration of neurotransmitter or other physiologically active substances.

The postsynaptic and presynaptic terminals exist as modules of molecules with highly structured interactions that produce the emergent physiological and behavioral properties in animals. The postsynaptic density includes a concentration of structural and functional proteins, including cytoskeletal, cell adhesion, and regulatory molecules that align the receptors and channels of the postsynaptic membrane with the active site of the presynaptic membrane (Ziff 1997). As it turns out, many of the components of the postsynaptic density, including the backbone scaffold proteins (Shank, Homer, and Discs-large), as well as some of the receptors associated with it (e.g., ionotropic and metabotropic glutamate receptors), have origins in the unicellular ancestors of animals (Alié and Manuel 2010). The apparent co-option of these protein modules by nervous systems makes these proteins interesting, but not ideal candidates for nervous system gene homology searches.

The presynapse includes the protein machinery that packages neurotransmitter and transports and docks synaptic vesicles with the presynaptic membrane. Again, several protein modules required for this are found outside of the Metazoa. For example, the SNAREs required for docking, priming, fusion, and release of neurotransmitter from the synaptic vesicle have homologs in fungi and non-opisthokonts (Burkhardt 2015), and even the SNAREs specifically required for synaptic function, sometimes called the “neuronal SNARE complex,” have orthologs in *Monosiga*



**Table 10.1** Emerging model systems of the non-bilaterian Metazoa

	Porifera	Ctenophora	Cnidaria	Placozoa
Species	<i>Amphimedon queenslandica</i>	<i>Mnemiopsis leidyi</i> ; <i>Pleurobrachia bachei</i>	<i>Nematostella vectensis</i> ; <i>Hydra magnipapillata</i> ; <i>Acropora digitifera</i> ; <i>Aiptasia pallida</i> ; <i>Renilla muelleri</i>	<i>Trichoplax adhaerens</i> <sup>a</sup>

All species have annotated nuclear genome sequences, and many have functional studies that have contributed to knowledge of nervous system origins

<sup>a</sup>*Trichoplax adhaerens* is the only well-described Placozoa

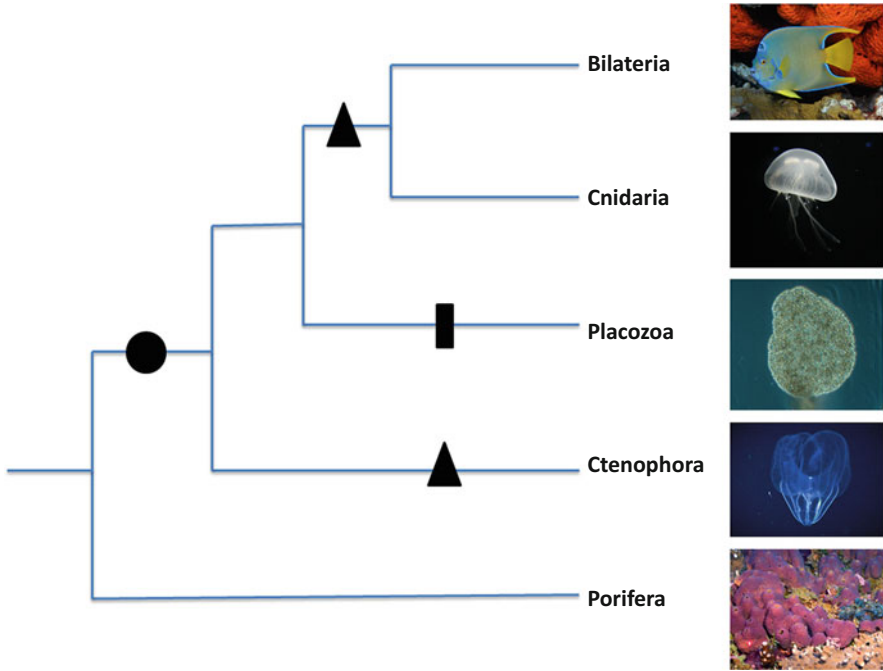
*brevicollis* (e.g., syntaxin-1 and SNAP25). Marlow and Arendt (2014) have suggested that the evolutionarily conserved core proteins of the presynaptic active zone could be a good test of the homology of synapses.

### 10.3 Uncertainty in the Metazoan Phylogeny

The use of phylogenetic tests to attempt to reconstruct the evolutionary histories of complex systems is both an incredibly powerful and challenging tool, fraught with issues of parsing orthologs versus paralogs and differences in evolutionary rates that must be dealt with through the application of complicated phylogenetic models.

In addition, taxon sampling has been a prevailing issue in the lower Metazoa due to the relative inaccessibility of, and difficulty in transporting and handling, many marine animals. Today, the field of animal phylogenetics is beginning to benefit from the increased sampling of more elusive marine organisms, and several genome projects have yielded a wealth of information about animals that were previously only subject to molecular phylogenies using a few conserved genes. These include members of the Porifera (sponges), Ctenophora (comb jellies, sea gooseberries), Cnidaria (jellyfish, corals, and sea anemones), and Placozoa (*Trichoplax adhaerens*) (Table 10.1).

With respect to describing the origin(s) and early evolution of the nervous system, there has been a fundamental problem with the uncertain phylogenetic positions of four phyla at the base of the metazoan tree: the Porifera (sponges), Ctenophora (comb jellies), Cnidaria (jellyfish, corals, and sea anemones), and Placozoa (e.g., Ryan et al. 2013; Moroz et al. 2014; Dunn et al. 2008; Philippe et al. 2009; Schierwater et al. 2009; Simion et al. 2017; Pisani et al. 2015). In particular, it has been unclear whether the base of the tree should be occupied by Porifera, animals without nervous systems, or Ctenophora, animals with nervous systems. From these conflicting evolutionary hypotheses derives two questions of particular interest: Did the canonical metazoan nervous system containing neurons, neurotransmitters, and synapses evolve more than once? And, after a lineage has acquired a nervous system, could the nervous system be lost (Fig. 10.1)?



**Fig. 10.1** Alternative hypotheses for the origins of the metazoan nervous system. Porifera is placed as the sister group to other animals as in Pisani et al. (2015) and Simion et al. (2017). The circle represents a common origin of the nervous system, while the triangles represent two distinct origins, one leading to the Ctenophora and one leading to the Cnidaria + Bilateria group. The rectangle represents the potential loss of nervous system features in the lineage leading to Placozoa (*Trichoplax adhaerens*). Bilateria, Cnidaria, Ctenophora, and Porifera photos courtesy of NOAA Photo Library CC-by-2.0. Placozoa photo courtesy of B. Shierwater from Eitel et al. (2013), CC-by-4.0

## 10.4 Nervous System Evolution at the Base of the Metazoan Tree

### 10.4.1 *The Proto-Synaptic Scaffolding of Sponges*

Sponges (Porifera) are a diverse group of aquatic animals, comprising at least 15,000 extant species that are asymmetrical or radially symmetrical, with a variety of shapes, colors, sizes, habitats, and distributions that may be colonial or solitary (Hooper and van Soest 2002). Sponges have approximately 16 cell types (Simpson 1984), and at least some groups (e.g., Demospongiae, Haplosclerida) have functional epithelia that occlude the passage of ions (Leys et al. 2009; Adams et al. 2010). Sponges do not have muscles or neurons, but they do receive signals through sensory cilia and produce coordinated effector responses that alter filter feeding behavior (Leys 2015).

Due to their anatomical simplicity, morphological studies have traditionally placed the Porifera as the earliest diverging branch of the animal tree, in agreement with their early appearance in the fossil record (Love et al. 2009; Yin et al. 2015). The sequencing of the first sponge genome, the Great Barrier Reef demosponge *Amphimedon queenslandica* (Srivastava et al. 2010), demonstrated a diverse genetic tool kit with developmental and biochemical pathways previously only associated with more complex animals, including analogs of genes that are expressed in the muscle and neural tissues of eumetazoans. Sakarya et al. (2007) showed the co-expression of a number of proteins from *Amphimedon* larval flask cells whose homologs interact in eumetazoan synapses, implicating a “proto-postsynaptic scaffold,” which predated the origin of nervous systems. The genome sequence of *A. queenslandica* contains genes for the postsynaptic density (PSD); postsynaptic neurotransmitter receptor homologs for metabotropic glutamate receptors (mGluRs); presynaptic vesicle proteins that allow calcium-regulated vesicle exocytosis, including the elements for vesicle docking used in synapses but not specific to them (e.g., the SNARE complex genes); and a high-voltage-activated (HVA) calcium channel (Ca<sub>v</sub>1) (Srivastava et al. 2010). These data, along with other genes found associated with neuropeptide and neurohormone processing and secretion (Table 10.2), seem to suggest that *Amphimedon* has molecular capability that is evolutionarily associated with synaptic transmission in other animals. Additionally, functional studies indicate a few possible neurotransmitters (glutamate, nitric oxide, and possibly GABA) are involved in signaling to effectors in sponges (Dunn et al. 2015; Elliott and Leys 2010; Ellwanger et al. 2007). Due to the presence of many of the same proteins in the unicellular eukaryote *Monosiga brevicollis*, it seems as though the origin of many “synaptic proteins” predated the acquisition of synapses or neuron-like cells (Alié and Manuel 2010; Moroz and Kohn 2015). This finding is consistent with the stepwise evolution of nervous systems and helps to confirm Porifera’s place at the base of the metazoan tree.

### 10.4.2 *The Ctenophore Nervous System*

Ctenophores, comb jellies and sea gooseberries (phylum Ctenophora), are an enigmatic group of exclusively marine animals, of which only about 150–200 species have been described (Mills 1998, electronic publication). Ctenophores typically have translucent, gelatinous bodies, which are characterized by eight rows of cilia (comb rows or comb plates) used for locomotion. Relatively little is known about their biology compared to other metazoans due to difficulties capturing many species without fragmenting their tissues. Due to their soft body, ctenophores were also poorly preserved in the fossil record (Dunn et al. 2015). Two species of fossil ctenophore have been found from the Late Devonian (400 MYA) (Stanley and Stürmer 1983, 1989). Ctenophora likely represents the earliest lineage of non-bilaterian animals possessing a nervous system and mesoderm-derived muscles; they have ectodermal and endodermal nerve nets and concentrations of sensory cells

**Table 10.2** Nervous system proteins for the postsynaptic density core, putative neurotransmitters/neuropeptides, putative receptors, voltage-gated ion channels, and presynaptic core active zone proteins from Porifera, Ctenophora, Placozoa, and Cnidaria

Phylum model animal	Postsynaptic density core proteins <sup>a</sup>	Putative neurotransmitters or neuropeptides	Putative postsynaptic neurotransmitter receptors	Voltage-gated ion channels <sup>a</sup>	Presynaptic core active zone proteins <sup>a</sup>
Porifera <i>Amphimedon queenslandica</i>	Shank; Discs-large (DLG); Homer	Glutamate; nitric oxide; GABA; serotonin; dopamine	mGluRs; GABA-BR; rhodopsin-like GPCRs (serotonin and dopamine)	Ca <sub>v</sub> (1)	Munc13; liprin-alpha-like protein; synaptotagmin; synaptobrevin; Snap23; syntaxin
Ctenophora <i>Mnemiopsis leidyi</i> / <i>Pleurobrachia bachei</i>	Discs-large; Homer	Glutamate; nitric oxide; possibly GABA; RFamide	iGluRs; mGluRs; GABBR2 (subunit 2); rhodopsin GPCRs; CNN1/ASIC-like; (Deg)/EINaC	Ca <sub>v</sub> (1)/Ca <sub>v</sub> (1); Na <sub>v</sub> (2)/Na <sub>v</sub> (2); K <sub>v</sub> (44)/K <sub>v</sub> (36)	Rab3; RIM; Munc13; ELKS; liprin-alpha; synaptotagmin; synaptobrevin; Snap23; syntaxin
Placozoa <i>Trichoplax adhaerens</i>	Discs-large; Homer	FMRFamides; endomorphin-like peptide (ELP); glutamate; nitric oxide; dopamine	mGluRs, iGluRs, GABBR2; ionotropic RFamide receptors	Ca <sub>v</sub> (3); Na <sub>v</sub> (3); K <sub>v</sub> (11)	RIM; Munc13; liprin-alpha-like protein; synaptotagmin; synaptobrevin; Snap25; syntaxin
Cnidaria <i>Nematostella vectensis</i>	Shank; Discs-large; Homer	Acetylcholine; glutamate; GABA; glycine; catecholamines; serotonin; RFamides; nitric oxide	GABA; mGluR; dopamine; epinephrine; serotonin, (5-HT); AMPA; NMDA; muscarinic Ach	Ca <sub>v</sub> (7); Na <sub>v</sub> (5); K <sub>v</sub> (50)	Rab3; RIM; Munc13; ELKS; liprin-alpha; synaptotagmin; synaptobrevin; Snap25; syntaxin

The list of proteins is central to the analysis of this review but not intended to be exhaustive

<sup>a</sup>Most data for pre- and postsynaptic complexes and ion channels summarized from the analysis of Moroz and Kohn (2015). Remaining data collected from Srivastava et al. (2008, 2010), Senatore et al. (2016), Ryan et al. (2013), Moroz et al. (2014), Watanabe et al. (2009), and Kass-Simon and Pierobon (2007). Caution should be noted that data taken from genomic analyses do not confirm the function of proteins

and neurons in the aboral region (aboral organ), which is unique to the group (Ryan et al. 2013; Moroz et al. 2014).

The first ctenophore genomes sequenced were from the lobate *Mnemiopsis leidyi* (Western Atlantic Ocean) (Ryan et al. 2013) and, the Pacific sea gooseberry, *Pleurobrachia bachei* (Moroz et al. 2014). These analyses have shown that many of the genes involved in the differentiation and formation of bilaterian synapses are present in sponges and ctenophores (Srivastava et al. 2010; Ryan et al. 2013; Moroz et al. 2014). Like other animals with nervous systems, ctenophores have chemical and electrical synapses that are formed by gap junction proteins (innexins) that connect the cytoplasm of adjacent cells for quick signal transduction (Moroz and Kohn 2015). They also contain a complement of voltage-gated ion channels (for calcium, sodium, and potassium) similar to other animals with a nervous system, although there are fewer kinds of sodium and calcium channels than in *Trichoplax adhaerens*, which has no nervous system (Moroz et al. 2014) (Table 10.2).

Unexpectedly, ctenophores lack many synaptic genes that are also missing in sponges, but are present in cnidarians and bilaterians (Ryan et al. 2013), showing that at least some of the proteins missing in sponges may not necessarily be required for the first evolutionary steps in canonical nervous system development. Key genes encoding the evolutionarily conserved core proteins of the presynaptic active zone (RIM, Munc13, RIM-BP,  $\alpha$ -liprin, ELKS) (Marlow and Arendt 2014), which dock and prime synaptic vesicles for exocytosis and place the active region of the presynaptic terminal opposite to the postsynaptic density (Südhof 2012), are present in ctenophores, while key functional components (RIM and ELKS) are absent in sponges (Jékely et al. 2015; Marlow and Arendt 2014) (Table 10.2).

Unlike most other animals with nervous systems, data collected from a long history of molecular and immunohistochemical analyses suggest ctenophores do not use serotonin, acetylcholine, dopamine, noradrenaline, adrenaline, octopamine, or histamine at the synapse (Moroz and Kohn 2015), although, like sponges, they do seem to use glutamate and have a great variety of glutamate receptors, including metabotropic and ionotropic glutamate receptors (iGluRs), the latter which are not found in sponges (Moroz et al. 2014; Moroz and Kohn 2015).

### 10.4.3 *The Placozoa*

*Trichoplax adhaerens*, the only well-described species of phylum Placozoa, is a flat, disk-shaped marine animal that may grow up to only a few millimeters in diameter. *Trichoplax* is composed of six distinct cell types (Smith et al. 2014) arranged in three layers. *Trichoplax* does not have muscles, neurons, or an internal gut cavity, and it feeds by phagocytosis as well as external digestion by its ventral epithelium. The ventral epithelium also contains neurosecretory gland cells that secrete endomorphin-like peptides (ELPs) that were shown to halt gliding behavior accompanied by ciliary arrest (Senatore et al. 2017). A subset of gland cells may also secrete FMRFamide-like neuropeptide (Schubert 1993; Smith et al. 2014).

Recent phylogenetic analyses have placed the Placozoa as the sister group to the Eumetazoa (Bilateria + Cnidaria) (Pisani et al. 2015; Simion et al. 2017), but their phylogenetic position has not been stable. The mitochondrial genome of *Trichoplax* is the largest known in the Metazoa (43 kb) and contains introns, unknown ORFs, and intragenic spacers that are not characteristic of the compact mitochondrial genomes of bilaterians. Phylogenetic analyses of *Trichoplax* mitochondrial proteins have placed it at the base of the Metazoa, which at first glance seems parsimonious with the animal's simple anatomy (Dellaporta et al. 2006). Phylogenies of nuclear genes, however, have placed the Placozoa as the sister group to the Bilateria (Collins 1998) or sister to Cnidaria + Bilateria (DaSilva et al. 2007; Pisani et al. 2015; Simion et al. 2017), renewing old ideas that the Placozoa are derived from more complex animals, suggesting they may have indeed evolved from an ancestor that possessed a functional nervous system.

The genome paper for *Trichoplax* describes several genomic elements associated with neuroendocrine function, including members of the calcium, sodium, and potassium voltage-gated ion channel families, genes for calcium-regulated vesicle transport, and metabotropic and ionotropic glutamate receptors. Many of the postsynaptic density proteins that are present in sponges are also found in *Trichoplax* (Srivastava et al. 2008) (but Shank has not been identified). Notably, the *Trichoplax* genome contains a variety of voltage-gated channels not seen in the Porifera and more isoforms of calcium and sodium voltage-gated channels than either of the tenophore genomes (Table 10.2).

#### 10.4.4 Cnidarian Nervous Systems

Cnidarians are a diverse group of over 11,000 species of marine and fresh water animals. Phylum Cnidaria is subdivided into two major clades: the Medusozoa (jellyfish, box jellies, and hydrozoans) and the Anthozoa (sea anemones and corals). Cnidarians may be free-swimming medusae or sessile polyps, both of which have radial symmetry, stinging cnidocytes, and a gastrovascular cavity. Due to the diversity of the group, it is not surprising there are many variations in their nervous systems, but in general, cnidarians have a diffuse nerve net with regionalization of nervous tissue in some groups (Watanabe et al. 2009), including neural rings, plexuses, or tracts with neurons organized with respect to neuropeptides (Koizumi et al. 2004). Additionally, some medusae may exhibit highly specialized sensory structures such as rhopalia containing complex eyes and statocysts for equilibrium (Garm and Mori 2009; Skogh et al. 2006).

Phylogenetic analyses resolve the Cnidaria as the sister group to Bilateria (Hejnol et al. 2009; Pick et al. 2010), and study of cnidarian nervous systems may help to reconstruct the nervous system of the last common ancestor of the group, which must have had an ancestral complexity of synaptic and other nervous system proteins (Bosch et al. 2017). Although the complement of nervous system genes is said to be nearly complete in the Cnidaria, and a spectrum of classical

neurotransmitters have been identified, including glutamate, GABA, serotonin, dopamine, epinephrine, norepinephrine, and acetylcholine, as well as a variety of RFamide neuropeptides (Kass-Simon and Pierobon 2007), many of the receptors have not been identified (Bosch et al. 2017). GABA, metabotropic glutamate, dopamine, epinephrine, serotonin, AMPA, NMDA, and muscarinic ACh receptors are a few that have been reported for both *Nematostella* and *Hydra* (Putnam et al. 2007; Chapman et al. 2010; Watanabe et al. 2009).

## 10.5 Conclusions

### 10.5.1 *Evolution of Synapses from Ancient Chemosensory Cells*

A point of critical importance to any understanding of the evolution of nervous systems seems to be the incredibly early emergence of genes used for the structure and function of synapses. Major pre- and postsynaptic elements, including the postsynaptic density, are not only found in the Porifera, but many are also found in the choanoflagellate *Monosiga brevicollis* (Burkhardt 2015). These components, then, were likely present in the ancestors of animals, over 600 MYA.

Although sponges do not have nervous systems, understanding of their genomes, proteins, and behaviors seems key to interpreting the possible mechanisms through which very small, sessile, or pelagic animals might have gained greater ability to sense and respond to their environments. Shaham (2010) hypothesized that neural synapses are derivative of ancient ancestral chemosensory organs. For example, a simple sensory receptive field specialized for detecting external stimulus could be easily co-opted to sense and respond to a similar internal stimulus. In his paper, Shaham discusses the Porifera as an example, noting that the postsynaptic density of *Amphimedon* is expressed in larval flask cells, which possess a sensory cilium (Leys and Degnan 2001). Marlow and Arendt (2014) further elaborated upon this idea, suggesting the possibility that co-option of simple chemosensory fields could also explain the recruitment of glutamate as the major neurotransmitter in the Ctenophore lineage: glutamate was already likely used as an intercellular signaling molecule in the ancestor of extant sponges, which are known to respond to glutamate with contraction of their canal systems (Elliott and Leys 2010).

### 10.5.2 *Origin and Evolution of the Nervous System*

By comparing nervous system genes from ctenophores with that of cnidarians and by comparing both ctenophore and cnidarian nervous system genes to vertebrates (data from Moroz and Kohn 2015), there is an overall trend toward increased

complexity, which appears to be at least partially due to a marked process of expansion and loss of preexisting genes or gene families through duplication and specialization of function. This holds true for many “neural” genes with pre-metazoan origins, for example, G-protein-coupled receptors (GPCRs) like the metabotropic glutamate receptor family undergo steady expansion and apparent diversification of function throughout the Metazoa (Krishnan et al. 2014).

The homology of many of the basic nervous system genes used across the Metazoa (Table 10.2) makes an argument for a common origin of the nervous system; and this hypothesis is supported by recent phylogenetic analyses of the Metazoa (Simion et al. 2017; Pisani et al. 2015). Overall, these data suggest that sponge genomes contain a molecular tool kit that preceded the nervous system origin, which was co-opted and used for neural function in a common ancestor of ctenophores and cnidarians. Did this common ancestor have neurons? The matter is still open to debate. Certainly, there is no argument that ctenophore nervous systems are unusual compared to those of other animals, and derived, and convergent, characters are numerous within the group (Moroz et al. 2014). Derived nervous system features do not eliminate the possibility of common ancestry. Further studies of the conserved structural (or developmental) genes of non-bilaterian nervous systems will be necessary to determine their expression, protein localization, and precise functions before we can finally declare whether nervous systems evolved more than once.

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# Chapter 11

## Nonprotein-Coding RNAs as Regulators of Development in Tunicates



Cristian A. Velandia-Huerto, Federico D. Brown, Adriaan Gittenberger, Peter F. Stadler, and Clara I. Bermúdez-Santana

**Abstract** Tunicates, or urochordates, are a group of small marine organisms that are found widely throughout the seas of the world. As most plausible sister group of the vertebrates, they are of utmost importance for a comprehensive understanding of chordate evolution; hence, they have served as model organisms for many aspects of the developmental biology. Current genomic analysis of tunicates indicates that their genomes evolved with a fast rate not only at the level of nucleotide substitutions but also in terms of genomic organization. The latter involves genome reduction, rearrangements, as well as the loss of some important coding and

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noncoding RNA (ncRNAs) elements and even entire genomic regions that are otherwise well conserved. These observations are largely based on evidence from comparative genomics resulting from the analysis of well-studied gene families such as the Hox genes and their noncoding elements. In this chapter, the focus lies on the ncRNA complement of tunicates, with a particular emphasis on microRNAs, which have already been studied extensively for other animal clades. MicroRNAs are known as important regulators of key genes in animal development, and they are intimately related to the increase morphological complexity in higher metazoans. Here we review the discovery, evolution, and genome organization of the miRNA repertoire, which has been drastically reduced and restructured in tunicates compared to the chordate ancestor. Known functions of microRNAs as regulators of development in tunicates are a central topic. For instance, we consider the role of miRNAs as regulators of the muscle development and their importance in the regulation of the differential expression during the oral siphon regeneration. Beyond microRNAs, we touch upon the functions of some other ncRNAs such as yellow crescent RNA, moRNAs, RMST lncRNAs, or spliced-leader (SL) RNAs, which have diverse functions associated with the embryonic development, neurogenesis, and mediation of mRNA stability in general.

## 11.1 Introduction

Tunicates are organisms characterized by a fast rate of genomic and developmental evolution. Some fast-evolving evolutionary changes include loss of synteny, fast changes in cis-regulatory sequences, and loss of several key regulatory developmental genes (Satou et al. 2008; Denoëud et al. 2010), such as several central or posterior Hox genes involved in AP patterning of metazoans (Ikuta and Saiga 2005) and Gbx involved in the establishment of the midbrain-hindbrain boundary in vertebrates (Yagi et al. 2003).

The role of noncoding RNAs in tunicate development has been studied since the 1990s, dating back to the seminal work by Swalla and Jeffery on the RNAs localized in the yellow crescent or myoplasm, a cytoskeletal domain in oocytes of the ascidian *Styela clava* (Swalla and Jeffery 1995). The yellow crescent (YC) RNA identified to be present throughout embryonic development was the first example of what is now a large and rapidly growing class of ncRNAs with important roles in growth and development in tunicates. This asymmetrically distributed ascidian RNAs were part of the set of many other RNAs known as maternally synthesized cytoplasmically localized RNAs, discovered first in oocytes of *Xenopus* (Bashirullah et al. 1998).

The current state of knowledge on ncRNAs in tunicates is far from comprehensive and complete. Nevertheless, in particular, microRNAs have been studied already in some detail in several tunicate species, such as *Oikopleura dioica*, both

*Ciona* species,<sup>1</sup> *Didemnum vexillum*, and *Salpa thompsoni*. These studies have revealed many losses of miRNA families that are very well conserved outside the tunicates. At the same time, many gain unique miRNAs among recently divergent lineages in the tunicates when compared to other groups of chordates (Fu et al. 2008; Velandia-Huerto et al. 2016). Relaxed constraints in the evolution of genomes and developmental trajectories in the tunicates may have been responsible for the plethora of reproductive strategies, morphologies, and life histories observed in the group (Holland 2015).

## 11.2 miRNA Families Origin and Evolutionary Perspective

### 11.2.1 Origins and Evolution of MicroRNAs

MicroRNAs (miRNAs) have been described in almost all animals and plants as well as unicellular eukaryotes. They are important posttranscriptional regulators of gene expression affecting a sizable fraction of all mRNAs (Ameres and Zamore 2013). Mechanistically, miRNAs depend on the presence of the evolutionarily even older RNA interference pathways (Cerutti and Casas-Mollano 2006; Shabalina and Koonin 2008) that leads to the suppression of double-stranded RNA molecules in a cell's cytoplasm.

Throughout animals, canonical miRNAs are processed through a well-characterized pathway. The primary precursor transcript (pri-miRNA) is transcribed by pol-II. While in most cases the pri-miRNA is a long noncoding RNA, some miRNAs are processed from protein-coding transcripts, where they are mostly derived from introns (Lin et al. 2006). In the next step, hairpin-shaped precursors, the pre-miRNAs, are excised while the RNA is still residing in the nucleus. These are exported into the cytoplasm (Lund et al. 2004) and then processed further into miRNA/miRNA\* duplexes. In the final step, the single-stranded mature miR or its complement, the miR\*, is incorporated into the RISC complex. Sequence complementarity of miR and mRNA ensures the targeting specificity (Bartel 2009). As a consequence, miRNAs share a set of structural characteristics, most importantly the extremely stable secondary structure of the precursor hairpin and the 2-bp overhang of miR and miR\* generated by Dicer processing. These features make it possible to reliably identify miRNAs from short RNA-seq data; see, e.g., Langenberger et al. (2010, 2012) and Friedländer et al. (2012).

Most animal microRNAs are among the most highly conserved genetic elements. The most stringent selection pressure acts on the mature miR sequence. This is a consequence of the fact that a single miR typically targets a large number of mRNAs.

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<sup>1</sup>In accordance with the prevalent use in the ascidian community, we use the term *Ciona robusta* reflecting that “morphological evidence that the molecularly determined *C. intestinalis* type A and type B are different species: *C. robusta* and *C. intestinalis*” (Brunetti et al. 2015).

Mutations in the mature sequence thus simultaneously affect many interactions and thus are almost always selected against. In conjunction with the stringent requirements on the secondary structure, the entire precursor is under strong stabilizing selection (Price et al. 2011), explaining the observed high levels of sequence conservation. As a consequence, even evolutionarily distant homologues of miRNAs can be readily detected despite the short sequence length. Most efficiently, *infernal* (Nawrocki and Eddy 2013) is used for this purpose, since it makes use of both sequence and structure comparison. The evolution of miRNAs can thus be traced back in time with high accuracy (Hertel et al. 2006).

Like other gene families, miRNAs form paralogs (Tanzer and Stadler 2004; Hertel et al. 2012) and hence often appear as families of homologous genes. This forms the basis of the miRBase nomenclature (Ambros et al. 2003). A series of investigations into the phylogenetic distribution of miRNA families showed that miRNAs are infrequently lost at family level and thus serve as excellent phylogenetic markers (Sempere et al. 2006; Heimberg et al. 2007, 2010; Wheeler et al. 2009), although the massive restructuring of the miRNA complement of tunicates is an important exception to this rule (Fu et al. 2008).

The innovation of new miRNA families is an ongoing process. Experimental surveys of the miRNA repertoire thus have reported a large number of very young and even species-specific miRNAs (Bentwich et al. 2005; Berezikov et al. 2006). The process was studied quantitatively in fruit flies, where innovation rate of as many as 12 new miRNA genes per million years has been estimated (Lu et al. 2008). This is consistent with the fact that stable hairpins are abundant structural elements in random RNAs, which makes it not only possible but actually quite likely that miRNA precursors appear by chance in transcribed genomic regions (Tanzer and Stadler 2004; Campo-Paysaa et al. 2011; Marco et al. 2013). Of course, only a tiny fraction of these fortuitously processed hairpins have a function and hence are subject to selection, and an even smaller subset is conserved over long evolutionary time scales. Detailed studies showed that evolutionarily young miRNA has comparably low expression levels. Initially, they go through a phase of relatively fast sequence evolution (Liang and Li 2009; Meunier et al. 2012), which slows down as the selective pressures from a gradual increase in the number of target sites increases. A large, diverse set of targets then protects against miRNA loss (Lee et al. 2007). The rate of gain of miRNA families that are retained essentially permanently amounts to only 1 per several million years. This number is consistent with divergence of the miRNA complements between animal phyla.

Many authors have observed that overall, the miRNA repertoire has been expanding throughout animal evolution in a manner that at least roughly correlates with morphological complexity (Hertel et al. 2006; Sempere et al. 2006; Niwa and Slack 2007; Prochnik et al. 2007; Lee et al. 2007; Heimberg et al. 2007; Peterson et al. 2009; Berezikov 2011). Several bursts of miRNA innovation have been observed (Hertel et al. 2006; Heimberg et al. 2007; Tanzer et al. 2010; Hertel and Stadler 2015), most notably at the root of the placental mammals, the ancestor of “free-living” nematodes, or the radiation of the drosophilids. Massive morphological simplification, on the other hand, is sometimes associated with a drastic loss of miRNA families. This has been observed most prominently for tunicates (Fu et al. 2008; Dai et al. 2009).

### 11.2.2 *miRNA Identification and Validation*

The first miRNA reported in any tunicate was let-7, which was first detected in *Ciona robusta* and *Herdmania curvata* (Pasquinelli et al. 2000). A previous study the same year in *C. elegans* had shown that small RNA let-7 (21 nt) was required for late larval to adult developmental transition (Reinhart et al. 2000). Small RNA let-7 was then shown to also be differentially expressed during the development of many distantly related animal taxa but was not detected in Porifera, Ctenophora, Cnidaria, and Acoelomorpha, suggesting that let-7 was involved in the regulation of late temporal transitions during development or in the evolution of complex life histories in the Nephrozoa (Pasquinelli et al. 2000, 2003).

The first systematic computational screen of a tunicate genome in 2005 followed the first large animal sequencing projects. Beginning with *C. robusta* and *C. savignyi*, a profile-based strategy was implemented in the ERPIN program (Legendre et al. 2005). This work detected a set of new miRNA candidates considered to be *C. robusta* specific, such as the members of the family miR-9 and miR-79 together with many other miRNA families that, as expected, were found to be homologous between both *Ciona* species. Among these were miR-124, miR-92, miR-98, miR-325, the miR310-313 group, and let-7. In the same year, a whole-genomic comparative approach in the urochordate lineage was performed on the species *C. robusta*, *C. savignyi*, and *O. dioica*. Using a computational screening of structured ncRNAs based upon homology between predicted precursor hairpin structures, 41 miRNA candidates were detected including let-7 and other six known candidates in *C. robusta* (Missal et al. 2005). The same group in 2007 implemented a structure-based clustering approach in *C. robusta* predicted the presence of 58 miRNAs, of which only let-7, miR-7, miR-124, and miR-126 coincided with the previously annotated miRNAs (Will et al. 2007).

Up to this point, efforts to map the miRNAs in urochordate lineages were mainly focused on computational approaches. With improved sequence technologies, new hybrid strategies were employed that combined computational and experimental studies to validate candidate families previously detected. For instance, the first bona fide records for *C. robusta* miRNAs were registered in mirBase only in Release 11 based on the data reported in Norden-Krichmar et al. (2007). This study searched the genomes of *C. robusta* and *C. savignyi* for conservation of the seed regions of the known mature miRNA sequences compiled in the 2006 release of mirBase release. Those miRNAs were aligned locally using the FASTA/ssearch34 program, retaining only matches with at least 90% identity. The characteristic hairpin structure and the relative positioning of the mature sequences were used as additional filters. After manual curation, a set of 18 miRNAs that appeared conserved in both *Cionas* was reported. The expression of 14 of these families, including let-7, miR-7, and miR-126, was then confirmed by Northern blot analyses in the adult tissue of *C. robusta*.

Until 2008, most of the miRNAs were annotated in *Cionas*, and few new miRNA families were discovered in other urochordates. The first repertory of miRNAs in

tunicate beyond the *Ciona* species was published in 2008 for the larvacean *O. dioica* (Fu et al. 2008). At that time, Fu et al. were studying the temporal-spatial expression patterns of conserved miRNAs in different developmental stages of oocytes, 1-cell zygote, 2–8 cell embryos, blastulas, gastrulas, tadpoles (in different stages), and 1–6-day-old adults from *O. dioica*. Small RNAs were isolated, amplified by RT-PCR by rapid amplification of cDNA ends (RACE) of the developmental stages, cloned, and sequenced. Blast searches using the sequences of cloned small RNA libraries were used to annotate small RNAs as miRNA candidates. In further steps, the recovered genomic flanking sequences in each side of those mapped candidates were used as input to predicted secondary structures by mfold v3.1. This step was used to detect candidates that fold like miRNA hairpins and aimed to decrease the set of false-positive potential miRNAs in *O. dioica*. Finally, for this set of potential candidates, a developmental miRNA array dot blot analyses were performed to detect miRNA expression. With this approach from 3066 sequenced small RNA clones, expression was detected for only 55 miRNAs. The authors suggested that these candidates were expressed throughout the short life cycle of *O. dioica* showing that some of them were stocked as maternal determinants prior to rapid embryonic development. The authors also identified a set of sex-specific miRNAs that appeared as male/female gonad differentiation became apparent and was maintained throughout spermatogenesis (Fu et al. 2008). Unexpectedly, the majority of the miRNAs loci in *O. dioica* were located in antisense orientations in the host genes, in contrast to the majority of the mammalian miRNAs known at the time.

Between the years 2009 and 2015, the majority of the studies of miRNAs in tunicates were focused on the validation of expression of computational predicted miRNAs in *Cionas* with the special focus on *C. robusta* as model organism of tunicates or on the test of new computational approaches such as miRTRAP, miRDeep2, and miRRim2, which used next-generation sequencing libraries of small RNAs derived from *C. robusta* to validate their algorithms. Then by the year 2016, the first comparative homology-based search strategy lets us to identify the repertoire on miRNAs and other ncRNAs in the carpet sea squirt *Didemnum vexillum* with a preliminary comparative analysis of gain and losses of miRNA families in chordates which included the *Cionas*, *O. dioica*, and the colonial tunicate *Botryllus schlosseri* (Velandia-Huerto et al. 2016). By the same year, from the preliminary genome sequence assembled for the Southern Ocean salp, *Salpa thompsoni* (Urochordata, Thaliacea), a set of miRNA families were detected (Jue et al. 2016), and in 2017, the prediction of miRNA families was reported for *Halocynthia roretzi* species (Wang et al. 2017).

### 11.2.2.1 High-Throughput Studies of *Ciona* miRNAs

At the end of the last decade, the application of next-generation sequencing technologies to sequence small RNA libraries changed the common way used to detect expression of miRNAs in many organisms including the tunicates. This technology became one of the most common approaches that supported methods like RT-PCR,



microarrays, or dot blotting which were previously used to validate miRNA expression in tunicates. In 2009, small RNA libraries prepared from various developmental stages including unfertilized eggs, early embryos, late embryos, and adults from *C. robusta* were assayed by high-throughput sequencing of cDNA with an Illumina 1G Genome Analyzer. These sequencing data revealed **80** miRNA families in *C. robusta*. Unexpectedly, a distinct species of small RNAs processed from the distal parts of the miRNA precursor hairpins was found to be abundantly expressed. These were termed miRNA-offset RNAs (moRs) (Shi et al. 2009). Later on, after extracting noncoding conserved regions of whole-genome alignments between *C. robusta* and *C. savignyi*, a set of 12 million sequences were computationally folded using RNAfold and mfold. Then after combining the following criteria—structure/sequence conservation, homology to known miRNAs, and phylogenetic footprinting—the authors detected a set of 458 candidate sequences (Keshavan et al. 2010). Then in order to validate those candidates, RT-PCR and PAGE were conducted to design a custom microarray. Two hundred forty-four of the 458 miRNA predictions were represented either in their microarray data or in the Illumina sequences from Shi et al. (2009), leading to an estimate of about **300** miRNA genes in *C. robusta*. It is worth noting that Keshavan et al. (2010) did not recover 39 previously characterized miRNAs. A novel computational strategy for the systematic, whole-genome identification of microRNA from high-throughput sequencing information was developed in 2010 in Hendrix et al. (2010) and applied to *C. robusta*. This method, miRTRAP, relies on the sequence patterns produced by mechanisms of microRNA biogenesis but also includes additional criteria regarding the prevalence and quality of small RNAs arising from the antisense strand and the neighboring loci. With that approach, nearly 400 putative microRNAs loci were detected. The miRTRAP approach depends crucially on the depth of the small RNAs mapped to a given locus and requires a very accurate assignment of small RNA sequences on their relative positions along the hairpin, that is, miR/miR\*, moR/moR\*, and loop (Hendrix et al. 2010). A further improvement in the analysis of miRNA sequencing data became available with miRDeep2, a revised version miRDeep (Friedländer et al. 2012). It was reported to identify with an accuracy of 98.6% and 99.9% canonical and noncanonical miRNAs in different species. A reanalysis of the small RNA data from Shi et al. (2009) with miRDeep2 reported 313 known and 127 novel miRNAs in *C. robusta*. In the same year, the program miRRim2 (Terai et al. 2012) was applied to the *C. robusta* genome, in which some candidates were identified from the work of Hendrix et al. (2010) and several novel promising candidates were detected.

In 2013, Kusakabe et al. (2013) investigated the expression patterns of the cluster miR-1 and miR-133 in *C. robusta* and in *C. savignyi*. RT-PCR amplification of miR-1/133 precursors was performed, and PCR products were subcloned and sequenced. Whole-mount in situ hybridization to detect cin-miR-1/miR-133 primary transcript was performed, and LNA Northern blotting was conducted on different developmental stages.

### 11.2.2.2 High-Throughput miRNA Searches in Other Urochordates

Since 2016, new approximations have increased our knowledge about new families in other tunicates, thanks to the sequence of new urochordate genomes of the species *D. vexillum*, *S. thompsoni*, and *H. roretzi*. A detailed homology-based computational survey of ncRNAs was performed for an early draft genome of *D. vexillum* (Velandia-Huerto et al. 2016). Blast and HMMer searches were performed with annotated small ncRNA sequences from metazoans and hidden Markov models from Rfam<sup>2</sup> to obtain the sort of candidates at sequence level. Structural alignments of those sequences were performed by infernal (Nawrocki and Eddy 2013), using metazoan-specific covariance models to annotate the small ncRNA collection, in which 57 families and 100 loci of miRNAs were found.

Small RNA libraries for the Southern Ocean salp *S. thompsoni* were sequenced with an Illumina HiSeq 2000 (Jue et al. 2016). After filtering data sets to 18–24 nt for miRNA and 28–32 nt for piRNA, the reads were aligned to *S. thompsoni* genome, and miRNA gene folding predictions were performed using RNAfold. In this initial survey of small RNAs, were revealed the presence of known, conserved miRNAs, as well as novel miRNA genes and mature miRNA signatures for varying developmental stages. Then in 2017, the prediction of 319 miRNA candidates in *H. roretzi* was obtained through three complementary methods. The experimental validation suggested that more than half of these candidate miRNAs are expressed during embryogenesis. The expression of some of the predicted miRNAs were validated by RT-PCR using embryonic RNA. In this approach, *C. robusta* small RNA-seq reads derived from *C. robusta* (Shi et al. 2009) (previously known as *C. intestinalis* today reclassified) were used to identify conserved miRNAs in *H. roretzi* (Wang et al. 2017).

Current repertoire of miRNAs (Fig. 11.8) is based on the final matrix of miRNA families from Hertel and Stadler (2015) and complemented by homology methods developed by Velandia-Huerto et al. (2016), and specifically for *S. thompsoni* and *H. roretzi*, blast searches with structural alignments were applied on the reported candidates in Jue et al. (2016) and Wang et al. (2017).

### 11.2.3 miRNA in Clusters

One of the most interesting aspects about the patterns of genomic locations of miRNAs is to know whether those loci are randomly distributed throughout the genome as single copies or if they are arranged on consecutive locations or in tandem copies clustered to be expressed from polycistronic primary precursors or to be transcribed independently (Tanzer et al. 2010). Interestingly in *O. dioica*, miRNAs are located in the antisense orientations of protein-coding gene and immediately

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<sup>2</sup><http://rfam.xfam.org/>

downstream of its corresponding 3'UTR region or even more frequently in the sense strand of introns (Fu et al. 2008). Nevertheless, after those conspicuous distributions, some clusters have been also identified in *O. dioica*. For instance, four miRNAs, miR-1490a, miR-1493, miR-1497d, and miR-1504, are reported by Fu et al. (2008) to be present as two copies, and miR-1497d-1 and miR-1497d-2 are included in the large miR-1497 cluster. The current structure of this cluster is shown in Table 11.1 although only one copy for the miR-1497 has been reported for *C. robusta* located in an intergenic region (Fu et al. 2008; Hendrix et al. 2010) and one in *C. savignyi* overlapped in an intron (Fu et al. 2008). By testing real-time PCR co-expression of some miRNAs, their host, and adjacent genes in *O. dioica* by Fu et al. (2008), it was discovered that in the case of the cluster miR-1487/miR-1488, there was no clear positive or negative correlation with the expression of its antisense hosting gene. The same authors showed that in males, this cluster expression was not associated with the expression of its adjacent ABCA3 gene.

In *C. robusta*, some miRNAs are located in introns, and a small class of miRNAs are derived from the exonic parts of mRNAs (Hendrix et al. 2010). In contrast, some miRNAs in *O. dioica* are expressed from loci on the opposite strand on protein-coding genes. An example is miR-2246, which also produces antisense miRNAs derived from miRNA loci (moRNAs). Only 44 loci appeared to be expressed as antisense products from the 300 miRNA loci predicted in 2010 by Hendrix et al. (2010). In *Cionas* miRNAs organized in clusters have been also detected; for example, in *C. robusta*, a putative cluster was detected by Keshavan et al. (2010) using microarray analysis that shows a similar loci organization to the cluster let-7/miR-125/miR-100 observed in *Drosophila*. The miR-1473 was later classified as the orthologue of miR-100 in the analysis derived from the comparison of the evolution of this cluster conducted by Griffiths-Jones et al. (2011). The authors suggested that mir-100, mir125, and let7 are clustered in most of the bilaterian genomes including the 1473, that is, an orthologue of mir-100.

Current analysis of this cluster shows that the distribution of miRNA families on this let-7 cluster is present in all the studied chordate species. Vertebrate species such as *D. rerio* and *L. chalumnae* contain more than one let-7 cluster, extending the loci definition which is not restricted only to one element but for a cluster of many loci with different length distributions. It is important to see that let-7 is organized sometimes with another let-7 locus or with another miRNA's loci families. The distribution of this cluster reported in amphioxus is composed of two let-7 and three mir-10 (one bfl-mir-100, one bfl-mir-125a, and one bfl-mir-125b); this cluster architecture is conserved in vertebrates that apparently inverted the order and split the relation between let-7 and mir-10, creating two different cluster order groups: let-7 + mir-10 and let-7 + other families. In this way, tunicates belong to the latter group, not including mir-10 on the cluster but including mir-233, mir-1473, or mir-125.

A second miRNA cluster consisting of the miR-182 and miR-183 was also detected in *C. robusta* in 2010 by Keshavan et al. (2010) which in the current predictions reported another member locus: the miR-96 organized in the middle of those loci, as is shown in the Fig. 11.7. Here, the authors also found five additional

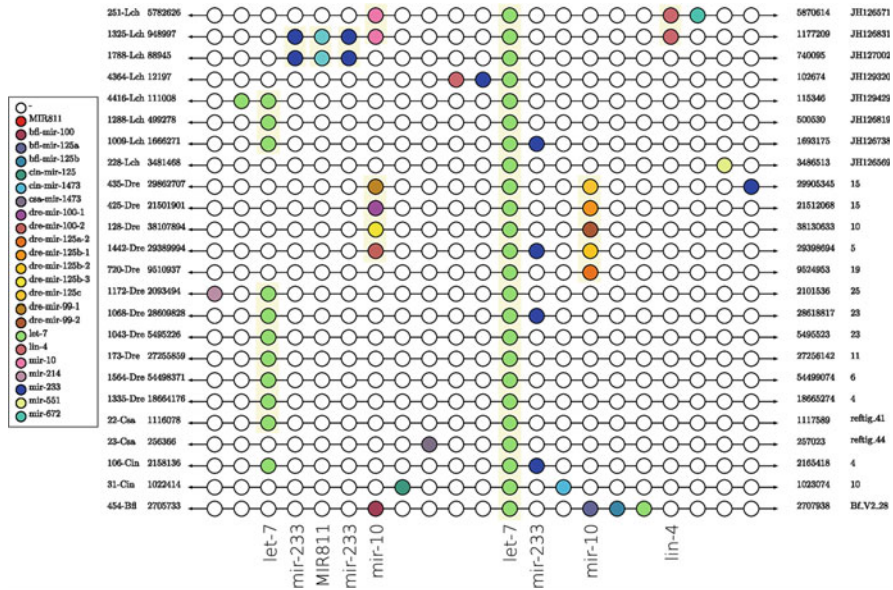
**Table 11.1** Details of the biggest miRNA cluster for chordate species

Specie	Chr	Start	End	Size (Mb)	No.	miRNAs detail
<i>B. floridae</i>	Bf_V2_118	216744	220351	3607	5	bfl-mir-4869, bfl-mir-4857, bfl-mir-4862, bfl-mir-4856b, bfl-mir-4856a
<i>O. dioica</i>	scaffold_3	2222857	2223714	857	6	odi-mir-1497e, odi-mir-1497d-2, odi-mir-1497d-1, odi-mir-1497c, odi-mir-1497b, odi-mir-1497a
<i>B. schlosseri</i>	chrUn	40003	41320	1317	2	mir-233, mir-10
<i>C. robusta</i>	7	4153284	4156782	3498	23	cin-mir-4006d, cin-mir-4006c, cin-mir-4001b-2, cin-mir-4000i, cin-mir-4006g, cin-mir-4001e, cin-mir-4001d, cin-mir-4000g, cin-mir-4006f, cin-mir-4006b, cin-mir-4001b-1, cin-mir-4000c, cin-mir-4006e, cin-mir-4000b-2, cin-mir-4001a-1, cin-mir-4000b-1, cin-mir-4002, cin-mir-4000d, cin-mir-4001h, cin-mir-4000a-2, cin-mir-4006a-2, cin-mir-4006a-3, cin-mir-4006a-1
<i>C. savignyi</i>	reftig_16	3924783	3925336	553	3	csa-mir-216b, csa-mir-216a, csa-mir-217
<i>C. savignyi</i>	reftig_1	1335375	1336487	1112	3	csa-mir-92b, csa-mir-92c, csa-mir-92a
<i>D. rerio</i>	4	28738556	28754891	16335	60	dre-mir-430a-18, dre-mir-430c-18, dre-mir-430b-4, dre-mir-430a-15, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430a-15, dre-mir-430c-18,

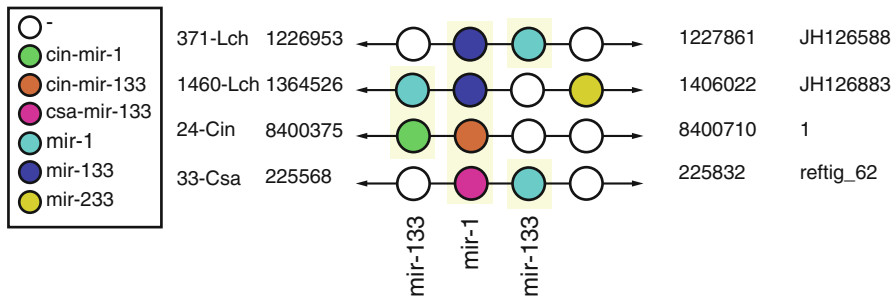
(continued)

**Table 11.1** (continued)

Specie	Chr	Start	End	Size (Mb)	No.	miRNAs detail
						dre-mir-430b-3, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-8, dre-mir-430a-15, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430a-17, miR-430, dre-mir-430b-20, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430i-3, dre-mir-430c-18, dre-mir-430b-3, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-8, dre-mir-430a-11, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430i-3, dre-mir-430c-18, dre-mir-430b-19, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430a-17, miR-430, dre-mir-430b-20, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430i-3, dre-mir-430c-18, dre-mir-430b-19, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-5, dre-mir-430a-15, dre-mir-430c-18, dre-mir-430b-3, dre-mir-430a-10, dre-mir-430c-18, dre-mir-430b-8, dre-mir-430a-15, dre-mir-430c-18, dre-mir-430b-5
<i>L. chalumnae</i>	JH126646.1	1529355	1882777	353422	7	mir-233, mir-233, mir-233, mir-598, mir-672, MIR535, mir-233



**Fig. 11.1** Multiple alignment of let-7 clusters. Specific names from annotations and homology predictions are described in the legend. Names from miRBase families are reported at the bottom of the aligned elements



**Fig. 11.2** mir-1/mir-133

paralogs of let-7 within a 1-kb stretch, but it is important to know that those elements had been identified on chromosome 4q on Ensembl release 54 version, and in the current version, only two of those elements have been identified by homology approaches (Fig. 11.1).

The cluster miR-1/miR-133, expressed specifically in *Cionas* muscle tissues, was also reported by Kusakabe et al. (2013). The authors reported that one copy of this cluster is presented in both *Cionas*. As shown in Fig. 11.2, a copy is also present in *L. chalumnae*. In 2012, a new cluster was proposed in *C. robusta* by Terai et al. (2012) located on the chromosome 10q and composed of the mir-4054 locus and the

mir-4091. In the current distribution of this cluster, a new annotated family, the mir-4008 with three paralogous families, is located on the middle of those loci. This current distribution is shown in Table 11.2; these loci were validated by Norden-Krichmar et al. (2007), Fu et al. (2008), Hendrix et al. (2010), and Terai et al. (2012). As was mentioned by Hendrix et al. (2010), it is not very common to find related miRNAs organized in clusters composed by closely related families that differ in just a single nucleotide in the seed sequence as was found on the cluster composed by nine Ci-mir-2200, seven Ci-mir-2201, and nine Ci-mir2203 which were previously reported under that putative names. The same authors also found a second large cluster composed of 11 miRNAs that gather into four paralogous families, 3 Ci-mir-2200, 3 Ci-mir-2201, 4 Ci-mir-2204, and 2 Ci-2217. Current distribution of miRNA families in *C. robusta* and curated annotations indicate that in other regions of the chromosome 7q, the miRNAs are organized in tandem copies of families. An example is the big cluster composed of the families miR4000, miR-4001, miR4002, and miR4006 located on chromosome 7q (Table 11.2). Another cluster is also located on the same chromosome composed by the families miR-4003, miR4005, and miR4077 in Table 11.2. Some other clusters are also found on the chromosome 1a, 10q, and 3p. See this structure in Table 11.2; most of them were validated by Hendrix et al. (2010) (Figs. 11.3, 11.4, 11.5, 11.6, 11.7, 11.8, 11.9).

Some other clusters shared between both *Cionas* are the cluster 92, 124, and 200 validated by Norden-Krichmar et al. (2007), Fu et al. (2008), and Hendrix et al. (2010), of which structure is shown in Table 11.2.

## 11.3 miRNAs and Its Role in Development

### 11.3.1 miRNA Discovery and Its Role in Development

Both microRNAs (miRNAs or miRs) and microRNA-offset RNA (moRNAs or moRs) are developmentally regulated as shown during *C. robusta* development (Shi et al. 2009). In spite of the considerably higher abundance of miRs and miRs\* in cells than their corresponding abundance of moRs, all three small RNA types have been shown to have regulatory roles in gene expression. Although a vast majority of miRNAs remain to be studied, there are already many cases of well-studied miRNAs (including many that are mentioned in this chapter that have been studied in tunicates) that are known to target mRNAs, modulate their levels of expression, and affect developmental processes both in plants and animals (Zhao et al. 2018). Only recently, two studies demonstrated for the first time that two moRs (viral moR-rR1-3-5p and moR-21) could also modulate gene expression and were not merely the by-product of miRNA biogenesis (Umbach et al. 2010; Zhao et al. 2016).

Table 11.2 Reported clusters in the literature

Specie	Chr	Start	End	miRNAs	Comments	Source DB	Ref.
Ciro	4q	2082260	2083286	<b>cin-let-7a-1, cin-let-7f, cin-let-7b, cin-let-7c, cin-let-7a-2</b>	Reported on miRBase and annotated on Ensembl	miRBase	Hendrix et al. (2010) and Fu et al. (2008)
Cisa	reflig_41	1114139	1117597	<b>csa-let-7c-1, csa-let-7b, csa-let-7a, csa-let-7c-2</b>	Reported on miRBase and does not detected by homology strategies	miRBase	Fu et al. (2008)
Ciro	10q	3226200	3228884	<b>cin-mir-34</b> , cin-mir-4091, cin-mir-4008a, cin-mir-4008c, cin-mir-4008b, cin-mir-4054	NA	miRBase, homology	Norden-Krichmar et al. (2007), Fu et al. (2008), Hendrix et al. (2010), and Terrai et al. (2012)
Ciro	7q	4828431	4835967	cin-mir-4077b, cin-mir-4003b, cin-mir-4005b, cin-mir-4077d, cin-mir-4003a-1, cin-mir-4003c, cin-mir-4077a, cin-mir-4003a-4, cin-mir-4003d	NA	miRBase, homology	Hendrix et al. (2010)
Ciro	3q	567478	571031	cin-mir-4001f, cin-mir-4000e, cin-mir-4001c, cin-mir-1502d, cin-mir-4018a, cin-mir-4019, cin-mir-1502b, cin-mir-1502a, cin-mir-4007, cin-mir-4000f, cin-mir-4001i, cin-mir-4018b, cin-mir-1502c	Inclusion of cin-mir-4019 and cin-mir-4007	miRBase, homology	Hendrix et al. (2010)
Ciro	HT000037.1	4884	5250	<b>cin-mir-367, cin-mir-4009c, cin-mir-4009b</b> , cin-mir-367, cin-mir-4009c, cin-mir-4009a, cin-mir-4009b	Non-highlighted names could be found in the current genome at HT000037.1 scaffold	miRBase, Homology	Hendrix et al. (2010)



Ciro	10q	3226200	3228884	<b>cin-mir-34</b> , cin-mir-4091, cin-mir-4008a, cin-mir-4008c, cin-mir-4008b, cin-mir-4054	NA	miRBase, homology	Hendrix et al. (2010)
Ciro	3q	884615	885508	cin-mir-92a, cin-mir-92d, cin-mir-92c	NA	miRBase, homology	Hendrix et al. (2010)
Cisa	refug_1	1335375	1336487	csa-mir-92b, csa-mir-92c, csa-mir-92a	NA	miRBase, homology	Fu et al. (2008)
Oidi	scaffold_1	3086369	3086586	odi-mir-92b, odi-mir-92a	NA	Homology	Fu et al. (2008)
Ciro	7q	4969691	4969912	cin-mir-124-1, cin-mir-124-2	NA	miRBase, homology	Hendrix et al. (2010) and Fu et al. (2008)
Cisa	refug_262	49392	49620	csa-mir-124-1, csa-mir-124-2	NA	miRBase, homology	Fu et al. (2008)
Ciro	HT000325.1	8331	8778	cin-mir-200, <b>cin-mir-3575</b> , cin-mir-141, <b>cin-mir-5611</b>	NA	miRBase, homology	Norden-Krichmar et al. (2007), Fu et al. (2008), Hendrix et al. (2010), and Friedländer et al. (2012)
Cisa	refug_613	31353	31949	csa-mir-200, csa-mir-141	NA	miRBase, homology	Fu et al. (2008)
Ciro	7q	4153284	4156782	cin-mir-4006d, cin-mir-4006c, cin-mir-4001b-2, cin-mir-4000i, cin-mir-4006g, cin-mir-4001e, cin-mir-4001d, cin-mir-4000g, cin-mir-4006f, cin-mir-4000h*, cin-mir-4006b, cin-mir-4001b-1, cin-mir-4006e, cin-mir-4001a-1, <b>cin-mir-4001a-2</b> , cin-mir-4002, cin-mir-4001h, cin-mir-	Elements marked with * are identified by homology strategies at the same cluster, but in another order reported by miRBase	miRBase, homology	Hendrix et al. (2010)

(continued)

Table 11.2 (continued)

Specie	Chr	Start	End	miRNAs	Comments	Source DB	Ref.
				4000a-2, cin-mir-4006a-2, cin-mir-4006a-3, cin-mir-4006a-1, <b>cin-mir-4006e</b> , <b>cin-mir-4001a-1</b> , <b>cin-mir-4006b</b> , cin-mir-4000c, <b>cin-mir-4006e</b> , cin-mir-4000b-2*, <b>cin-mir-4001a-1</b> , cin-mir-4000b-1*, <b>cin-mir-4001a-2</b> , <b>cin-mir-4002</b> , cin-mir-4000d*, <b>cin-mir-4001h</b> , <b>cin-mir-4006a-3</b> , <b>cin-mir-4006a-1</b> , <b>cin-mir-4000a-1</b>			

Bold text represents those miRNA elements that are currently annotated and validated but could not be detected by homology strategies  
**Ciro**, *C. robusta*; **Cisa**, *C. savignyi*; **Oidi**, *O. dioica*

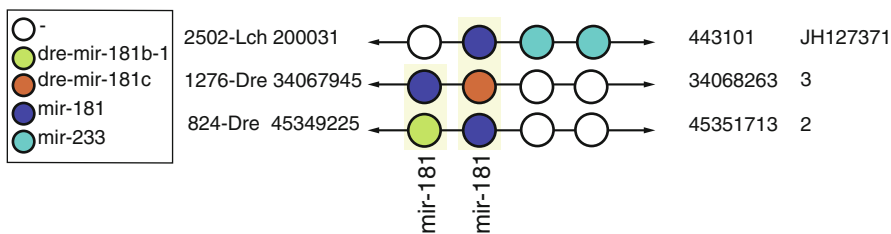


Fig. 11.3 mir-181

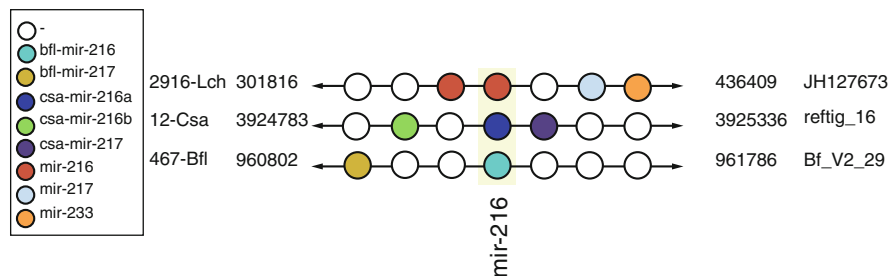


Fig. 11.4 mir-216/mir-217

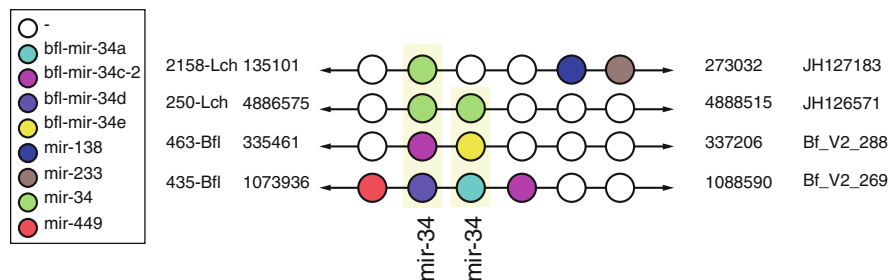


Fig. 11.5 mir-34

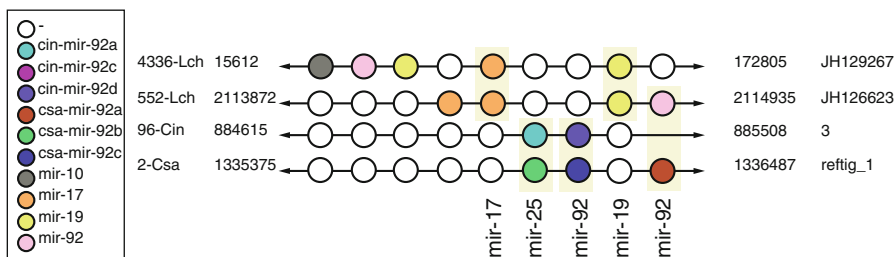
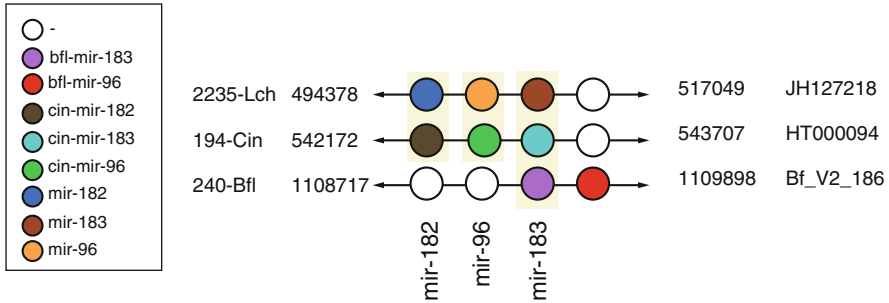


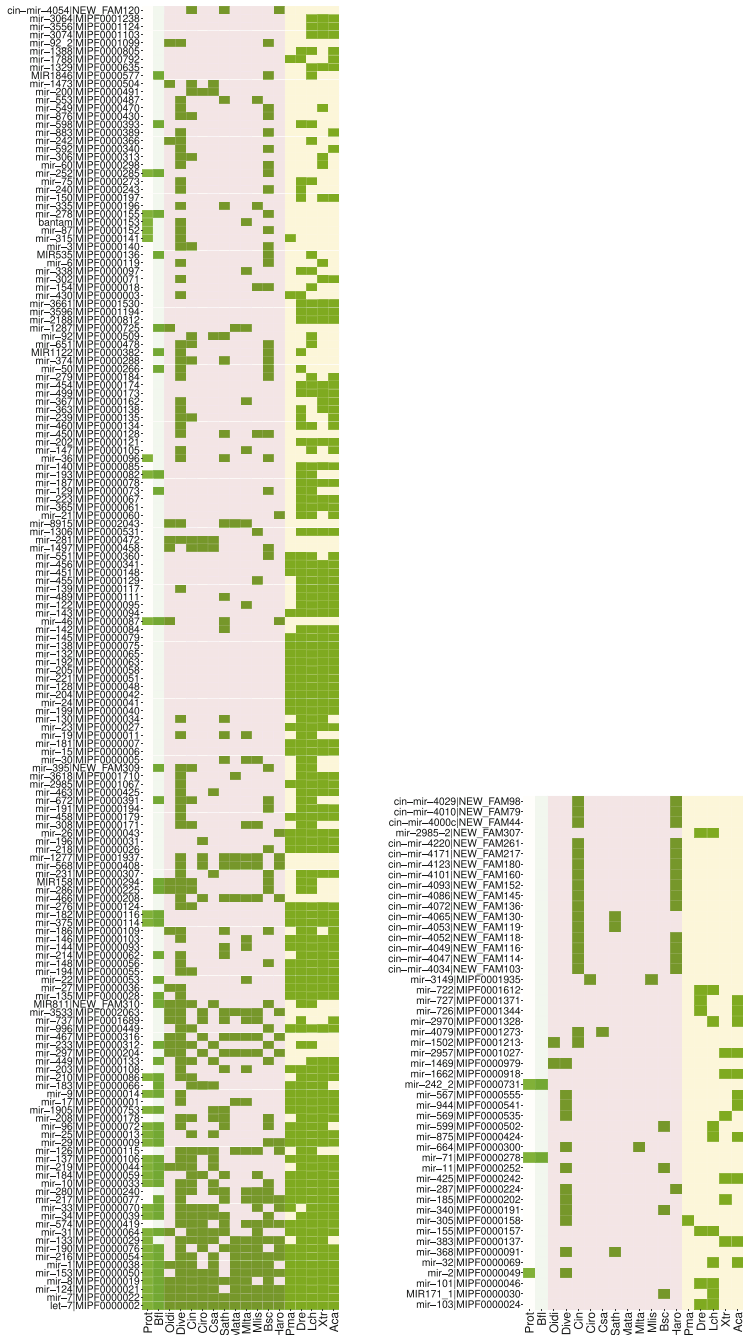
Fig. 11.6 mir-92



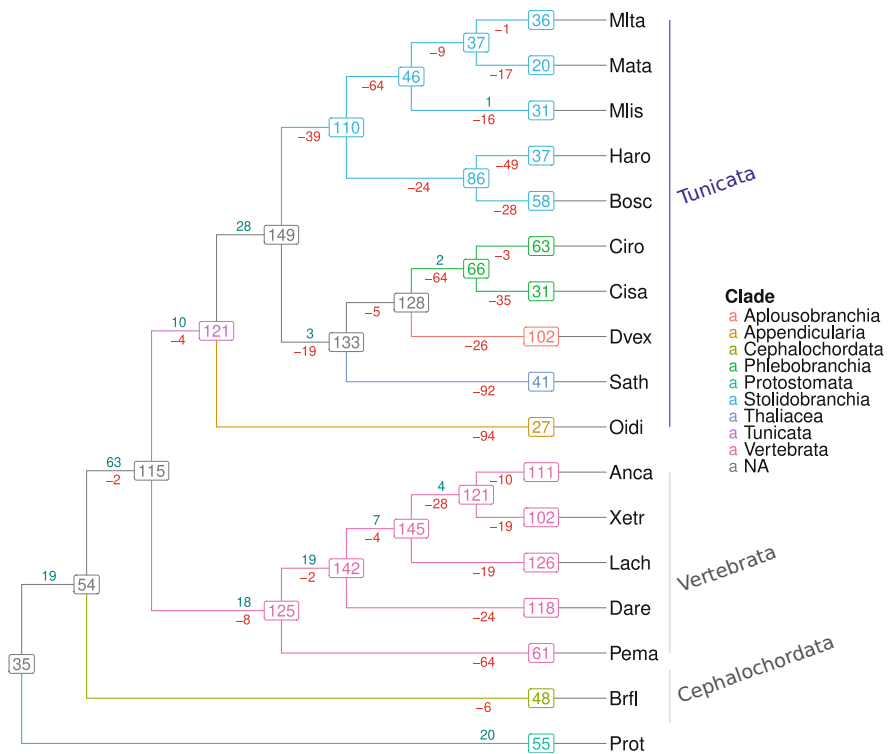
**Fig. 11.7** mir-182/mir-96/mir-183

### 11.3.2 Neuronal Fate Determination and Regulation by miR-124

The miRNA miR-124 is expressed in the nervous system of many animals, including *Drosophila* (Aboobaker et al. 2005), *C. elegans* (Clark et al. 2010), and humans (Sempere et al. 2004). As was first observed in in vitro studies of mouse brain cells, low expression of miR-124 was related to neural stem cell maintenance, whereas high expression of miR-124 induced the differentiation of neuronal cell types (Cheng et al. 2009). A regulative role of miR-124 in nonneural vs. neural fate decisions was further investigated by embryonic experiments in vivo (Chen et al. 2011) and by theoretical and in silico modeling analyses in *C. robusta* (Chen et al. 2014). These studies showed that miR-124 promotes nervous system development by feedback interactions with Notch signaling. During nervous system development of *C. robusta*, cells in the dorsal and ventral midline epidermis of the tailbud embryo take either an epidermal sensory neuron (ESN) or peripheral nervous system (PNS) fate, a decision mediated by lateral inhibition using a classical model of feedback loop regulation Notch-Delta signaling in neighboring cells (Collier et al. 1996; Chen et al. 2014). Cells that take an ESN fate showed low expression of miR-124 presumably by Notch inhibition, whereas cells that take a PNS fate expressed high levels of miR-124, which in the latter case was shown to target and repress nonneuronal genes (e.g., neuronal repressors SCP1 and PTBP1) downstream of Notch signaling (Chen et al. 2011). In addition, expression of miR-124 in larval epidermal cells was sufficient for ectopic neural specification, which resembled mis-expression experiments using Pou4, an important transcription factor for sensory neuron specification (Chen et al. 2011; Joyce Tang et al. 2013). Whereas miR-124 targeting to SCP1 is thought to have evolved in the vertebrates + tunicates, miR-124 targeting to PTBP1 may be conserved among bilaterians except for ecdysozoans (Chen et al. 2011) suggesting that the miRNA regulatory logic in lateral inhibition models of Notch-Delta signaling may have broader implications in other organisms yet to be studied (Chen et al. 2014). The research team also showed that miR-124 acted at the gastrula stage and targeted other nonneural genes such as muscle determinant Macho-1 and notochord determinant Brachyury to allow for ectodermal fate specification (Chen et al. 2011).



**Fig. 11.8** The absence/presence matrix of miRNA families along bilaterian species. **Prot**, Protostomata; **Brfl**, *B. floridae*; **Oidi**, *O. dioica*; **Dvex**, *D. vexillum*; **Ciin**, *C. robusta*; **Cisa**, *C. savignyi*; **Ciro**, *C. robusta*; **Sath**, *S. thompsoni*; **Mata**, *M. oculata*; **Mlta**, *M. occulta*; **Mlis**, *M. occidentalis*; **Bosc**, *B. schlosseri*; **Haro**, *H. roretzi*; **Pema**, *P. marinus*; **Dare**, *D. rerio*; **Lach**, *L. chalumnae*; **Xetr**, *X. tropicalis*; and **Anca**, *A. carolinensis*



**Fig. 11.9** Dollo parsimony of miRNA family distribution in some chordate genomes. **Prot**, Protostomata; **Brfl**, *B. floridae*; **Oidi**, *O. dioica*; **Dvex**, *D. vexillum*; **Ciro**, *C. robusta*; **Cisa**, *C. savignyi*; **Sath**, *S. thompsoni*; **Mata**, *M. oculata*; **Mlta**, *M. occulta*; **Mlis**, *M. occidentalis*; **Bosc**, *B. schlosseri*; **Haro**, *H. roretzi*; **Pema**, *P. marinus*; **Dare**, *D. rerio*; **Lach**, *L. chalumnae*; **Xetr**, *X. tropicalis*; and **Anca**, *A. carolinensis*. The phylogenetic distribution of this species was obtained from Delsuc et al. (2017) and Kocot et al. (2018). Red numbers represent losses and green gains of miRNA families

### 11.3.2.1 Muscle Development and the Polycistronic miR-1/miR-133 Cluster

A well-studied case of miRNA regulation in muscle development is the miR-1/miR-133 polycistronic cluster. Whereas miR-1 promotes differentiation of muscle, miR-133 promotes proliferation of muscle precursors (Chen et al. 2005). In the chordates, these two miRNAs are encoded in an antisense direction in a relatively close localization (3–11 kb apart) within the gene *mind bomb 1* (MIB1) and transcribed as a single primary (i.e., polycistronic) transcript. Except for *Drosophila* and ambulacrarians (i.e., echinoderms and hemichordates), a close proximity of these two miRNAs has been documented in most animal taxa suggesting some form of functional regulatory constraint of a condensed miR-1/miR-133 cluster for the bilaterians (Campo-Paysaa et al. 2011). During *C. robusta* development, the

polycistronic transcription can be detected in the nuclei of presumptive tail muscle cells from the gastrula stage onward, and its transcription is regulated by an **850** bp sequence upstream of the transcript start site (Kusakabe et al. 2013). Differential expression of the two miRNAs in muscle tissues was only detected in the adult, where body wall muscle expressed similar levels of miR-1 and miR-133 and heart muscle expressed significantly higher levels of miR-1 (Kusakabe et al. 2013).

### 11.3.2.2 miRNA Expression During Oral Siphon (OS) Regeneration

Three stages of regeneration have been proposed that reconstruct main events of regeneration that match expected miRNAs expression profiles in the corresponding timeframes (Knapp et al. 2013). The three phases correspond to (a) wound healing, (b) transition, and (c) redevelopment. The miRNA-mRNA transcriptional profiling using a correlation network correlated differential expression of mRNAs to miRNA profiles during the three regeneration windows mentioned above in *C. robusta* oral siphon regeneration (Spina et al. 2017). In the first phase, i.e., wound healing, miRNA target clusters of miR 4178b-5p and miR 4\_20211 were found to be correlated to the differential expression of genes involved in the following Gene Ontology (GO) term functional classifications: immune response, stress response, and apoptosis. In the second phase, i.e., transition, miR 4008c-5p, miR 4123-5p, miR 4178-5p, miR 2\_15911, miR 4\_20211, and miR 11\_7539 were correlated and known to target Wnt, TGFb, and MAPK pathway genes that may be regulating the proliferative state characteristic of this particular timeframe. In the third phase, i.e., redevelopment, miR4008c-5p, miR 10\_4533, and miR 11\_6940 known to target ECM peptidase inhibitors are correlated with the characteristic extracellular matrix remodeling that occurs at the final phase of regeneration and which resembles the original developmental processes. In contrast, other miRs were found expressed throughout the regenerative process. MiRNA miR 10\_4533 known to target IGF and IGFb was found expressed presumably regulating the proliferation of progenitor cells. Also miR-9 was found expressed throughout regeneration and is known to be essential for neural development and function, presumably by targeting and regulating genes involved in cytoskeleton and cell cycle functions (Galderisi et al. 2003; McBeath et al. 2004), instead of targeting Notch or Hes-1 (Spina et al. 2017).

### 11.3.2.3 miRNA Expression During *O. dioica* Development

A most thorough study of the miRNA repertoire expressed during development has been published for the larvacean *O. dioica* (Fu et al. 2008). Using a miRNA array approach with **55** candidate miRNAs and **10** developmental stages for analyses, some general patterns of miRNA occurrence emerged. MicroRNAs were expressed throughout the life cycle of the animal and were deposited in eggs as maternal determinants for early zygotes. Expression of zygotic miRNAs, such as miR-1487 and miR-1488, was observed starting in the blastula stage (1.5 h postfertilization).

Most miRNAs analyzed showed developmental regulation (for specific miRNAs that were differentially expressed at each stage, see Fu et al. (2008)), except for some such as miR-1497 that was expressed throughout all stages (Fu et al. 2008). From this study, the first sex-specific miRNAs were revealed: miR-1478 was expressed in 6-day-old females in the oocytes, whereas miR-1487/88 was expressed in 6-day-old males. Interestingly, the compact genomes of *O. dioica* showed one single copy of most miRNA loci, except for miR-1490a, miR-1493, miR-1497d, and miR-1504 that were in two copies (Fu et al. 2008).

## 11.4 Other ncRNAs Associated with Development

### 11.4.1 *Yellow Crescent RNA*

Yellow crescent RNA, i.e., YC RNA, consists of an about 1.2-kb-long polyadenylated RNA, which can be present throughout the embryonic development of ascidians (Swalla and Jeffery 1995). Its name refers to the fact that in situ hybridization YC RNA is localized in the yellow crescent region of one-cell zygotes. The YC transcripts are actually already found in the cortex of unfertilized eggs, segregating with the myoplasm to the yellow crescent after fertilization (Swalla and Jeffery 1995). Subsequently, most YC transcripts enter the primary muscle cell lineage after cleavage and are also present in the secondary muscle cell lineage (Swalla and Jeffery 1995). YC RNA was first discovered in the club tunicate *Styela clava* (Swalla and Jeffery 1995). As the presence of the 1.2-kb RNA in oocytes and early cleaving embryos indicates that it is a maternal transcript, YC RNA is considered to be a maternal RNA (Swalla and Jeffery 1995). It is associated with the cytoskeleton and segregates to the muscle cells during ascidian embryogenesis. Although the YC ORF encodes for a putative polypeptide of 49 amino acids, this protein is relatively small and does not show any significant homology to any known proteins. As the YC RNA shows various features indicating that it actually functions as an RNA rather than as a protein-coding molecule, it is considered to be a noncoding RNA that may play an important role in growth and development (Swalla and Jeffery 1995).

### 11.4.2 *MicroRNA-Offset RNAs*

MicroRNA-offset RNAs, i.e., moRNAs, are about 20 nucleotides long RNAs that lie adjacent to pre-miRNAs. They can originate from both ends of these pre-miRNAs, although prevalently they are derived from the 5' arm (Bortoluzzi et al. 2011). During a study focused on identifying miRNAs in the simple chordate *C. robusta*, moRNAs were first discovered (Shi et al. 2009). Unexpectedly, half of the *C. robusta* miRNA loci that were detected in this study turned out to encode the previously uncharacterized small RNAs, in addition to conventional miRNA and miRNA\*



products. This new class of RNAs was hereafter referred to as “moRNAs,” for miRNA-offset RNAs. It became clear that these moRNAs are probably produced by RNase II-like processing and are observed, like miRNAs, at specific developmental stages (Shi et al. 2009). These results and subsequent studies gave rise to the hypothesis that moRNAs represent a new class of functional regulators whose qualitative alteration and/or expression dysregulation might even impact human diseases (Bortoluzzi et al. 2011). Evidence supporting this hypothesis is still fragmentary however. After the discovery in *Ciona*, moRNAs were also found in human cells by deep sequencing analysis. Hereby, it was reported that moRNAs from 78 genomic loci were weakly expressed in the prefrontal cortex (Langenberger et al. 2009). Additional indications that moRNA have a distinct function include the fact that some moRNAs are as conserved as miRNAs and are in fact conserved across species to the extent that correlated with expression level (Shi et al. 2009). The expression level of certain moRNAs can even be greater than for their corresponding miRNA (Umbach and Cullen 2010). Finally, it can be argued (Bortoluzzi et al. 2011) that it is likely that moRNAs might represent a functional class of miRNA-related agents as moRNAs are prevalently produced by the 5' arm of the precursor, independent of which arm produces the most expressed mature miRNA (Langenberger et al. 2009; Umbach and Cullen 2010). What functions moRNAs may have vary. For example, moRNA expression was recorded in solid tumors, together with other small RNAs (Meiri et al. 2010). In addition, the fact that an 18-fold enrichment of moRNAs was observed in the nucleus (Taft et al. 2010) indicates that at least some moRNAs may have functions related to nuclear processes (Bortoluzzi et al. 2011). Recently, a specific class of moRNAs (moRNA-21) has been associated with posttranscriptional gene regulation, proliferation of vascular smooth muscle cells (VSMC), and mediated gene downregulation in a process mediated by Ago2 (Zhao et al. 2016). Although these studies do provide good indications, the potential functional roles that moRNAs can play remain to be still largely unknown.

### ***11.4.3 Long Noncoding RNA RMST***

Long noncoding RNAs, i.e., lncRNAs, are abundantly found within mammalian transcriptomes. One of the known groups of lncRNAs includes the rhabdomyosarcoma 2-associated transcript (RMST), which is indispensable for neurogenesis (Ng et al. 2013). Human RMST was shown to be responsible for the modulation of neurogenesis as its expression is regulated by the transcriptional repressor REST, while it increases during neuronal differentiation (Ng et al. 2013). Hereby, it was found that RMST is actually necessary for the binding of SOX2 to promoter regions of neurogenic transcription factors. SOX2, a transcription factor known to regulate neural fate, in combination with RMST was actually found to coregulate a large pool of downstream genes implicated in neurogenesis, i.e., more than 1000 genes were differentially expressed upon RMST knockdown (Ng et al. 2013). These results illustrated the role of RMST as a transcriptional coregulator of SOX2 and a key

player in the regulation of neural stem cell fate (Ng et al. 2013). A further confirmation of the importance of RMST came with the discovery of a homologue of this lncRNA in the simple chordate *D. vexillum*, i.e., the carpet sea squirt (Velandia-Huerto et al. 2016). While homologues of “human” lncRNAs are rarely found across all chordates due to their low levels of sequence conservation, a plausible homologue of RMST 9, the conserved region 9 of the rhabdomyosarcoma 2-associated transcript known for its interaction with SOX2, was found in *D. vexillum*. Subsequently, putative homologues were also found in the genomes of the ascidians *C. robusta*, *C. savignyi*, and *B. schlosseri* and the Florida lancelet *B. floridae*, illustrating that RMST lncRNA is thus conserved across chordates, making them one of the best conserved lncRNAs known to date (Velandia-Huerto et al. 2016).

#### 11.4.4 Spliced-Leader RNA

mRNA 5′ leader trans-splicing is a mode of gene expression in which the 5′ end of a pre-mRNA is discarded and replaced by the 5′ segment of a spliced-leader (SL) RNA (Vandenberghe et al. 2001). Spliced-leader RNAs, i.e., SL RNAs, hereby consist of a 5′ exon and a 3′ intron with a conserved consensus 5′ splice donor site at the exon-intron boundary (Ganot et al. 2004). SL RNA trans-splicing has not only been described for euglenoids, kinetoplastids, cnidarians, nematodes, and Platyhelminthes (Ganot et al. 2004) but also for deuterostomes like the simple chordate *C. robusta* (Vandenberghe et al. 2001) and the appendicularian *O. dioica* (Ganot et al. 2004). Hereby, *O. dioica* was shown to not only trans-splice SL RNAs to mRNAs, as does *C. robusta*, but also to use trans-splicing in resolving polycistronic transcripts (Ganot et al. 2004). During trans-splicing, the capped SL RNA exon moiety is covalently linked to the 5′ ends of mRNAs, forming a leader sequence ranging from 16 nt in *C. robusta* to 41 nt in trypanosomatids (Ganot et al. 2004). The role of SL trans-splicing is still unknown in many cases. SL trans-splicing may potentially have functions varying from the mediation of mRNA stability or translatability (Maroney et al. 1995) and the resolution of polycistronic pre-mRNAs (Agabian 1990; Blumenthal 1995) to the production of functional mRNAs from RNA polymerase I transcripts (Lee and der Ploeg 1997).

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**Part III**  
**Differentiation, Regeneration and Stemness**



# Chapter 12

## Differentiation and Transdifferentiation of Sponge Cells



Maja Adamska

**Abstract** Over 100 years of sponge biology research has demonstrated spectacular diversity of cell behaviors during embryonic development, metamorphosis and regeneration. The past two decades have allowed the first glimpses into molecular and cellular mechanisms of these processes. We have learned that while embryonic development of sponges utilizes a conserved set of developmental regulatory genes known from other animals, sponge cell differentiation appears unusually labile. During normal development, and especially as a response to injury, sponge cells appear to have an uncanny ability to transdifferentiate. Here, I argue that sponge cell differentiation plasticity does not preclude homology of cell types and processes between sponges and other animals. Instead, it does provide a wonderful opportunity to better understand transdifferentiation processes in all animals.

### 12.1 Introduction

Sponges (Porifera) are exclusively aquatic animals, with all but a few species found in marine environments (Hooper and van Soest 2002). They are morphologically simple, with no neurons, muscles, or digestive tracts. Remarkably, sponges exhibit a spectacular capacity for regeneration, which appears to be driven by a combination of cell motility and unusual plasticity of cell differentiation. These features have attracted cell biologists to the study of sponges since the nineteenth century (James-Clark 1867; Saville-Kent 1880; Wilson 1907; reviewed by Simpson 1984).

Sponges have traditionally been considered the oldest of surviving animal phyla (reviewed by Dohrmann and Wörheide 2013). While this position has been hotly debated throughout the past decade, the most recent analyses provide substantial support for the view that sponges are indeed the sister clade to all other animals

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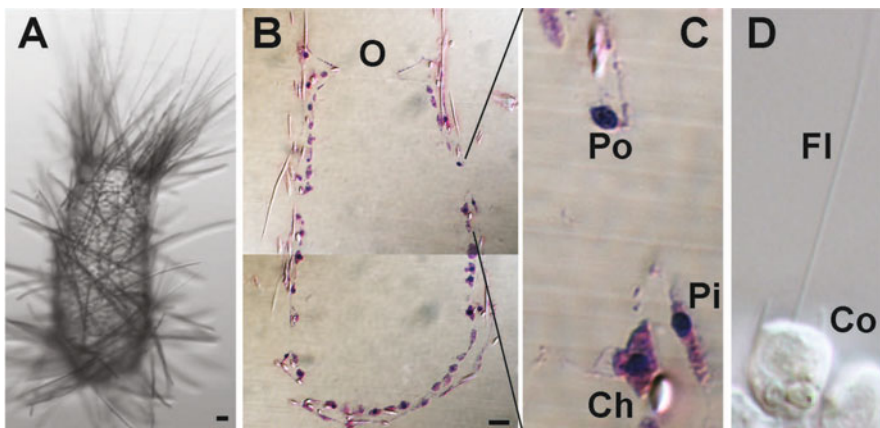
M. Kloc, J. Z. Kubiak (eds.), *Marine Organisms as Model Systems in Biology and Medicine*, Results and Problems in Cell Differentiation 65,

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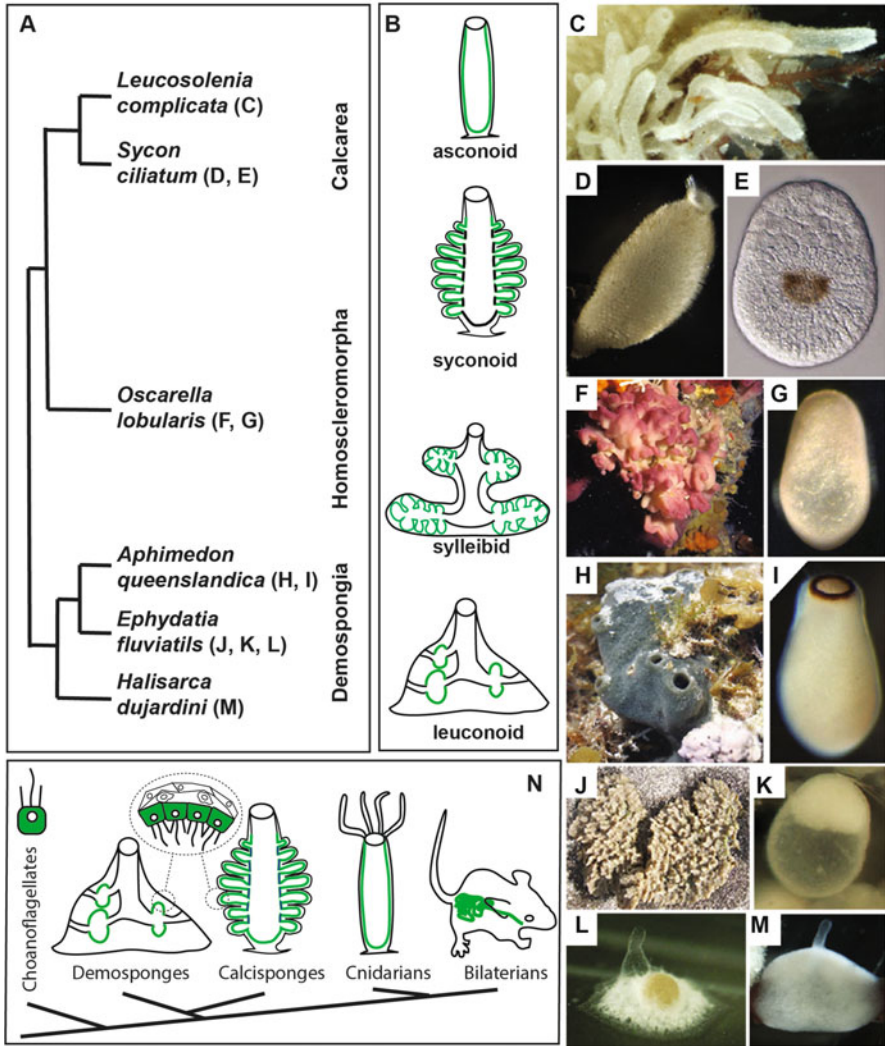
(e.g., Whelan et al. 2017; Feuda et al. 2017; reviewed by King and Rokas 2017). Given their phylogenetic position, study of sponge cells holds a promise of uncovering the evolutionary history of the mind-boggling diversity of animal cell types. However, it is important to keep in mind that sponges are not “living fossils,” as they have continued to evolve independently of the eumetazoans (complex animals such as cnidarians and bilaterians) for over 700 million years. Therefore, no feature of sponge cells can be automatically considered “ancestral” to features of eumetazoan cells. On the other hand, careful analyses comparing sponges with unicellular and colonial relatives of animals, as well as a range of eumetazoans, have generated numerous insights and—sometimes conflicting—hypotheses on the evolution of cell differentiation mechanisms in animals (recently reviewed by Seb -Pedr s et al. 2017; Brunet and King 2017).

## 12.2 Cell Types in Sponges: Ancient Characters?

Sponges are almost universally filter feeders, although a handful of species abandoned this lifestyle and its accompanying morphological features to become predators (Vacelet and Boury-Esnault 1995). The term Porifera (pore bearing) refers to innumerate minute openings (ostia), which pierce the surface of juvenile and adult sponge bodies. Ostia allow water to enter chambers formed by choanocytes (collar cells), which are characterized by the presence of an actin-based microvilli collar surrounding a single motile flagellum (Figs. 12.1, 12.2). Propelled by beating of the flagella, water flows through the chambers and is expelled through larger and less



**Fig. 12.1** Juvenile calcisponge *Sycon ciliatum*, an example of the simplest (asconoid) sponge morphology. (a) Live specimen, (b) hematoxylin- and eosin-stained resin section, (c) magnification of (b) showing cell details, and (d) isolated live choanocyte. *Ch* choanocyte, *Co* collar, *Fl* flagellum, *Pi* pinacocyte, *Po* porocyte, *O* osculum. Scale bar represents 10  $\mu\text{m}$ . (a–c) modified after Leininger et al. (2014)



**Fig. 12.2** Sponge model species and their body plans. **(a)** Phylogenetic relationships between the most commonly used sponge model species. **(b)** Schematic representations of sponge body plans; **(c–m)** images of live adult **(c, d, f, h, j)**, juvenile **(l, m)**, and larval **(e, g, i, k)** specimens; **(n)** proposed evolutionary relationship between sponge and eumetazoan body plans. Green color represents cells and tissues involved in food digestion. **(d–l, n)** are reproduced, with modifications, from Adamska et al. (2011) and Adamska (2016a) respectively

numerous (sometimes single) exhalant openings (oscula). As the water passes through the chambers, food particles (predominantly bacteria) are captured by collars of choanocytes (and, to a lesser extent, their cell bodies as well as some other cell types) (Leys and Eerkes-Medrano 2006).

In addition to propelling water, capturing and digesting the food, choanocytes are the major proliferating cell type in sponges. Choanocyte proliferation is often accompanied by shedding—loss of non-apoptotic cells. This high rate of cell turnover is hypothesized to maintain tissue homeostasis (Alexander et al. 2014). As will be discussed below, choanocytes can also readily transdifferentiate into other cell types.

This multitude of choanocyte functions is reminiscent of “multitasking” by single-cell organisms, contrasting with the often highly specialized metazoan cells. Furthermore, choanocytes are morphologically virtually indistinguishable from choanoflagellates, which are single cell or colonial protists, and the nearest relatives of animals. This similarity has been noted early (James-Clark 1867; Saville-Kent 1880) and is generally considered to reflect an evolutionary link between choanocytes and choanoflagellates (King 2004; Maldonado 2004; Richter and King 2013; Brunet and King 2017, but see Mah et al. (2014) for an alternative point of view).

Pinacocytes are the second major cell type in sponges. In contrast to broadly cuboid choanocytes forming the innermost layer of the sponge bodies (the choanoderm), pinacocytes are extremely flat and thin and make up the external surface of the sponges (Figs. 12.1, 12.2). Intriguingly, no records of proliferation in pinacocytes have been published so far. For a long time, sponges have been said to lack “true epithelia” (reviewed by Leys et al. 2009). However, this view was changed by the discovery of a pinacoderm-associated collagen IV-containing basement membrane (Boute et al. 1996) and observation of the pinacoderm’s sealing capacity (Adams et al. 2010).

Depending on sponge species, a large diversity of cells can be found in the non-epithelial layer (mesohyl) sandwiched between the pinacoderm and the choanoderm (reviewed by Simpson 1984). Two particularly well-studied cell types present in the majority of sponges are archeocytes and sclerocytes. Archeocytes are defined as amoeboid, motile mesohyl cells with nucleolate nucleus (Simpson 1984; Ereskovsky 2010). They are proliferative (although less so than choanocytes) and are generally considered to be pluripotent (or totipotent) stem cells (reviewed by Funayama 2010). Sclerocytes are responsible for secretion of spicules—sponge skeletal elements. Spicules can be siliceous [formed intracellularly, in vacuoles, reviewed by Uriz et al. (2003)] or calcareous [formed cooperatively by several sclerocytes, reviewed by Sethmann and Wörheide (2008)].

### 12.3 Sponge Body Plans: Is There Similarity to Other Animals?

The overwhelming majority of adult sponges are constructed from the same “building blocks”: pinacocytes, choanocytes, and a selection of mesohyl cells. However, sponge body plans vary widely among and within the four distinctive sponge classes: Calcarea, Homoscleromorpha, Demospongiae, and Hexactinellida. This

chapter focuses on three classes including the most extensively used sponge model species. I omit the fascinating but highly derived and difficult to work with Hexactinellida (glass sponges), which have syncytial organization (Leys et al. 2007).

In the simplest case, a sponge body can be an exquisitely simple filter unit composed of two epithelial layers: the external pinacoderm (exopinacoderm) and the internal choanoderm. The two epithelia are connected by ring-shaped porocytes forming the ostia (Fig. 12.1). Sandwiched between the pinacoderm and the choanoderm, a narrow mesohyl layer contains sclerocytes and, in adults, oocytes and developing embryos. This simple body plan is referred to as asconoid and is found only among the Calcarea (calcareous sponges, calcisponges), which are characterized by calcite spicules. While not speciose (Hooper and Soest 2002), calcisponges display the largest diversity of body plans among all sponge classes. Adult asconoid calcareous sponges grow through budding and formation of additional, sometimes anastomosing, tubes, as, for example, in *Leucosolenia complicata* (Fig. 12.2c). In addition to the asconoid body plan, calcareous sponges can be syconoid, sylleibid, or leuconoid.

In the syconoid body plan, ostia lead directly to choanocyte chambers, which open to a central atrium. The central atrium is lined by endopinacocytes and crowned by the osculum. Syconoid sponges usually maintain radial symmetry of the body, having only one osculum through their adult life, as it is the case of *Sycon ciliatum* (Fig. 12.2d). In the sylleibid body plan, pinacocyte-lined canals collect water from several choanocyte chambers before opening into the atrium. In the most complex sponge body plan (leuconoid), a system of endopinacocyte-lined channels connects the ostia and oscula; choanocyte chambers branch from these channels into a thick mesohyl layer (Fig. 12.2b).

Similarly to their sister group (the calcisponges), homoscleromorphs are generally small and exclusively marine (Hooper and Soest 2002; Ereskovsky 2010). In contrast to calcisponges, they never have calcareous skeletal elements, but some species possess siliceous spicules. Homoscleromorphs have sylleibid or leuconoid bodies, losing axial symmetry after the juvenile stage as they spread along the substrate (see *Oscarella lobularis*, Fig. 12.2f). Their mesohyl is usually thin and cell poor. Uniquely among sponges, all homoscleromorph pinacocytes are equipped with flagella, although in contrast to the flagella of choanocytes, these are not motile (reviewed by Ereskovsky 2010).

Demosponges are the most numerous of poriferan lineages, both in terms of number of species and total biomass. While all are leuconoid, they display a spectacular diversity of body sizes (from the minute to the almost 2 m tall giant barrel sponges), shapes, and colors. Demosponges from the family Spongillidae, such as *Ephydatia fluviatilis* (Fig. 12.2j–l), have secondarily invaded freshwater habitats and are found across the globe in streams, rivers, and lakes (recently reviewed by Manconi and Pronzato 2016). Demosponge mesohyl is particularly rich in cells including archeocytes. In demosponges containing inorganic skeletal elements, sclerocytes produce silica-based spicules, sometimes of immense beauty (Hooper and van Soest 2002).

At first sight, the adult body plans of sponges might appear to bear no resemblance to other animal body plans. However, Ernst Haeckel, comparing asconoid

and syconoid sponges to cnidarian polyps, proposed an evolutionary link between sponges and eumetazoa. According to his view, there is homology between the choanoderm and the endoderm and the exopinacoderm and the ectoderm, with the osculum corresponding to the mouth of the polyp (Haeckel 1870). Originally based solely on morphology and function of the germ layers and their derivatives, this view fell out of favor. However, Haeckel's proposed homology has enjoyed a resurgence following recent analyses of eumetazoan axial patterning and endoderm specification gene expression in sponges. For example, Wnt ligands are expressed within the posterior end of bilaterians, the oral end of cnidarians, and around the osculum of sponges (Leininger et al. 2014; Borisenko et al. 2016). At the same time, transcription factors associated with endomesoderm specification, such as Brachyury and GATA, are expressed in the choanoderm (Leininger et al. 2014; Nakanishi et al. 2014, reviewed by Fortunato et al. 2015). If Haeckel was correct, choanocytes could be considered a direct evolutionary link between endoderally derived eumetazoan cells and the choanoflagellate-like ancestors of animals (Adamska 2016a, b; Peña et al. 2016, Fig. 12.2n).

## 12.4 Germline and Stem Cell Systems in Sponges

In the majority of animal model systems (including the fruit fly *Drosophila melanogaster* and the roundworm *Caenorhabditis elegans*, both representatives of the Ecdysozoa, as well as all studied vertebrates), germ cell specification is one of the earliest developmental events. Strict segregation between the potentially immortal germline and the mortal soma has been long considered the hallmark of animal evolution (Weismann 1892). However, more recent studies utilizing a broader range of models—including flatworms and cnidarians—demonstrated that this strict segregation has likely evolved independently in the ecdysozoan and vertebrate lineages, with many other animals relying on a system of stem cells to produce both germ and somatic cells (reviewed by Juliano and Wessel 2010; Solana 2013).

Sponges have no gonads, and appear to have no segregated germline, instead deriving gametes from somatic cells with stem cell properties, such as archeocytes. As reviewed by Ereskovsky (2010), electron microscopy observations are consistent with choanocytes giving rise to both oogonia and spermatogonia, even in species that do possess archeocytes. In *Ephydatia fluviatilis*, spermatogonia appear to be produced by unequal division of choanocytes, resulting in the renewal of the choanocyte (remaining within the continuous choanoderm epithelium) and release of the spermatogonium into the mesohyl (Paulus and Weissenfels 1986). Spermatogonia then continue mitotic divisions, and become surrounded by “dendritic cells” of unclear origin, which form walls of sperm cysts wherein meiosis and sperm maturation occur synchronously (Paulus and Weissenfels 1986). In sponges lacking archeocytes, such as some homoscleromorpha and calcareans, both oocytes and sperm cells are derived from choanocytes (e.g., Franzen 1988; reviewed by Ereskovsky 2010). In contrast, in carnivorous demosponges lacking choanocytes,

both types of gametes are derived from archeocytes (Riesgo et al. 2007), demonstrating evolutionary plasticity of gametogenesis in sponges.

An open question is how the genome of choanocytes—which are intensively proliferating and constantly exposed to environmental insults—is protected against deleterious mutations? It is generally assumed that protection of the genome from harmful mutations, including those due to activity of transposable elements, is the key reason for early germline/soma segregation, as well as the slow cycling of the stem cells. “Genome guardian” proteins, including many RNA-binding proteins (such as piwi, vasa, p110, and nanos), have been found to be expressed in a variety of germ and stem cells across the animal kingdom (reviewed by Solana 2013). Expression of many of these genes has been recently analyzed in reproductive specimens of *Sycon ciliatum* (Leininger et al. 2014) and *Oscarella lobularis* (Fierro-Constaín et al. 2017), revealing moderate and uniform expression levels in choanocytes and elevated expression in gametes. It therefore appears that choanocytes are not only functioning as sponge germline but are also protected by the same genome-guarding mechanisms as are germ cells in the eumetazoans. In line with that, in demosponges which have both choanocytes and archeocytes, such as *Ephydatia fluviatilis*, “genome guardian” genes including *Piwi* are expressed in both cell types, although their expression in archeocytes is higher (Funayama et al. 2010; Alié et al. 2015).

Direct transformation of archeocytes to choanocytes is well documented in *Ephydatia fluviatilis*. This species produces gemmules—overwintering structures containing thousands of resting archeocytes packed within a coat built of specific spicules and collagen. Upon activation, archeocytes crawl out of the coat, differentiate into all somatic cell types, and build a juvenile sponge with a single osculum (Fig. 12.21). Dedifferentiation of choanocytes into archeocytes in *Ephydatia* has also been suggested (Funayama 2013) and has been recently demonstrated in *Amphimedon queenslandica* (Nakanishi et al. 2014 and see below). Therefore, it appears highly plausible that, as proposed by Funayama (2013), choanocytes and archeocytes together constitute the stem cell system in sponges.

## 12.5 Cell Differentiation and Transdifferentiation During Embryonic Development and Metamorphosis: Diversity and Plasticity

As in other animals, sponge embryos are produced after fertilization—either external in “free spawners” or internal in “brooding” sponges. In an overwhelming majority of cases, embryonic development ends by formation and release of a motile larva, with a small number of species being direct developers (reviewed by Maldonado 2006). Larvae of some species are simple, such as the hollow, completely ciliated cinctoblastulae of the Homoscleromorphs and calciblastulae (Fig. 12.2g) of the Calcineans. Slightly more complex are the amphiblastulae of the Calcaroneans (Fig. 12.2e). On the other end of the spectrum are the highly complex demosponge

parenchymellas, which can be equipped with sensory organs (the pigment ring of *Amphimedon*) or “ready to plug in” choanocyte chambers of *Ephydatia* (Fig. 12.2i, k).

Sponge larvae are always lecithotrophic (i.e., they are not feeding but rely on nutrients provided by the mother) and usually free-swimming in the open water, with some crawling along the substrate (Maldonado 2002, 2006). In all cases, the motility depends on movement of cilia, which can cover the entire surface of the larva (cinctoblastulae, calciblastulae, some parenchymellae) or only the anterior region (amphiblastulae). In the most complex cases, such as the parenchymella of *Amphimedon queenslandica*, the ciliated cells cover most of the larva, with a small patch of large unciliated cells at the anterior pole and a large patch on the posterior pole. The posterior area of unciliated cells is surrounded by a conspicuous ring, formed by pigmented cells and cells with long cilia. The ring acts as a simple sensory organ, as it reacts to light by changing the position of the long cilia, resulting in change of the swimming direction (Leys and Degnan 2001). It has been shown that the light sensitivity depends on cryptochrome, transcripts of which are expressed during formation of the ring (Rivera et al. 2012). Much less is known about photoreception in other sponges. For example, neither opsin nor cryptochrome has been found in the genome of calcaronean sponges (Fortunato et al. 2014b), even though the calcaronean amphiblastulae were shown to be phototactic (Elliot et al. 2004). The amphiblastulae possess four cells of unknown function (the cross cells), which have been proposed to act as sensory cells (Tuzet 1973) and which, during embryonic development, express a number of genes associated with neuronal and sensory cell specification in eumetazoans (Fortunato et al. 2014a, b, 2015, 2016).

Metamorphosis, which in at least some species is triggered by nitric oxide (NO) signaling (Ueda et al. 2016), is dramatic and results in complete reorganization of the body plan. Depending on the type of larvae, fate of larval cells upon metamorphosis is either relatively stereotypic or appears to depend solely on position of cells. The remarkable diversity of sponge development has been extensively covered elsewhere (Ereskovsky 2010), and therefore only three well-studied and diverse examples will be described here.

### **12.5.1 Formation and Metamorphosis of the Amphiblastula: Embryonically Determined Cell Fate?**

Embryonic and postembryonic development of calcaroneans, all of which have amphiblastula type larvae, has been studied in several species, with molecular studies limited to *Sycon ciliatum* (Ereskovsky 2010; Amano and Hori 1993; Franzen 1988; Leys and Eerkes-Medrano 2005; Eerkes-Medrano and Leys 2006; Fortunato et al. 2014a, b; Leininger et al. 2014). Oocytes are uniformly distributed across the mesohyl and are shaped like flattened spheres, with the short axis perpendicular to the surrounding epithelia (Fig. 12.3a). Fertilization is assisted by a complex of



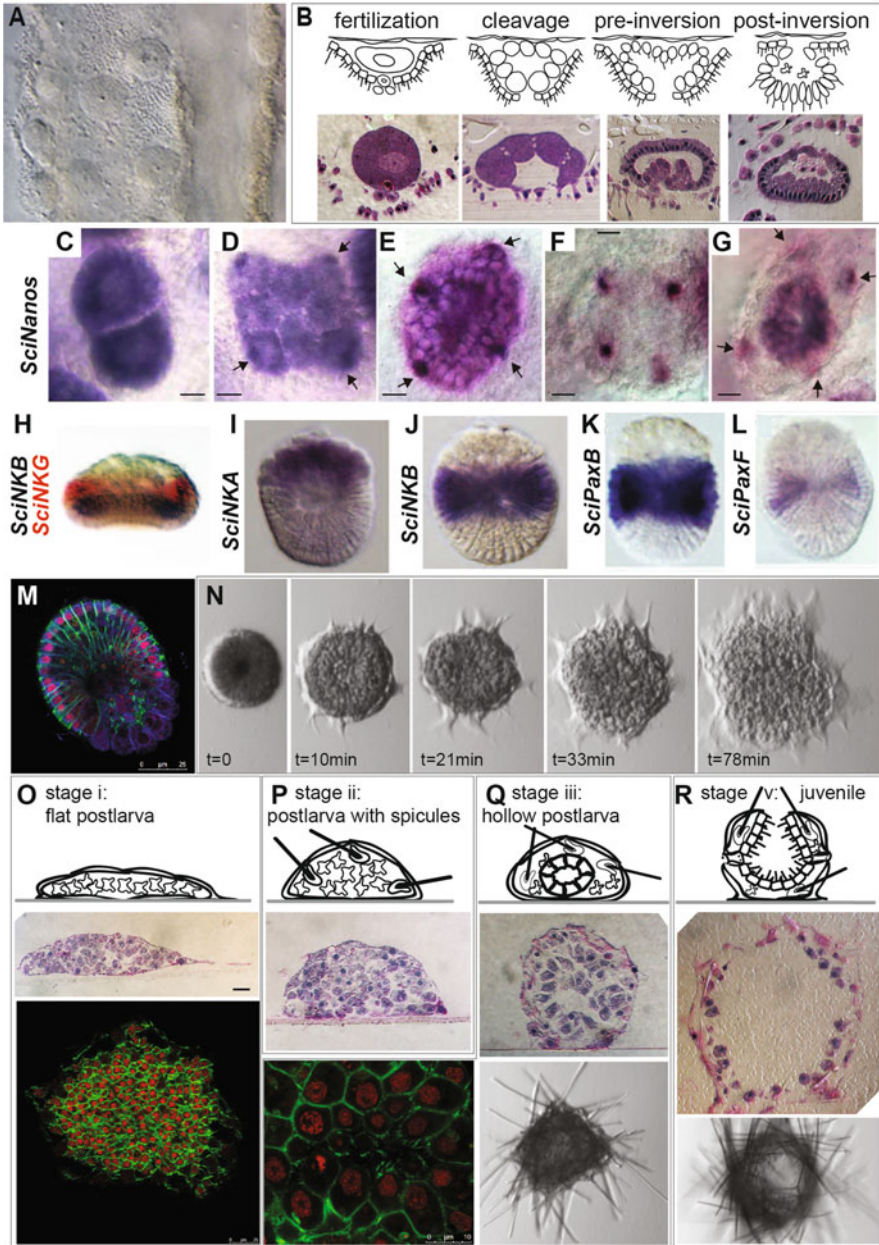
accessory cells, which are assumed to be derived from choanocytes laying directly above the center of the oocyte. The first two cell division planes are perpendicular to the long axis of the zygote and produce four identical blastomeres, which remain connected with each other via cytoplasmic bridges (Fig. 12.3b–d). The third division is oblique, producing two types of cells—four internal cells which are closer to the pinacoderm and four “corner” cells, which are closer to the choanoderm. Cytoplasm of the latter cells is not homogenous, and transcripts of several genes (e.g., *SciNanos*) were found to be enriched in a small area in each of the four corner blastomeres (Fig. 12.3d). As the cell divisions progress, the *nanos*-rich cytoplasm becomes restricted to the forming cross cells (Fig. 12.3d–g). Gene expression among the remaining blastomeres remains uniform throughout the cleavage stages.

Cell differentiation produces two additional larval cell types: ciliated micromeres, generated predominantly from the internal cells, and larger, nonciliated macromeres, generated from the “corner” cells. As no cell tracing experiments have been conducted during calcarean embryogenesis, this cell lineage is deduced from gene expression studies as well as shape and relative position of the blastomeres and larval cells. Cell movement is unlikely due to maintenance of cytoplasmic bridges throughout the cleavage stages, and the cross cells provide clear reference points. While gene expression among the macromeres has so far been shown to be always uniform, expression domains of several transcription factors reveal that the equatorial and central micromeres are not equivalent (Fig. 12.3h–l).

At the end of embryonic cell differentiation, a cup-shaped embryo—known as stomoblastula—is formed, with the “mouth” (a central opening between macromeres) communicating with choanocytes. The embryo subsequently everts through the opening to form the single-layered larva, with ciliated cells in the anterior half, nonciliated cells in the posterior half, and four cross cells on the equator. Intriguingly, a few maternal cells enter the larval cavity upon eversion.

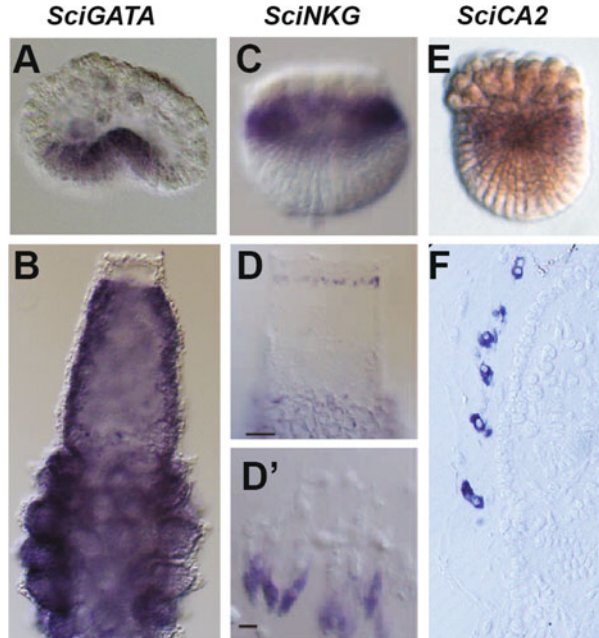
After a period of swimming, initially at the surface and then around substrates, the larvae settle on their anterior pole (as defined by their swimming direction). The macromeres maintain their epithelial character and envelop the micromeres, which undergo epithelial-to-mesenchymal transition, lose their cilia, and become ameboid and motile. As the metamorphosis progresses, the majority of the former micromeres develop collars and flagella, and align to form the choanocyte chamber (Fig. 12.3m–r). Subsequently, the osculum opens at the apical pole (corresponding to the former posterior pole of the larva), and porocytes become apparent giving rise to a functional, asconoid juvenile.

Fate of larval cells and the origin of the adult cell types in calcareous sponges are only partly understood. Observations of metamorphosing specimens, including ultrastructural studies, clearly show that exopinacocytes are derived from larval macromeres, while the cross cells and maternal cells degenerate. On the other hand, while choanocytes and mesohyl cells (such as the early differentiating sclerocytes) are both derived from larval micromeres, it is not entirely clear whether fate of the micromeres depends on their relative position within the larvae. Gene expression studies provide clues that this might be the case (Fig. 12.4). For example, *SciGATA* is expressed in central (but not equatorial) micromeres and subsequently in



**Fig. 12.3** Embryonic and postembryonic development in *Sycon ciliatum*. (a) Mature oocytes in fixed and cleared body fragment; (b) cartoon representations and histological sections of key embryonic development stages; (c–f) during cleavage, expression of *SciNanos* marks cytoplasm becoming partitioned into cross cells; (g) in pre-inversion stage, *SciNanos* expression continues in the cross cells and appears de novo in the macromeres; (h–l) patterns of transcription factors in late post-inversion stage embryo and larvae; (m) released larva, micromeres are toward the top; (n) time series of larval settlement; (o–r) cartoon representations, histological sections, and confocal or live images of metamorphosis stages. In all confocal images, actin filaments are labeled green and DNA is labeled

**Fig. 12.4** Gene expression provides clues to cell lineage in *Sycon ciliatum*. (a, b) *SciGATA* in anterior micromeres and choanocytes; (c, d) *SciNKG* in posterior micromeres and sclerocytes; (e, f) *SciCA2* (carbonic anhydrase 2) in posterior micromeres and sclerocytes. (a, c, e) Late post-inversion stage embryos and larvae; (b, d) cleared whole young adults (d' is magnification of d); (f) resin section of adult radial chamber. Reproduced with modifications from Leininger et al. (2014) (a, b), Fortunato et al. (2014a) (c, d), and Voigt et al. (2014) (e, f)



choanocytes (Leininger et al. 2014). At the same time, posterior-most micromeres and then sclerocytes express homeobox gene *SciNKG* (Fortunato et al. 2014a) and a key biomineralization enzyme *SciCA2* (Voigt et al. 2014). These results suggest that fate of the micromeres might be determined during embryonic development, but lineage tracing experiments are needed to resolve this question.

### 12.5.2 Formation and Metamorphosis of the Cinctoblastula: Position-Dependent Cell Fate?

A simple, completely ciliated cinctoblastula larva is characteristic for homoscleromorpha sponges (reviewed by Ereskovsky 2010). In all species studied, embryogenesis occurs in endopinacocyte-derived individual follicles, which are embedded in the mesohyl. Cleavage is total and equal and results in formation of a solid stereoblastula without any apparent polarity. The next step of development is unique among metazoans: the internal blastomeres migrate toward the surface in a process named “multipolar egression” (Ereskovsky and Boury-Esnault 2002),



**Fig. 12.3** (continued) red; in (m) microtubules are additionally labeled blue. Images are reproduced, with modifications, from Leininger et al. (2014) (a, b), Fortunato et al. 2014a (c–j, p–r), and Fortunato et al. (2014b) (k, l)

producing a single-layered epithelium. As in the calcaronean larvae, maternal cells can reside within the larval cavity. The surface of the epithelium increases through proliferation, resulting in folding within the follicle restricting its volume; the completely ciliated larva “stretches out” upon release from the follicle. At release, the larvae have clear polarity, with differently sized and shaped cells occupying the anterior, middle, and posterior regions (Ereskovsky 2010). Ereskovsky et al. (2007) carried out detailed, ultrastructure-based examinations of metamorphosing larvae of a range of homoscleromorpha species. As in other sponges, the larvae settle and attach to the substrate on their anterior pole. In contrast to amphiblastulae, the larval epithelium remains continuous, and only a few cells migrate into the larval cavity to become mesohyl cells. The majority of larval cells differentiate into pinacocytes and choanocytes without losing cell junctions and cilia. Intriguingly, the fate of the larval cells does not appear to depend directly on their position (and therefore morphology) in the larvae. Upon settlement, a diversity of epithelial folding patterns (apical, middle, or basal invagination) can be observed among progeny of the same parent even if metamorphosing in the same conditions. The fate of larval cells is linked to the position in which they end in the juvenile, rather than their original position. For example, choanocytes can be derived from anterior or posterior pole cells, depending on whether the invagination was basal or apical.

On the ultrastructural level, the corresponding cells of larvae which will undergo different modes of metamorphosis are indistinguishable, although it is possible that they already express different sets of genes affecting their behavior during metamorphosis. Another possibility is that the folding of the metamorphosing larval epithelium is a stochastic process, and the cells differentiate according to the relative position in which they find themselves in the postlarvae. Given the plasticity of cell differentiation observed in other processes (see below), this explanation appears more likely. Gene expression studies throughout development and metamorphosis of cinctoblastulae should resolve this matter.

### ***12.5.3 Formation and Metamorphosis of the Parenchymella: Lability of Cell Fate?***

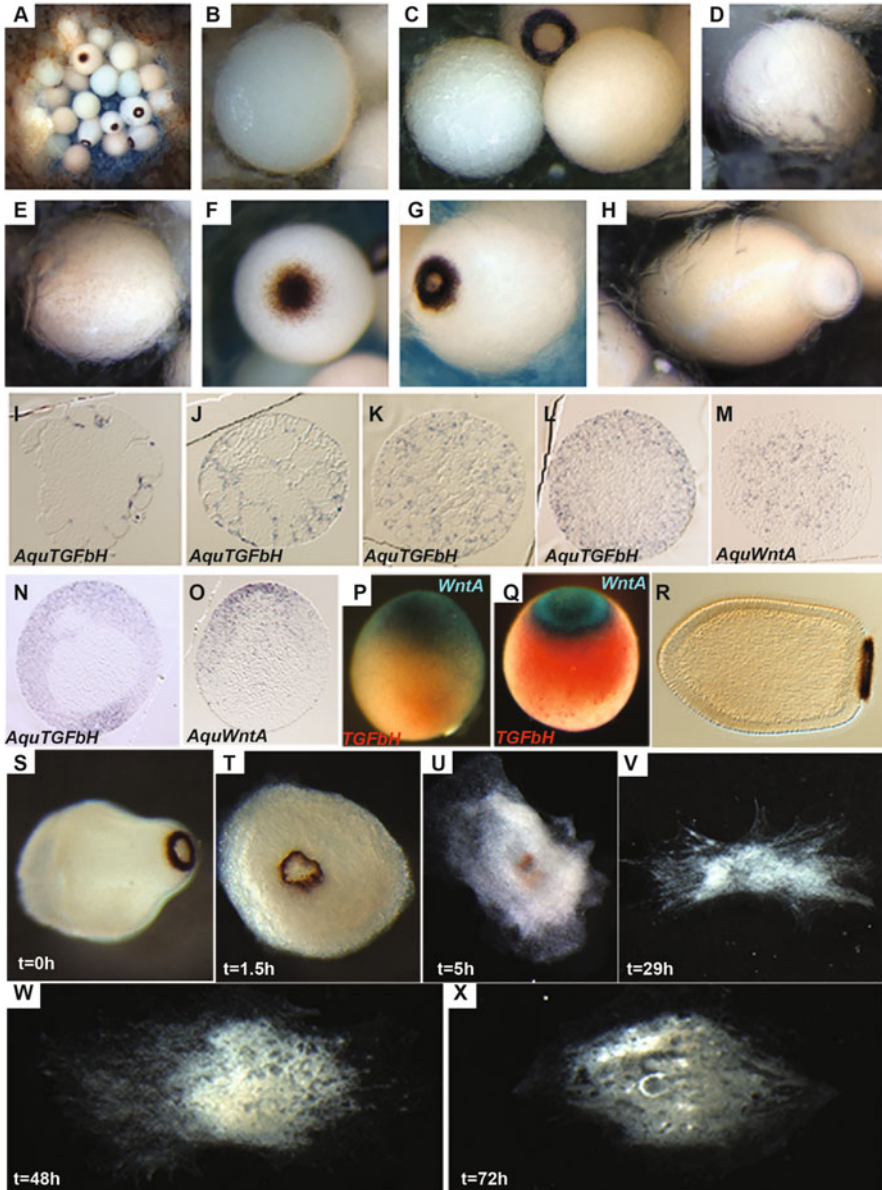
While a spectacular array of larval types exists among demosponges, development of *Amphimedon queenslandica*, the first sponge to have its genome sequenced, has been studied most extensively (see Degnan et al. (2015) for the most recent review). In contrast to amphiblastulae and cinctoblastulae, which are tiny (100  $\mu\text{m}$ ), single-layered and composed of just 3–5 cell types, the parenchymella larvae of *Amphimedon* are relatively large (over 500  $\mu\text{m}$ ) and complex. The oocytes and embryos are found in brood chambers, each containing tens of embryos of different ages, with each embryo enveloped in a follicle layer (Fig. 12.5a–h). The initial divisions are chaotic, unequal, and often incomplete, generating differently sized cells without any apparent polarity. Transcripts of many developmental regulatory

genes, including signaling molecule  $TGF\beta H$ , are detectable in tiny micromeres which cleave off large, yolky macromeres (Fig. 12.5i–k). Cell tracing experiments suggest that no significant cell movement happens as the cleavage progresses. Instead, the resulting stereoblastulae have no apparent polarity, with cells expressing  $TGF\beta H$  and another regulatory gene,  $WntA$ , distributed in a salt-and-pepper manner. Significant cell movement is observed in the next step of development—sometimes referred to as gastrulation, as it results in formation of an asymmetric, bi-layered embryo (Adamska et al. 2010; Degnan et al. 2015). By the end of this stage, while live embryos appear superficially uniform, the smaller cells (many of which  $TGF\beta H$ -positive) form an outside layer, and the larger cells constitute the inner cell mass. At the same time,  $WntA$ -positive cells become concentrated in one region of the embryo which will become the posterior pole of the larva (Fig. 12.5n–p). Pigment cells, which by this stage have differentiated among the outer layer of the embryo, migrate toward the  $WntA$  domain to form a spot and then sensory ring of the larva (Fig. 12.5q). Mature larvae have three distinguishable layers: the inner cell mass (containing archeocytes, sclerocytes, and a diversity of other cell types), the subepithelial layer (composed of large spherulous cells and some smaller cells), and the surface epithelium (composed of ciliated cells responsible for larval motility as well as a smaller number of flask cells and globular cells, which are likely to be sensory cells of the larvae) (Fig. 12.5r, reviewed by Degnan et al. 2015).

Fate of the larval cells has been studied extensively in *Amphimedon*. By fluorescent labeling of the outer larval epithelium, Leys and Degnan (2002) found that the ciliated larval cells differentiate predominantly into choanocytes (as well as amoeboid cells). Nakanishi et al. (2014) have confirmed that majority of the larval epithelial cells become choanocytes but have also demonstrated that some of these cells give rise to exopinacocytes (although it is not clear whether the exopinacocytes might be derived from a specific population of cells residing in the epithelium, such as flask or globular cells). By labeling of the proliferative archeocyte population in the larvae, the authors have also shown that many choanocytes and exopinacocytes are derived from larval archeocytes. Therefore, there appears to be no strict correspondence between larval and adult cell types in this species.

## 12.6 Cell Differentiation and Transdifferentiation During Growth and Asexual Reproduction

Fascinatingly, the lability of cell differentiation appears to be a feature that lasts throughout the entire life of sponges, which seem to be in continuous process of tissue rearrangements, aptly called “chronic morphogenesis” (Gaino and Burlando 1990). Sponge cells continue to move extensively in adults (Bond 1992), and the adult morphology is often environmentally plastic (Palumbi 1984; Kaandorp 1999; Mendola et al. 2008; Manconi and Pronzato 1991; reviewed by Bosch et al. 2014). In addition to “global” changes of morphology, sponges have been shown to



**Fig. 12.5** Embryonic and postembryonic development in *Amphimedon queenslandica*. (a) brood chamber; (b–g) embryonic development stages from cleavage to pigment ring; (h) larva hatching from the follicle membrane (anterior pole view); (i–m) gene expression in non-polarized embryos, from early cleavage to blastulae; (n–p) gene expression in polarized, bi-layered embryos; (q) gene expression is spot stage embryo; (r) larva; (s–x) time series of metamorphosis. (a–h, s–x) are images of live specimens, (r) is a fixed and cleared specimen, (i–o) are resin sections of embryos stained for expression of *TgfbH* or *WntA*, (p, q) are whole embryos stained for expression of both genes. (a–h) are reproduced from Adamska et al., 2010, (i–o) are reproduced from Adamska et al. (2007), (q) is from Adamska et al. (2011)

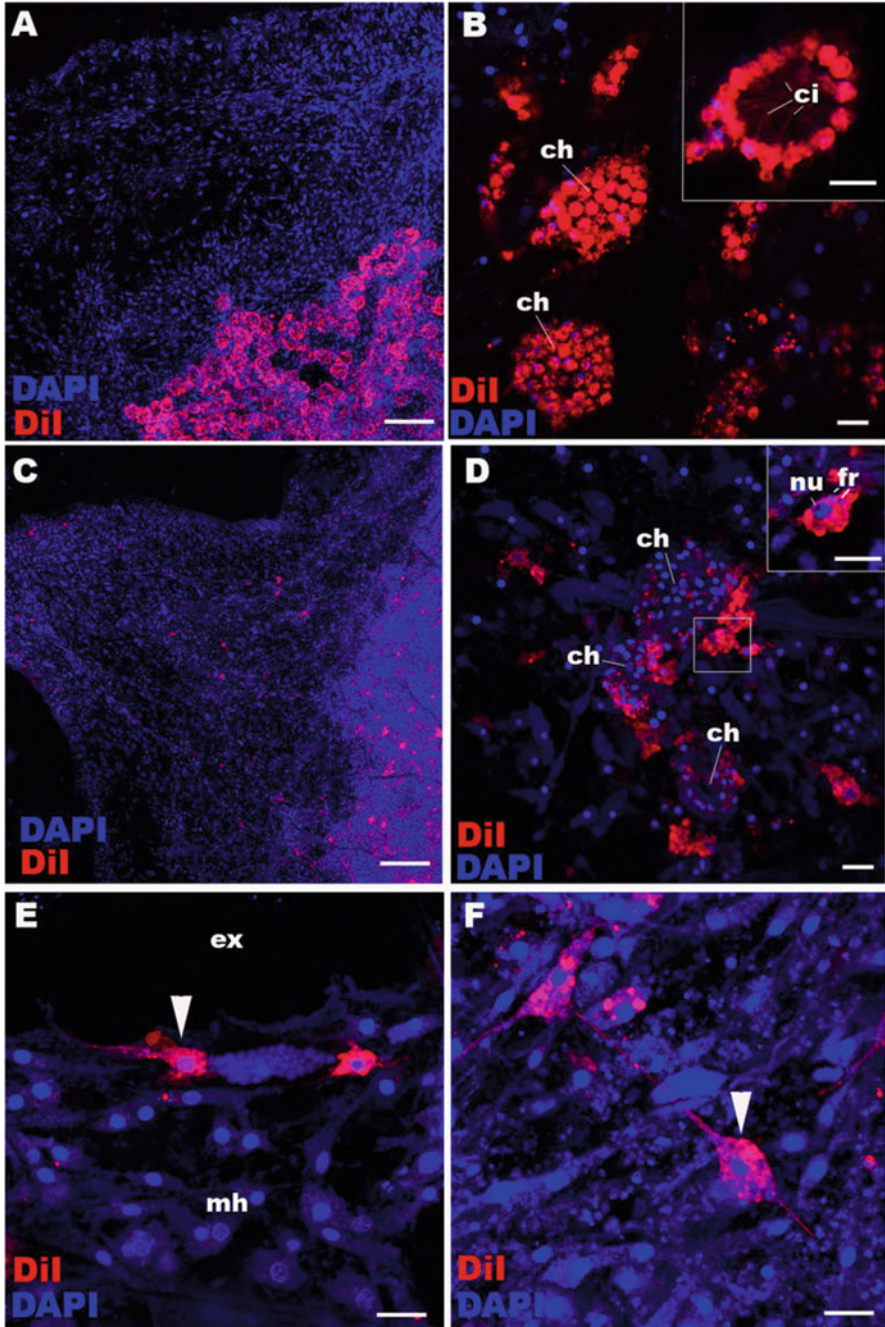
directionally rearrange their canal system in response to change of the water flow, either by differential growth or by “replumbing” of the existing canals to change their polarity (McDonald et al. 2003; Mendola et al. 2007).

As only choanocytes and archeocytes (where present) have been shown to proliferate in adult sponges, other cell types, including pinacocytes and sclerocytes, must therefore be derived from choanocytes and/or archeocytes. The differentiation routes and its mechanisms remain mostly unclear: do cells transdifferentiate directly, or is there a dedifferentiation step between two adult cell types (e.g., choanocyte and pinacocyte)? Recently, Nakanishi et al. (2014) demonstrated that choanocytes of *Amphimedon* juveniles can transdifferentiate into both exopinacocytes and sclerocytes (Fig. 12.6). The authors assume that archeocytes are an intermediate in these processes. This is consistent with studies of *Ephydatia* gemmule-derived juveniles, in which archeocytes have been shown to be the direct source of sclerocytes, as evidenced by expression of silicatein (the key enzyme used in siliceous spicule formation) in a fraction of archeocytes (Funayama et al. 2005; Mohri et al. 2008). In contrast to archeocytes differentiating into choanocytes, which maintain expression of Piwi in addition to expression of the choanocyte markers (e.g., annexin), archeocytes committed to the sclerocyte fate lose Piwi expression (reviewed by Funayama 2010). As fate of sclerocytes after they have completed synthesis of the spicule remains unknown, it is possible that these cells are terminally differentiated.

Even less is known about the origin of cells other than choanocytes in calcareous sponges. Woodland (1905) suggested that sclerocytes (and also porocytes) are derived from pinacocytes; this view has been shared by Simpson (1984) based on ultrastructural similarity between pinacocytes and sclerocytes. Only choanocytes (and gametes) express “genome guardian” genes in calcisponges, in line with the possibility that choanocyte to pinacocyte transition is a “one way” process, as is likely the case in demosponges. But this might not be universally true. Gaino et al. (1987) have shown that during growth of *Oscarella lobularis*, exopinacoderm invagination results in formation of small pockets, cells of which transdifferentiate first into endopinacocytes and then choanocytes to expand the aquiferous system. Therefore, in homoscleromorphs, pinacocytes are capable of transdifferentiation into choanocytes, suggesting that no cell differentiation is terminal in these sponges.

## 12.7 Cell Differentiation and Transdifferentiation During Regeneration

The ease with which sponge cells transdifferentiate is likely the driving force behind sponges’ amazing capacity for regeneration (reviewed by Wulff 2010), although cellular mechanisms involved in this process vary significantly among the studied species. In the demosponge *Halisarca dujardini*, surgical removal of a fragment of



**Fig. 12.6** Transdifferentiation during normal development of *Amphimedon queenslandica* juvenile. (a, b) Fluorescently labeled choanocytes of a juvenile sponge; (c–f) 7 days after labeling, red fluorescence shows cells derived from choanocytes: archeocytes (d, inset), exopinacocyte (e, arrowhead), and sclerocyte (f, arrowhead). *ch* choanocyte chambers, *ex* exopinacocyte, *fr* fragments of phagocytosed nuclei, *mh* mesohyl, *nu* nucleus. Reproduced from Nakanishi et al. (2014)

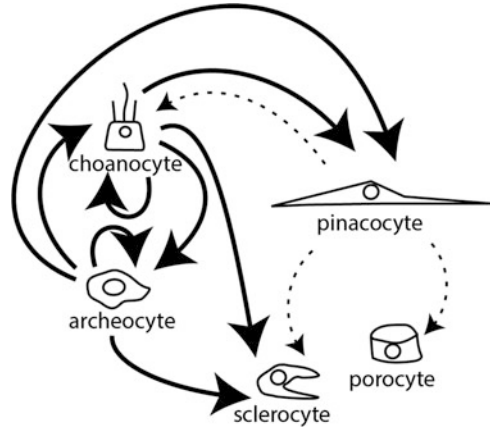


exopinacoderm results in disorganization of the underlying choanocyte chambers, followed by migration of dedifferentiated choanocytes and archeocytes toward the wound. Former choanocytes and archeocytes subsequently redifferentiate into exopinacocytes and choanocytes to rebuild the epithelia of the aquiferous system (Borisenko et al. 2015). In contrast, direct transdifferentiation of cells in intact epithelia has been demonstrated in the homoscleromorpha *Oscarella lobularis* (Ereskovsky et al. 2015) and the calcareous sponge *Leucosolenia complicata* (Ereskovsky et al. 2017). In both cases, an exposed sheet of choanocytes directly transdifferentiates into pinacocytes, without undergoing epithelial-to-mesenchymal transition, dedifferentiation, or proliferation.

Strikingly, sponges can not only easily regenerate lost body fragments but can reform after complete cell dissociation (an ability found also in some cnidarians; see Holstein et al. (2003) for review). Already in 1907, Wilson mechanically dissociated cells of *Microciona prolifera* by squeezing tissue pieces through fabric. Placed in seawater, the cells migrated (either by amoeboid movement or using flagella in case of choanocytes) and formed aggregates, from which within several days, complete and functional small sponges were rebuilt. Several researchers, including Huxley (1911) and Galtsoff (1925), replicated Wilson's work using variety of sponge species. It appeared that the fate of aggregates differs among the species, with some capable of producing complete functional sponges and some "stuck" along the way [recently revisited by Eerkes-Medrano et al. (2015) and Lavrov and Kosevich (2016)]. Aggregates unable to produce sponges either perish within days or form long-living (but not feeding), well-organized structures called "primmorphs" (Custodio et al. 1998; Sipkema et al. 2003). The ability to fully regenerate from dissociated cells appears to be individual species-dependent rather than have deeper phylogenetic association: *Sycon raphanus*, but not *S. coactum*, have been shown to be able to rebuild their bodies completely after dissociation (Huxley 1911; Eerkes-Medrano et al. 2015).

As is common in sponge cell biology, these observations raise more questions than answers. Do dissociated cells maintain their identity, and does the new body form by reorganization? Do they dedifferentiate and redifferentiate—in which case, is their fate somehow restricted by predissociation identity? Are some cells more capable than others to produce complete new bodies? From the limited data available so far, it appears that answers to these questions are model system-dependent. In all cases where aggregates persist (whether to produce primmorphs or functional sponges), pinacocytes are found to be completely covering the surface within days of aggregation. Huxley (1921), studying *Sycon*, interpreted this outcome as depending upon cell migration, with pinacocytes crawling to the surface to cover the choanocytes remaining inside. But in *Lubomirskia baicalensis*, cell migration combined with transdifferentiation of choanocytes and archeocytes into pinacocytes is responsible for formation of the continuous pinacoderm layer on the surface of primmorphs (Ereskovsky et al. 2016). In line with their accepted stem cell roles, pure archeocyte fractions of *Ephydatia* have been shown to be able to generate all adult types to rebuild the complete sponge (Buscema et al. 1980).

**Fig. 12.7** Summary of cell differentiation and transdifferentiation routes observed in juvenile and adult sponges. Dashed lines indicate transitions which have been proposed or were reported only once in the literature



## 12.8 Do Sponges Have Differentiated Cells and What Can We Learn from Them?

The lability of sponge cell differentiation (Figs. 12.6, 12.7), and in particular the striking ability of choanocytes to transdifferentiate into pinacocytes, has been seen as a strong reason to reject Haeckel’s hypothesis that sponge choanoderm is homologous to the endoderm while pinacoderm to the ectoderm (Nakanishi et al. 2014). According to this view, in light of the apparent lack of terminal differentiation, sponge cell types could not be homologous to eumetazoan cell types. To go one step further, one could argue that the different morphologies and function of sponge cells should be considered states rather than types. Perhaps differentiation of sponge cells is temporal rather than spatial, as proposed for the ancestor of all animals (Mikhailov et al. 2009). Assuming that the lability of sponge cell fate reflects the ancestral condition in animals, we could still use sponges to uncover molecular mechanisms (epigenetic chromatin changes, gene regulatory networks) from which “true” cell differentiation evolved in animals. Under this evolutionary model, we would expect to find a dramatic, qualitative difference in how sponge and eumetazoan cells differentiate.

But is the lability of cell differentiation in sponges really so different to cell differentiation in other animals? One of the most enduring and still useful metaphors of cell differentiation is Waddington’s “epigenetic landscape,” in which the progressive (and often irreversible) restriction of cell fate choices accompanying differentiation is pictured as a marble rolling down a complex landscape (Waddington 1957; recently reviewed by Fusco et al. 2014). The lability of cell differentiation in sponges—which appear to be able to “jump” easily from one valley to the next, often without re-tracing their steps to dedifferentiate first—is certainly more extreme than expected for “normal” differentiation observed during animal development.

However, research in the last few decades demonstrated that transdifferentiation is more widespread than imagined in Waddington’s times. Transdifferentiation has

been observed and analyzed during normal development, regeneration as well as disease states in various animals, and has been given significant attention in recent years (reviewed by Thowfeequ et al. 2007; Ladewig et al. 2013; Tata and Rajagopal 2016; Merrell and Stanger 2016). Perhaps the most striking example is transdifferentiation of a hindgut epithelial cell into a neuron during normal development in *Caenorhabditis elegans* (Jarriault et al. 2008; Zuryñ et al. 2014). Among mammals, development of the adrenal glands involves a perhaps less dramatic but well-documented conversion of zona Glomerulosa to zona Fasciculata cell identity (Freedman et al. 2013). Finally, during metamorphosis of many indirectly developing marine invertebrates, the differentiated cells of larvae dedifferentiate and subsequently redifferentiate into adult cell types (reviewed by Arenas-Mena 2010).

Transdifferentiation during regeneration is even more common (reviewed by Schmid et al. 1982; Sugimoto et al. 2011). Cnidarian cells have been shown to transdifferentiate in response to injury, allowing regeneration of complete organs from striated muscle (Alder and Schmid 1987). One of the most dramatic examples involving massive transdifferentiation can be observed during medusa-to-polyp transformation (“life cycle reversal”) in *Turritopsis nutricula* (Piraino et al. 1996). In vertebrates, regeneration of a complete lens from pigmented retina cells observed in newts (Wolff 1895) is now known as Wolffian regeneration. During this process, which depends on upregulation of expression of transcription factor Six3 (which is involved in eye development), retina cells dedifferentiate and subsequently redifferentiate into lens cells (Grogg et al. 2005). Transdifferentiation is also observed during regeneration of liver, where epithelial cells can give rise to hepatocytes (Choi et al. 2014; He et al. 2014).

Induced transdifferentiation (reprogramming) has recently emerged as an attractive potential therapeutic tool, especially for regenerative medicine (reviewed by Slack 2007; Graf and Enver 2009; Jopling et al. 2011; Sancho-Martinez et al. 2012; Eguizabal et al. 2013; de Lázaro and Kostarelos 2016). It appears that expression of a number of transcription factors can drive direct lineage conversion, even crossing germ layer boundaries. For example, mesodermally derived fibroblasts can give rise to hepatocytes (which are normally of endodermal origin) by induced expression of Hnf4 $\alpha$ , Foxa1, Foxa2, and Foxa3 or to neurons (which are normally of ectodermal origin) by induced expression of Ascl1, Brn2, and Myt1 (reviewed by Sancho-Martinez et al. 2012).

These findings have two key implications for sponge cell biology studies. One, they allow to see the ease with which sponge cells transdifferentiate not (only) as evidence of the “primitive” (i.e., pre-eumetazoan) character but as a marvelous adaptation to the risk of injury, allowing unparalleled efficiency of regeneration. Two, they suggest that sponges might be excellent models for studies of transdifferentiation with potential far-reaching medical benefits. Recent explosion of single-cell genomic methodologies is now allowing large-scale analyses of differentiation and transdifferentiation processes even in non-model systems such as sponges, and multiple laboratories are indeed taking advantage of these technologies. And even if no medical benefits can be gained from these studies in the near future, we can be sure to gain deeper insights into the fascinating life of sponge cells.

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# Chapter 13

## Holothurians as a Model System to Study Regeneration



José E. García-Arrarás, María I. Lázaro-Peña, and Carlos A. Díaz-Balzac

**Abstract** Echinoderms possess an incredible regenerative capacity. Within this phylum, holothurians, better known as sea cucumbers, can regenerate most of their internal and external organs. While regeneration has been studied in several species, the most recent and extensive studies have been done in the species *Holothuria glaberrima*, the focus of most of our discussion. This chapter presents the model system and integrates the work that has been done to determine the major steps that take place, during regeneration of the intestinal and nervous system, from wound healing to the reestablishment of original function. We describe the cellular and molecular events associated with the regeneration processes and also describe the techniques that have been used, discuss the results, and explain the gaps in our knowledge that remain. We expect that the information provided here paves the road for new and young investigators to continue the study of the amazing potential of regeneration in members of the Echinodermata and how these studies will shed some light into the mechanisms that are common to many regenerative processes.

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## 13.1 Introduction

### 13.1.1 *Echinodermata*

The phylum Echinodermata comprises animals with some fascinating characteristics. For one they are invertebrate members of the Deuterostomata clade, sharing similar embryological development with members of the Chordata. Even though their adult pentaradial symmetry might be perceived as a primitive trait, their embryological development identifies them as members of the vertebrate evolutionary branch. Moreover, modern genetic analyses have confirmed their close phylogenetic position to vertebrates (Cannon et al. 2014; Holland 2015; Perseke et al. 2013; Satoh et al. 2014). Only one other phylum, the Hemichordata, shares with them the characteristics of being non-chordate deuterostomes. In fact, their phylogenetic position can be considered as ideal for the comparative analyses necessary to understand the evolution of vertebrates. There are five classes within the phylum Echinodermata: Crinoidea (sea lilies), Asteroidea (sea stars), Ophiuroidea (brittle stars), Echinoidea (sea urchins), and Holothuroidea (sea cucumbers). Echinoderms are marine animals and can be found in all marine habitats from the coastal areas to the benthic depths and from the tropical to the polar regions.

Echinoderms are unique in many other ways. They possess a calcium endoskeleton that is very developed in some groups such as in sea urchins while less so in holothurians. They have a water vascular system, whose major function is thought to be nutrient transport and gas exchange. They display a mutable collagenous tissue (MCT) that has “muscle-like” properties, allowing for the reversible changes of tensile strength under the control of the nervous system; for review see Wilkie (2002). Finally, the focus of this chapter is that echinoderms feature an incredible regenerative capacity.

### 13.1.2 *Regeneration in Echinoderms*

All echinoderms have the capacity to regenerate lost body parts. In some groups, the regenerative capacity is far more developed than in others. For example, sea urchins, where regeneration capacity is less developed, can still regenerate their damaged spines and tube feet and can repair holes in their test. Sea stars and brittle stars are well known for being able to regenerate their arms and in some species for being able to regenerate a complete organism from a severed arm. However, it is in holothurians where the regenerating capacity is most developed. The species *Leptosynapta crassipatina* (Holothuroidea, Apodida) serves as a fitting example to highlight the regenerative capacity of holothurians. Organisms of this species can regenerate a whole organism from a disk from the oral complex (Smith 1971b). Many holothurian species can regenerate two organisms from one that has been cut in half. This process can occur in nature (fission) and has been studied in the

laboratory by scientists interested in the regenerative response. In fact, many holothurians use fission as their means of asexual reproduction. This process has been thoroughly documented in the holothurian *Cladolabes schmeltzii* (Holothuroidea, Dendrochirotida) using electron microscopy (Kamenev and Dolmatov 2015, 2017; Kamenev et al. 2013). When animals are ready to reproduce, they divide into two regions of about the same size, and each region regenerates the missing part. Therefore, the anterior part can regenerate the posterior part and vice versa.

The holothurian *Thyone briareus* (Holothuroidea, Dendrochirotida) can autotomize into two segments, both of which can regenerate a complete animal (Pearse 1909). However, in other species only one end of the animal has been shown to survive and regenerate a complete animal. This is the case for *Leptosynapta crassipatina* (Smith 1971a, b). A systematic analysis of the minimal region required to regenerate the organism was done, shedding some light into the likely progenitor cells required to undergo this process. In the case of *Leptosynapta*, if cut in half, only the anterior part will regenerate and form a whole organism (the missing posterior region), while the posterior part is not able to regenerate.

A more complete review of the autotomy process and the regeneration capacities of different holothurian orders can be found in a previous review (Garcia-Ararras and Greenberg 2001). In summary, these experiments highlight that the regenerating region must contain the necessary cells and gene activation network information necessary to rebuild the cells and tissues of the entire lost component. However, the results of many of these experiments, performed years ago, must be analyzed with the prism of our accumulated knowledge on stem cells and dedifferentiation to fully understand the regenerative mechanisms of echinoderms.

## 13.2 Digestive Tract Regeneration

Most studies on digestive tract regeneration, particularly those in vertebrate, refer strictly to the regeneration of the mucosa or luminal layer. There are important reasons for this. First, the luminal epithelial layer is known to be constantly formed and regenerated. Second, problems in the renewal of this layer cause a series of diseases, such as ulcers or cancers. However, whole organ regeneration, such as regeneration of the intestine or the stomach, has not been widely studied, probably for an obvious reason: not many organisms can achieve this task. Thus, it is only a selected group of animals where this event has been documented: planaria, tunicates, the echinoderms, and in some amphibians. Here we focus on the regeneration of the complete digestive tract in holothurians. For this we provide the available information on the cellular and molecular processes that bring about this process.

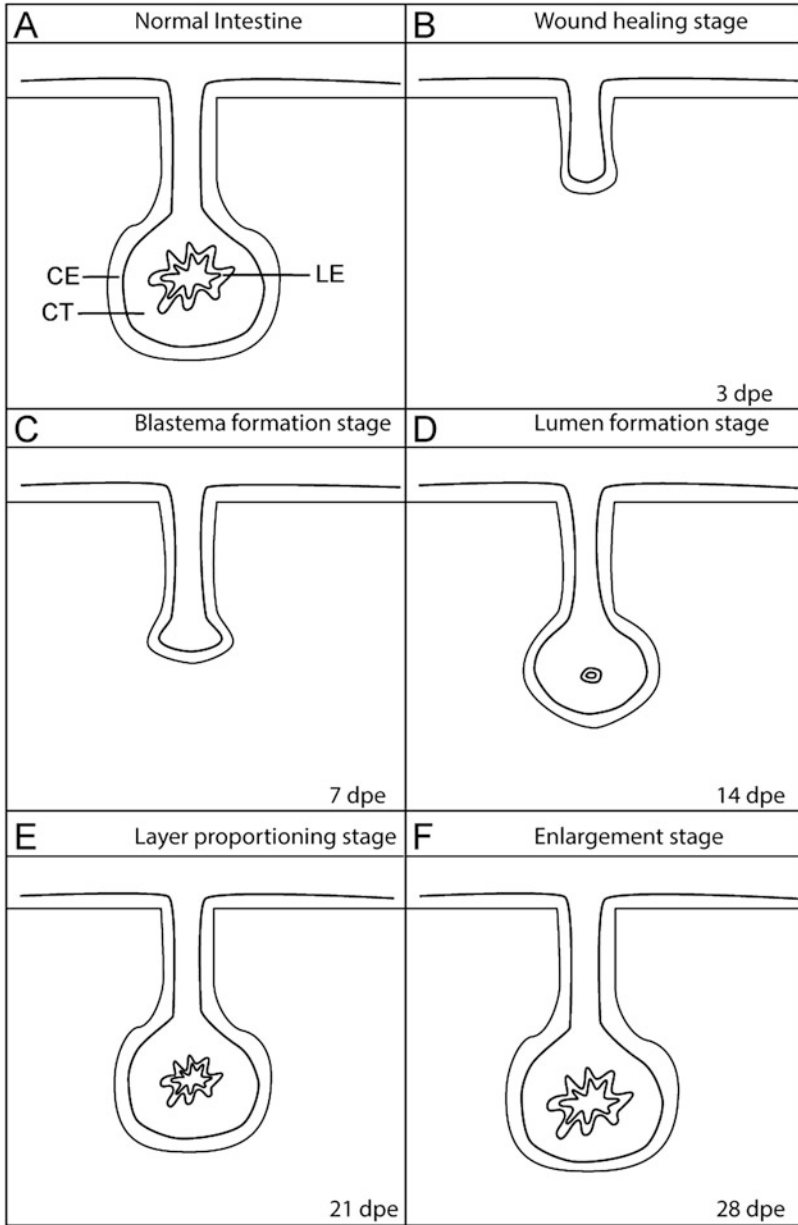
The regeneration of the digestive tract, following evisceration, has been studied in some species. The original studies were performed early last century by Fausta Bertolini at the Stazione Zoologica in Naples, Italy. In her work, using various

holothurian species, she described the formation of the new intestine following evisceration (Bertolini 1930, 1932). Even with the technical limitations of the time, she was able to illustrate some of the cellular events that underlie the formation of the new intestine at the free end of the remaining mesentery. Later histological studies, using different species, confirmed the mesentery as the main tissue involved in intestinal regeneration and extended the description of the regenerative processes (Bai 1971; Dawbin 1949; Kille 1935; Leibson 1992; Mosher 1956; Smith 1971a; Tracey 1972). In the 1990s our laboratory revisited the use of holothurians, focusing on the species *Holothuria glaberrima* (Holothuroidea, Aspidochirotida), to study intestinal regeneration, and began applying modern technologies to answer some of the remaining questions. Since then we have developed and extended this model system to probe into the cellular and molecular basis of regenerative processes. Our laboratory has published various reviews that provide additional details on sea cucumber evisceration and on various aspects of the regenerative processes that might be of interest to investigators (Garcia-Ararras and Greenberg 2001; Mashanov and Garcia-Ararras 2011; Mashanov et al. 2014a).

### 13.2.1 *Holothurian Digestive Tract*

Prior to describing the intestinal regeneration model, it is necessary to describe the anatomy/histology of *H. glaberrima* digestive tract. At the macroscopic level, the digestive tract consists of a short esophagus adjacent to the circumoral ring that opens to the mouth at the anterior region. At the posterior end of the esophagus begins a long curving intestine. This can be subdivided into three segments: a descending intestine, an ascending intestine, and a second descending intestine. The first two sections show a smaller diameter and have been labeled as descending and ascending small intestines, while the last segment is larger in diameter and has been described as the descending large intestine. The main difference between the descending and the ascending small intestine lies in the latter association with two other visceral organs: the hemal system and the right respiratory tree. The large intestine continues into the cloaca at the posterior end of the animal. The digestive tract is attached to the body wall by mesenteries, in a similar manner to the vertebrate digestive tract. The anterior part of the tract, the esophagus and descending intestine are attached by a dorsal mesentery, the ascending small intestine by a lateral mesentery, while the descending large intestine is attached by a ventral mesentery. In reality, the three mesenteries are all a continuous structure that changes the localization of its attachment to the body wall as one moves along the digestive tract.

At the histological level, the digestive tract is made up of three main tissue layers: the mesothelium, a connective tissue layer, and the luminal epithelium (Fig. 13.1). The mesothelium itself can be divided into two tissue types; the most external layer is made up of monociliated coelomic cells named peritoneocytes that are attached to each other via specialized cell junctions and extend processes that anchor them in the underlying basal lamina. The most internal layer of the mesothelium is a muscle



**Fig. 13.1** Holothurian digestive system and stages of the regenerating digestive system. (a) Diagram of a cross section through the echinoderm posterior intestine demonstrating its basic organization. Cross section through the stages of regeneration of the posterior intestine at (b) 3 dpe, (c) 7 dpe, (d) 14 dpe, (e) 21 dpe, and (f) 28 dpe. *DPE* days post-evisceration, *CE* coelomic epithelium, *CT* connective tissue, *LE* luminal epithelium

layer that can have both longitudinal and circular components and also lie above the basal lamina. This basal lamina separates the mesothelium from the underlying connective tissue layer. The connective tissue layer corresponds to the submucosa of vertebrates and as the name implies is characterized by having a large amount of connective tissue where few, scattered cells can be found. The luminal epithelium corresponds to the mucosa of vertebrates. This is a pseudostratified epithelium that is mainly formed by columnar cells or enterocytes whose apical ends face the luminal cavity. However, other cell types can be found among the enterocytes, including goblet-like cells and peptide-containing cells similar to vertebrate enteroendocrine cells (García-Arriarás et al. 1998; García-Arriarás et al. 2001).

The mesothelial layer is continuous with the mesothelial layer of the mesentery as is the connective tissue layer. Thus, the mesentery is formed by two mesothelial layers separated by a small amount of connective tissue. The mesentery mesothelium is itself continuous with the mesothelium that forms the coelomic epithelia of the peritoneum lining the coelomic cavity.

Neuronal cells have been described in all intestinal layers (Diaz-Balzac et al. 2007, 2010b, 2012, 2014, 2016; García-Arriarás et al. 2001). Cell bodies in the mesothelium appear to be primarily associated with the innervation of the mesothelial muscle. These cells are not associated into the ganglion-like structures found in the myenteric plexus of vertebrates but are isolated or grouped in small numbers and dispersed along the intestinal length. Neuron-like cells have also been identified within the connective tissue of the intestine and have been associated with the modulation of intestinal motility. Neuron-like cells have also been described within the luminal layer, and some of these appear to extend long processes into the connective tissue layer, suggesting that they might represent sensory cells in the luminal epithelia that convey information to the animal. Neuron-like cells have also been detected within the mesothelium of the mesentery. These are mainly found among large number of parallel fiber tracts that extend from the body wall to the intestine (Tossas et al. 2014). Although glial or support cells might be found in the enteric nervous system, their presence has not been determined due to a lack of appropriate markers that specifically identify this putative cell population.

The digestive tract is very similar in composition throughout all of its regions. However, the size of the layers varies within each region. For example, the esophagus has a very developed muscle layer that is much reduced in the rest of the digestive tract. In contrast, the connective tissue layer in the large intestine is the widest when compared to the rest of the digestive tract.

### ***13.2.2 Evisceration***

Many holothurian species activate an evisceration process when stressed or in danger (Hyman 1955; Emson and Wilkie 1980). This process involves the forceful ejection of their viscera, either anteriorly by the loss of the anterior part of the animal including tentacles, nerve ring, and Aristotle's lantern or the posterior evisceration of

the intestinal system through the cloaca. The organs that can be eviscerated include the esophagus, intestines, hemal system, respiratory trees, nerve ring, gonads, and others associated with the aquapharyngeal complex. However, the eviscerated organs depend on the species and its particular evisceration mode. Most of the organs eviscerated are those associated with the digestive tract. In holothurians, the digestive tract is principally made up of an extended intestinal system; thus this system comprises the main component of the eviscerated contents. The digestive system is also the first organ to be regenerated. Visceral evisceration is often mistaken with the ejection of Cuvierian tubules. These tubular structures are found in the posterior end, attached to the base of the respiratory trees in the cloaca and are filled with an adhesive substance. They are ejected through the cloaca when the organism feels threatened and form a sticky network that can deter the aggressor or predator. Cuvierian tubules are then regenerated, and this regenerative process has been documented by various investigators (VandenSpiegel et al. 2000).

While the ejection of the Cuvierian tubules is strictly a defense mechanism, the purpose of the evisceration reaction is less clear. Some investigators suggest it is a defense mechanism against predators; however, evisceration often can occur when animals are exposed to stressful situations such as high temperatures or low oxygen concentrations. Whatever evisceration is intended to achieve, the process, together with the ensuing regeneration, has been exploited by researchers to study organ regeneration.

Our laboratory has developed the use of the sea cucumber *Holothuria glaberrima*, a local species found in the rocky intertidal zone throughout the Caribbean, as a model system to study intestinal regeneration. *H. glaberrima* can be easily induced to eviscerate by intracoelomic injections of 0.35 ml KCl. They eviscerate posteriorly, via the cloaca, and eject their intestinal system (small and large), the hemal system, the right respiratory tree, and, when in season, most of the gonads. The rupture sites occur between the descending small intestine and the esophagus and between the large intestine and the cloaca. Autotomy of the intestine-mesentery connections occurs close to the intestine; thus, the ejected intestine contains a very small portion of the mesentery, while most of the mesentery remains attached to the body wall at one end and free within the coelomic cavity at the other end.

Intestine regeneration in this species provides an excellent biological system for studying regeneration. Advantages of this model include the following:

- (i) Evisceration is easily induced by KCl injections and follows a fixed pattern that diminishes the individual variability due to surgical amputations required by other regeneration models.
- (ii) The pattern of regeneration can be divided into several stages and involves the organization of specific cells and tissues into a functional organ.
- (iii) Regeneration occurs along the torn edge of the mesentery, physically isolated from other tissues of the organism.
- (iv) A complex organ in an adult organism is formed in less than 3 weeks.



- (v) The size of the organism is small enough for maintenance in indoor aquaria but large enough to obtain the quantities of tissue needed for the cellular and molecular experiments.
- (vi) The organism has a soft body wall that facilitates dissection.
- (vii) It is extremely abundant in local waters.

### 13.2.3 *Regeneration of the Digestive Tract*

#### 13.2.3.1 **Wound Healing**

Following evisceration there are three regions of the digestive tract that would need some type of immediate repair. Two of these are the area of the esophagus where the small intestine was attached and the area of the cloaca where the large intestine was attached. These regions are points of contact with the external environment and need to be sealed off rapidly to prevent the influx of bacteria or other pathogens into the coelomic cavity. The third region is the rupture margin of the mesentery where the intestine, both small and large, was attached. The wound healing response following evisceration has not been studied in detail in *H. glaberrima*. However, information from other echinoderm species provides a glimpse of what could be occurring.

#### 13.2.3.2 **Cellular Mechanisms**

We have shown that, following autotomy of the digestive tube, regeneration begins with the formation of a thickening at the free end of the remaining mesentery. This thickening grows in size until it forms a solid rod of tissue resembling a blastema that extends from the esophagus on the oral side of the animal to the cloaca on the posterior end. Intestinal regeneration involves cellular dedifferentiation (Candelaria et al. 2006), cell division (Garcia-Arraras et al. 1998, 2011), apoptosis (Mashanov et al. 2010b), cell migration (Cabrera-Serrano and Garcia-Arraras 2004; Garcia-Arraras et al. 1998), and ECM remodeling (Quinones et al. 2002) among other events associated with regenerative processes.

*Dedifferentiation* The first morphological indication of the regeneration process is the dedifferentiation of the muscle cells in the mesentery adjacent to the injury/autotomy site (Candelaria et al. 2006; Garcia-Arraras et al. 2011). In this process, muscle cells become fragmented, and the contractile apparatus is degraded and ejected from the cell in membrane-bound structures that have been named spindle-like structures (SLSs) because of their morphology (Garcia-Arraras and Dolmatov 2010). This dedifferentiation occurs in a temporal and spatial sequence. From its initiation at the tip of the mesentery at about days 1–2, it peaks at days 5–7 following evisceration. More interestingly, the dedifferentiation process occurs in a gradient, beginning at the tip of the mesentery and moving toward the body wall. Thus, at about 3 days post-evisceration (dpe), the mesenterial area near the injury site has

many SLSs, while the rest of the mesentery has the usual muscle fiber component. A few days later, the mesenterial area adjacent to the now larger rudiment is devoid of muscle cells and SLSs, the middle of the mesentery has few muscle fibers but an abundant amount of SLSs, while the area near the body wall has the normal-looking muscle fibers and few if any SLSs. We have hypothesized that these dedifferentiated muscle cells provide the precursors for the formation of the new intestine.

*Cell death and cell proliferation* Two other processes are associated with the initial formation of the rudiment: cell death (apoptosis) and cell proliferation. An increase in apoptosis has been documented using the TUNEL method, from 3 dpe (the earlier stage measured). Cell division has been documented using BrdU. Although some cell division is observed 24 h after evisceration, it is not until a few days later (5–7 dpe) when the peak in cell division is observed, most of it taking place in the mesothelium of the growing rudiment.

Dedifferentiation, apoptosis, and proliferation occur within some spatial differences. While dedifferentiation takes place within the mesenterial tissue and, as explained above, spreads in a gradient farther and farther from the regenerating structure, both apoptosis and proliferation mainly occur within the regenerating structure or in the mesenterial region close to it. A more discrete spatial difference has also been documented between apoptosis and cell proliferation at 7 dpe, where areas of the regenerating rudiment with high levels of apoptosis tend to be the same areas where less cell proliferation occurs and vice versa (Mashanov et al. 2010b).

Our working hypothesis of these initial events associated with the early formation of the intestinal rudiment is the following: evisceration induces the dedifferentiation of the muscle cells of the mesentery close to the injury site. This triggers a feed forward process where dedifferentiating cells induce other cells to dedifferentiate, thus producing the gradient in dedifferentiation that begins at the mesentery tip and extends toward the opposite end of the mesentery. Dedifferentiated cells then migrate toward the growing intestinal rudiment where they enter a proliferative state (some proliferation takes place during the migration, though it is within the mesothelium of the rudiment where it peaks). Apoptosis, activated by the injury, and possibly by the remodeling process, serves to provide the rudiment with a particular morphology and, as has been documented in other species/regenerative processes, might be serving as an inducer of cell proliferation.

*Epithelial to mesenchymal transition (EMT)* As the regenerating rudiment grows in size, a process of epithelial to mesenchymal transition is observed (Garcia-Arraras et al. 2011). The basal lamina of the intestinal rudiment appears discontinuous and is not visible at certain sites. At the same time, cells of the rudiment mesothelium dissociate and ingress into the rudiment connective tissue, forgoing their epithelial phenotype for a new roundish mesenchymal-type cell. The initial EMT takes place at the tip of the mesentery, that is, at the opposite end to the rudiment attachment to the mesentery; however, as time passes and the rudiment continues to grow, some cells appear to ingress in many areas of the rudiment and are not circumscribed to the tip. The final destination or the role of these incoming cells is unclear, although some

have been observed to migrate and apparently settle at the connective tissue side of the luminal epithelium basal lamina.

*Extracellular matrix remodeling* Concomitant with the increase in growth of the intestinal rudiment, there are also drastic changes in the composition of the extracellular matrix. This remodeling primarily involves the activation of matrix metalloproteases and the loss of the collagen fibrils, in a process that closely follows the dedifferentiation gradient (Quinones et al. 2002). Thus, collagen fibers are first lost within the connective tissue of the rudiment, and as time goes on, fibers are lost from the connective tissue of the mesentery that is adjacent to the intestinal rudiment. Similar to the wave of dedifferentiation, the remodeling of the ECM continues within the mesentery with loss of collagen in the central region, and in some cases the loss can extend to areas close to the body wall. Other components, such as fibronectin and glycosaminoglycan while losing some of their organization, remain within the mesentery and growing rudiment throughout the regeneration process (Quinones et al. 2002; Vazquez-Velez et al. 2016). Functional studies where ECM remodeling was slowed down by metalloproteinase inhibitors or where cell-ECM interactions were disrupted by ERGD peptides caused a negative effect in the process of inhibition (Cabrera-Serrano and Garcia-Arraras 2004; Quinones et al. 2002). These experiments highlight the important role played by the ECM in the regeneration of the intestine.

*Cell migration* Two cell migration events have been proposed to occur during intestinal regeneration. First, dedifferentiated mesothelial cells forming the coelomic epithelium are thought to migrate as an epithelial sheet toward the forming intestinal rudiment. These cells eventually form part of the regeneration epithelium of the rudiment, undergo cell division, and eventually differentiate into the mesothelium of the new intestine. The evidence for this migration is admittedly rather weak, and the phenomenon needs to be better studied with live action imaging and migration markers. The second migration event is the migration of cells along the mesentery connective tissue. Cells undergoing this migration are either coming from the body wall connective tissue or might be coelomocytes from the coelomic cavity. The event has been shown to take place using immunocytochemical markers that identify a spherulocyte population (Garcia-Arraras et al. 2006) and has been shown to be disrupted by ERGD peptides that interfere with the binding of cells to ECM molecules (Cabrera-Serrano and Garcia-Arraras 2004). This event, however, occurs relatively late in regeneration and is probably associated with the formation of the lumen either with the restoration of the ECM or with the activation of the immune system to protect the tissue from microorganism found in the newly forming luminal cavity.

*Lumen formation* By the end of the first week of regeneration, the free margin of the mesentery has become a solid rod of tissue that extends along the anterior-posterior axis of the organism. At the anterior end, the rudiment connects with the (uneviscerated) remnants of the esophagus, and at the posterior end, it connects

with the cloaca. This sets the stage for the formation of the luminal layer, which takes place during the second week of regeneration.

The formation of the luminal epithelial layer brings about the formation of the lumen. This process takes place as luminal cells from the cloaca and esophagus proliferate and invade the connective tissue of the regenerating intestine rudiment (Fig. 13.1). It is possible that, as has been shown in other species, the dividing luminal cells are partly dedifferentiated and lose their connection to the basal lamina, although keeping their connection with their neighbors (Odintsova et al. 2005; Shukalyuk and Dolmatov 2001). In experiments using BrdU, we have quantified the level of cell proliferation at the invading tip and shown it to be almost twice the proliferation of the luminal epithelial cells where the lumen has already formed. The cell migration in the connective tissue has two immediate consequences: first they open up the cavity that will become the intestinal lumen and second, they form the luminal cell layer. The incoming cells from the esophagus move posteriorly, while those from the cloaca move anteriorly so that in no more than a week, the two tips of incoming cells meet medially and form the complete lumen of the regenerated intestine. Thus, by 14 dpe the lumen has formed, and the mucosal epithelium has regenerated, establishing the basic layout of the intestine.

*Cellular differentiation* The early regenerating rudiment is composed of two tissue layers that are contiguous with the mesentery layers where they originated: a connective tissue layer that has increased in size as regeneration proceeds and an epithelial layer that, although contiguous with the mesentery mesothelium, differs dramatically from it. During the early stages of regeneration, the cells in the epithelial layer of the regenerating rudiment are rounded or spherical cells that show a high proliferative index. These cells are at least partially dedifferentiated, having lost muscle and peritoneocyte markers. But they retain other markers, such as immunoreactivity to the antibody MESO1, an undetermined epitope that identifies most, if not all, cells of the mesothelium (Garcia-Arraras et al. 2011). Nonetheless, soon after the formation of the rudiment, some of these epithelial cells begin the process of redifferentiation. The process of myogenesis has been studied in some detail (Murray and Garcia-Arraras 2004). In this process, the coelomic epithelial cells in the regenerating rudiment move toward the basal lamina and begin expressing muscle markers. This is a step-by-step process that is accompanied by morphological changes, where cells elongate, express the proteins of the contractile apparatus, and eventually form the muscle fibers. Thus, cell movement toward the basal lamina begins at about 4 dpe, when these cells begin to express some muscle cell markers, but organized contractile apparatus filaments, as determined by phalloidin binding, do not appear until a few days later, once cells have acquired the elongated, muscle-like morphology (Murray and Garcia-Arraras 2004). BrdU labeling has shown that newly formed muscle cells originate from dividing precursors and that most of the muscle cells are born during the second week of regeneration.

A similar process is assumed to occur in the formation of neurons of the enteric plexus (Tossas et al. 2014). However, this differentiation has not been studied in

detail, in part due to the smaller number of neurons (when compared with muscle cells) in the organ and to the difficulty in identifying them. The appearance of neurons in the mesothelium enteric nervous system occurs later than the appearance of muscle cells; neuronal phenotypes begin appearing at about 10 dpe and continue for the next 2 weeks.

A fast transition between cell proliferation and beginning of differentiation has also been documented in the luminal epithelium. Here cells with a high proliferating index are found at the tip of the migrating cells that originated from the esophagus. These cells do not show any of the available differentiated cell markers. Nonetheless, cells in the luminal epithelium that have formed a few days before from the rapidly proliferating/migrating cells already show markers that identify them as neuroendocrine cells (unpublished observation).

*Tissue layer proportioning* Once the lumen has formed, the basic layout of the intestine is completed. The three tissue layers, mesothelium, connective tissue, and luminal epithelium, are formed, and a hollow tube extending from the esophagus to the cloaca is ready for the passage of the food/nutrients. None of the studies have address the recovery of intestinal function; nonetheless, once the continuous lumen is formed, mud, algae, and other particles have been detected within the lumen of regenerating animals, implying that the animals have ingested these particles and are in the process of digestion.

This three-layer structure continues to be modified even after the lumen has formed. We have previously named this stage the stage of tissue layer proportioning (García-Arriarán et al. 1998). For example, although all tissue layers are present, their contribution to the complete organ does not clearly represent what is found in the normal intestine. Additionally, the initial intestine that forms resembles a small intestine throughout its length, and it takes some additional time for the width of the connective tissue in the posterior part of the organ to acquire the proportions of a large intestine. Similarly, changes are observed within the muscle component of the mesothelium (Murray and García-Arriarán 2004). As cells differentiate forming the muscle precursors and eventually the muscle fibers, a single layer of the mainly circular-oriented fibers is formed. This muscle layer continues growing in width, but a clear organization of its fibers is not discernible. It is not until about 4 weeks into regeneration that the two muscle layers that constitute the normal intestine (circular and longitudinal) can be identified.

*Intestinal enlargement* Roughly 1 month following evisceration, the intestinal system of the holothurian has formed what can be considered a smaller copy of the original organ. Arguably, not all tissue components are identical to those of the normal pre-eviscerated intestine, but all identifiable cell types and structures are present in a closely similar organization and proportional size. Once this stage is acquired, the next step is a general growth in the size of the structure in order for it to acquire the expected size. This process has not been studied in the laboratory. We have hypothesized that in the normal environment, the later processes of regeneration might occur at a faster pace than in the aquaria due to access to nutrients and

other environmental inputs that might be lacking in the lab setting; however, this has not been documented.

### 13.2.3.3 Species Differences

Intestinal regeneration following evisceration is a common process exhibited by holothurians. Some species, such as *Apostichopus japonicus* (Holothuroidea, Synallactida), follow a process of evisceration and regeneration very similar to that observed in *H. glaberrima* (Leibson 1992; Shukalyuk and Dolmatov 2001). However, the evisceration route as well as the regeneration process can show some differences depending on the species. For example, both *Sclerodactyla briareus* (previously known as *Thyone briareus*) and *Eupentacta fraudatrix* eviscerate anteriorly ejecting all their viscera together with their anterior nerve ring, introvert, and tentacles. While regeneration of the posterior intestinal segment in *E. fraudatrix* follows a pathway similar to that described for *H. glaberrima*, the regeneration of the anterior segment is somewhat different. The lumen epithelia in this segment are formed by transdifferentiation of the coelomic epithelial cells (Mashanov et al. 2005). This itself is an interesting process where the luminal epithelium has two different origins: an endodermal origin in the posterior intestine and a mesodermal origin in the anterior intestinal segment.

### 13.2.3.4 Molecular Basis of Intestinal Regeneration

As the cellular processes that bring about intestinal regeneration are elucidated, research focus has been directed at characterizing the underlying molecular events. The primary focus of this research has been to identify the genes that are differentially expressed between regenerating and normal (non-regenerating) intestines. The techniques that have been used in this approach have dramatically changed over the last two decades, as better and more sensitive methods are made available. Early studies were initially done using a selected candidate approach looking at particular genes that, because of their involvement in embryological development or in other wound healing or regenerative processes, seemed good candidates to be involved in intestinal regeneration. Example of this approach was the characterization of holothurian *Hox* genes (Mendez et al. 2000).

Another early approach was the use of differential display to identify transcripts that were preferentially expressed in regenerating tissues (Roig-López et al. 2000). Using this technique several holothurian genes were identified that could be playing various roles in the intestinal regeneration process. These genes include actin isoforms (Roig-López et al. 2000) and serum amyloid A (Santiago et al. 2000).

As methodologies improved, the next advance was the preparation of cDNA libraries of intestines at various stages of regeneration, the cloning and random sequencing of hundreds of genes (Rojas-Cartagena et al. 2007), and the preparation of “in-house” microarrays (Ortiz-Pineda et al. 2009). The random sequencing of

clones produced by itself new candidate genes that could be probed for their role in the regenerative process. Thus, ependymin (Suarez-Castillo et al. 2004; Suarez-Castillo and Garcia-Arraras 2007), ubiquitin-proteasome system genes (Pasten et al. 2012a, b), Centaurin (Rojas-Cartagena et al. 2007), and melanotransferrin (Hernandez-Pasos et al. 2017; Rojas-Cartagena et al. 2007) were among the genes characterized and whose expression was further studied using PCR to show their differential levels of expression at particular regeneration stages. However, it was the gene expression profiling using custom-made microchips that produced a surprising result, showing that hundreds of genes (which accounted to over 70% of the studied genes) were differentially expressed during regenerative stages (Ortiz-Pineda et al. 2009). Many of the differentially expressed genes showed no similarities to genes in the databanks (at the time of publication), while others were related to known genes involved in regeneration-related processes, wound healing, cell proliferation, differentiation, morphological changes, cell survival, stress response, immune activation, and neoplastic transformations. The differentially expressed genes included genes associated with developmental processes such as growth factors (TGF- $\beta$ , BMP, WNT, myotrophin) and transcription factors (homeobox, Krueppel-like, forkhead), with the cytoskeleton (actin, tubulin myosin, gelsolin) and with extracellular matrix (matrix metalloproteases, laminin, echinonectin, collagen, tenascin). Among the differentially expressed genes that were validated were Wnt9, homeobox genes, metalloproteinases, actins, tenascin, as well as several unknowns.

More recently new technologies of transcriptome analyses and RNAseq have been applied to our system and to other species of regenerating holothurians increasing exponentially the number of genes that are associated with regenerative responses. In *A. japonicus*, next-generation sequencing of regenerating body wall and intestine identified almost 7000 genes, and hundreds of these were found to be differentially expressed (Sun et al. 2011). Further studies by the same group using RNAseq reported over 2400 genes to be upregulated and over 1000 genes to be downregulated when compared to the normal intestine (Sun et al. 2013).

Nonetheless, in future studies, the large number of differentially expressed genes could be tailored down to those that might really play a significant role in regeneration. At present, most of the comparisons made to identify differentially expressed genes were made by comparing the regenerating intestine to the normal intestine. However, these two organs are comprised of different tissues and/or of disproportionate contributions of tissue layers to the whole organ. For example, the normal intestine has a large amount of luminal epithelium, while the early rudiment of the regenerating intestine lacks completely this tissue layer. Thus, a gene preferentially expressed in luminal epithelial cells will then appear as underexpressed in a RNAseq comparison. To minimize this problem, we are now comparing early regenerating intestine to the normal mesentery, since most of the tissue component of the early regenerating intestine is the mesentery that remains from the eviscerated intestine. By doing so, we have (1) identified genes that appeared to be differentially expressed between the normal and regenerating intestine but are not differentially expressed between the normal mesentery and the regenerating intestine, suggesting that these are genes that are preferentially expressed in the mesentery, (2) reduced the number

of differentially expressed genes, and (3) obtained a listing of genes that appear to be highly expressed from very early in the mesentery that will give rise to the intestine (Garcia-Arraras et al. unpublished results).

Two recent developments will also serve to advance future studies in intestinal regeneration using holothurians: first, the establishment of in vitro culture systems for isolated cells and organ explants of holothurians (Bello et al. 2015), and second, the increasing availability of echinoderm genomes and gene sequences (Long et al. 2016). In fact, the first holothurian genome (of *A. japonicus*) has recently been published (Zhang et al. 2017). In this study, investigators identified a specific tandem-duplicated prostatic secretory protein of 94 amino acids (PSP94)-like gene family and proposed that the genes in this family are somewhat involved in the remarkable regenerative capacities of the holothurians.

### 13.2.3.5 Gene Expression Patterns

Several gene products have been localized in the regenerating intestine: proteins using immunohistochemistry and mRNA by in situ hybridization. The expression of these gene products provides important information on the cellular events and the cellular changes that are underpinning the regenerative process. Among the gene products that have been identified are survivin, mortalin, and translationally controlled tumor protein (TCTP) (Mashanov et al. 2010b, 2012). These genes, associated with processes of cell proliferation and cell death, are expressed abundantly in the mesothelium of the growing intestinal rudiment at stages of regeneration that correlate with the peak of cell proliferation and apoptosis. In situ hybridization signals of the genes which encode soluble signaling proteins (WNT9 and BMP1/TLL) are also observed in the mesothelium of the intestinal rudiment and in the mesothelium of the mesentery adjacent to the growing rudiment during the early stages of regeneration. Although each gene has a particular temporal and spatial expression pattern, they all are highly expressed in the rudiment mesothelium at 7 dpe (not necessarily in the same cell types).

We have also identified the expression of the holothurian orthologues of the four so-called Yamanaka factors, *Myc*, *SoxB1*, *Klf1/2/4*, and *Oct1/2/11*. These are transcription factors that can induce pluripotency when expressed in mammalian somatic cells. In the early regenerating mesentery (2 dpe), only *Myc* is extensively expressed. *Klf1/2/4* and *Oct1/2/11* are only expressed in scattered cells, while no *SoxB1* expression is observed. At later times (7 dpe) the expressions of *Klf1/2/4* and *Oct1/2/11* increase in the mesothelium, but *SoxB1* expression remains low or undetectable.

These studies provide evidence that the gene expression profile of the mesothelial cells within the growing intestinal rudiment differs significantly from the mesothelium of the normal mesentery. This correlates with what is observed in terms of tissue organization, where the mesothelium in the normal mesentery is composed of externally localized coelomic epithelium with an underlying muscle layer and with scattered neuronal cells, all organized above a basal lamina, while during



regeneration this mesothelium becomes a simple epithelium of specialized cells without any muscle layer. This fits with our working hypothesis that as the mesentery cells dedifferentiate and migrate into the growing rudiment, they also undergo partial reprogramming, expressing genes that are associated with embryological and oncological processes. Thus, the mesothelial cells in the rudiment can be considered a regenerative epithelium with very distinct characteristics.

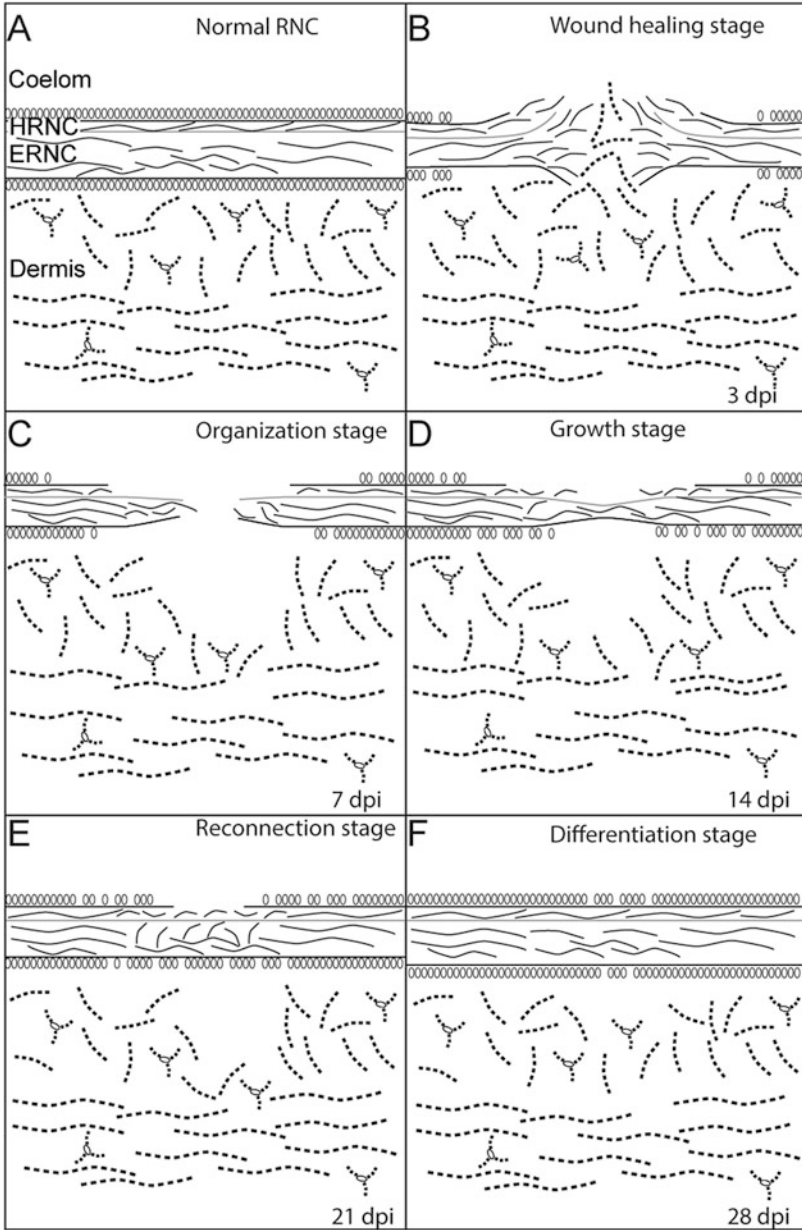
### 13.2.3.6 Functional Studies

We have used pharmacological tools to determine the functional role of gene products. In this respect, the focus has been to expose animals to available modulators (both inhibitors and/or activators) known to modify the activity of a protein or signaling pathway. These drugs are usually injected intracoelomically, during the regeneration period, and the outcome, in terms of rudiment growth or other parameters, is determined. Thus, the effect of modulators on cell division, cell death, cell dedifferentiation, and ECM remodeling can be determined. Inhibitors of proteinase activity, both MMPs (Quinones et al. 2002) and the proteasome degradation pathway (Pasten et al. 2012b), inhibit the regenerative process, causing a delay in the formation of the intestinal rudiment, determined by a smaller rudiment being formed. More recent experiments have shown a role of WNT in the early stages of regeneration, where injections of EGCG, a WNT pathway inhibitor, cause a reduction in the size of the regenerating rudiment, while LiCl, a WNT pathway activator, causes an increase in the rudiment size (Bello et al. unpublised results).

## 13.3 RNC Regeneration

### 13.3.1 *Holothurian Nervous System*

Echinoderm nervous systems follow the pentamerous body organization of the animal's body plan. In holothurians, the nervous system is composed of an anterior circumoral nerve ring from which five radial nerve cords (RNCs) extend posteriorly. The ring and radial nerve cords comprise the central nervous system (CNS) (Fig. 13.2). Each RNC is physically divided by a thin connective tissue layer into two components, the ectoneural radial nerve cord (ERNC) and the hyponeural radial nerve cord (HRNC). This connective tissue boundary contains several short neural bridges that allow the passage of neuroepithelia and fibers from one component to the other (Mashanov et al. 2006). This physical subdivision also correlates with a functional difference for each component. The ectoneural component of the RNC innervates the body wall, podia, and viscera. On the other hand, the hyponeural component of the RNC innervates the longitudinal (LM) and circular muscles (CM). This gives the HRNC a majorly motor function, while the ERNC has both sensory and motor functions. Peripheral nerves that arise from both the hyponeural and the



**Fig. 13.2** Holothurian nervous system and stages of the regenerating RNC. (a) Diagram of a longitudinal section through the echinoderm radial nerve cord and body wall demonstrating its basic organization. Cross section through the stages of regeneration of the radial nerve cord and body wall at (b) 3 dpe, (c) 7 dpe, (d) 14 dpe, (e) 21 dpe, and (f) 28 dpe. *DPI* days post-injury, *ERNC* ectoneurial radial nerve cord, *HRNC* hyponeurial radial nerve cord, *RNC* radial nerve cord

ectoneural components connect the CNS to other organs and structures. These peripheral nerves, plus the nervous component associated with different organs, comprise the peripheral nervous system (PNS) (for review see Mashanov et al. 2016). Among the PNS components that have been best studied are the podial nerves (Diaz-Balzac et al. 2010a), the enteric nervous system (Diaz-Balzac et al. 2007, 2014, 2016; Diaz-Miranda et al. 1995; 1996; Garcia-Arriaras et al. 1991a, b, 2001; Garcia-Arriaras and Viruet 1993), and the mutable connective tissue plexus (Diaz-Balzac et al. 2007, 2012, 2016).

The RNCs are ganglionated nerves, meaning that they have both neuronal bodies and fibers within them. They present a similar organization throughout the CNS, where the neuronal somata are found in the outer borders of the RNC, while the interior of the structure is a neuropil made by the fibers originating from the neurons. Radial glia, with elongations that extend from basal to apical ends, provide mechanical support. The glial bodies are interspersed among the neuronal soma. The nerve ring is itself a sort of ganglionated nerve, although only representing the ectoneural component. The neurons appear to be homogeneously distributed along the CNS components; there are neither ganglia nor large accumulations of neuronal bodies, although the nerve ring appears to have a slightly larger proportion of neurons than the RNCs (Mashanov et al. 2006, 2009, 2010a). The neurons express a wide range of neurotransmitters, from classical neurotransmitters, such as acetylcholine, catecholamines, and GABA (Diaz-Balzac et al. 2014, 2016), to neuropeptides such as GFSKLFYaide, a neuropeptide within the SALMFamide neuropeptide family (Diaz-Miranda et al. 1992; Elphick et al. 1991; Diaz-Miranda and Garcia-Arriaras 1995). Within the neuropil, evidence for the presence of neural tracts, analogous to those in the vertebrate NS, has recently been provided (Diaz-Balzac et al. 2016).

### 13.3.2 Radial Nerve Cord Transection

The methodology used to transect the RNC of two sea cucumber species differs as a reflection of their regenerative potential. Specimens of *E. fraudatrix* can be transected by cutting the RNC with scissors from the external part of the body (Mashanov et al. 2008). The position of the RNC can be determined by the rows of podial feet that lie external to the RNC. Once lesioned, the animals contract the body wall, closing the injury site and proceed to heal the injured area.

Specimens of *H. glaberrima*, in contrast, are not able to heal a wound that traverses the animal body wall and exposes the coelomic space to the outside environment. Therefore, in this species, a more complex method needs to be used to transect the RNC. Animals are eviscerated and then anesthetized. Once the animals become flaccid, the body wall is pushed from the outside through the cloaca, using a glass rod. This partial eversion of the body wall exposes the coelomic side of the body wall with its clearly defined pairs of longitudinal muscles. The RNCs lie within the body wall between the longitudinal muscle pairs and can be sectioned using a razor blade taking the precaution not to cut open the body wall to the outside

of the animal. Once the transection is done, the body wall is brought back through the cloaca, and the animals are left to regenerate. It is important, particularly for experiments that might last weeks, to label the body wall next to the transection with India ink or some other permanent stain, since it might be difficult to localize the transected area once the regenerative process has advanced.

### 13.3.3 Regeneration of the Nervous System

Holothurians are known to be able to regenerate most of their body parts and thus can regenerate the neural components within those body parts. Such regenerative prowess is exemplified by the sea cucumber *Sclerodactyla briareus*, previously known as *Thyone briareus*. These organisms can eviscerate their anterior portion or introvert which includes, among many other organs, their nerve ring (Garcia-Arraras and Greenberg 2001; Kille 1935). A new nerve ring is regenerated and reconnected to the RNCs in a few weeks. The original experiments describing regeneration of the anterior end of this species were done in the early part of last century. Thus, they lack the details of cellular and molecular processes that can be gained with the use of modern techniques.

More recent studies have focused on the regeneration of transected RNCs. It has been documented that the reconnection of the transected RNCs occurs without scarring a fact that contrast with the extensive scarring and lack of reconnection that takes place in mammals. This capacity has been documented in at least two species, *H. glaberrima* (San Miguel-Ruiz and Garcia-Arraras 2007; San Miguel-Ruiz et al. 2009) and in *Eupentacta fraudatrix* (Mashanov et al. 2008). This process is characterized by its timing and precision, since it only takes about a month to complete, and the final process is a RNC that is practically indistinguishable from its uninjured counterpart. The initial studies done in *E. fraudatrix* (Mashanov et al. 2008) and *H. glaberrima* (San Miguel-Ruiz et al. 2009) are complimentary to each other. The former provides a detailed anatomical description of the process, and the cellular components involved, at the electron microscope. The latter provides information on cellular events that take place during the regeneration process and in particular on neuronal cells. The *H. glaberrima* model has been further explored in terms of the presence and role of the glial component and most importantly of the molecular changes that underlie the regenerative response.

#### 13.3.3.1 Neurodegeneration

In both studied species, following transection, the cut edges of the RNC swell out and protrude into the coelomic cavity. However, even in the face of this discontinuity in the RNC, both ectoneural and hyponeural components retain their organization. Nonetheless, signs of neurodegeneration are observed: less densely packed nerve processes, swollen or distorted nerve fibers, secondary lysosomes, and

apoptotic cells. The latter are particularly abundant in the stump edges (Mashanov et al. 2013). In addition, spherule-containing cells, which are rarely observed within the RNC of normal animals, infiltrate the wounded area (San Miguel-Ruiz et al. 2009).

### 13.3.3.2 Initial Growth

The initial steps in regeneration are characterized by two interconnected events, the accumulation of ECM in the wound edges and the formation of an elongation from the ectoneural portion of the RNC. The exposed RNC is slowly covered by the new ECM and by the coelomic epithelium, although it is still separated by the injury gap. It is not clear which cell types are responsible for the deposition of the new ECM or what is the component of the new connective tissue. Nonetheless, transcriptomic analyses have shown that genes responsible for ECM molecules comprise some of the most upregulated genes during the early process of RNC regeneration (Mashanov et al. 2014b).

The origin and formation of the elongations that extend from the ectoneural region of the RNC of both stumps have been better studied. These are the result of an active process of cell dedifferentiation that originates at the RNC stump and eventually extends more distally from the injury site. Most of the dedifferentiating cells are the radial glia, although additional dedifferentiation of other cell types (i.e., neurons) has not been excluded. The dedifferentiation process has been described in detail using electron microscopy and immunohistochemical markers. In brief, the cells remain within the adluminal region of both ectoneural and hyponeural component and remain attached to each other by intercellular junctions. The glial cytoskeleton undergoes radical changes, where the intermediate filaments are fragmented and either broken down internally or phagocytized by the adjacent cells. These dedifferentiated glial cells make up most of the tubular outgrowth from the ectoneural component that extends into the newly formed ECM to bridge the injury gap and reconnect with ectoneural extension originating from the opposite stump. The migrating dedifferentiated glial cells at the tip of the extension are closely followed by the growing neuronal fiber possibly originating from neurons whose fibers have been severed by the transection.

Cell proliferation which plays a minor role in the initial healing process of the nerve stump increases during this stage. Dividing cells are observed in the area that is extending from the ectoneural tissue as well as in adjacent areas. The dedifferentiated glial cells are thought to be the main cells undergoing proliferation.

It is important to state that there are no clear delimitations between stages. Some cell events are continuous but can change either temporally and/or spatially at different stages. For example, signs of neurodegeneration continue to be found even as regenerative processes begin. These neurodegenerative events spread from the areas close to the cut stumps to more distal areas. In this scenario, cellular apoptotic cells are observed from early regeneration near the exposed stumps, possibly in injured cells that were too damaged to survive, and then increase in

number in more distal areas at later stages of regeneration. It is thought that the initial apoptosis is a direct effect of the lesion-caused damage, while the later apoptotic event might be due to remodeling of the cellular architecture of the RNC.

### 13.3.3.3 Reconnection

Eventually, as the tubular growths from both nerve stumps elongate and meet, they form the initial bridge joining the two RCM through the injury gap. This is probably the most complex step given the difficulty of reconnecting two nerve stumps to recreate a RNC without creating a scar or abnormal connection. This process required not only a complex signaling of cellular factors but also of ECM and interactions between the neurons, glia, and their surrounding tissues.

At this stage three different but contiguous areas can be found: first, the space within the injury gap which is mainly formed by dedifferentiated glial cells and the newly regrowth of neuronal fibers; adjacent to this is the area undergoing dedifferentiation, which can extend to about 500 $\mu$ m from the injured stumps; finally, the area of the RNC that does not undergo any visible change in its structure or cell composition following the injury. This spatial order reflects the temporal process that is taking place for the formation of the new RNC.

It is during this stage that the peak of cell proliferation takes place, being the injury site area the place where the highest numbers of dividing cells are observed. Cellular proliferation is also documented in the adjacent area that is undergoing dedifferentiation but rarely in areas of the RNC distal to the injury site. Cellular proliferation takes place in both ectoneural and hyponeural RNC subdivisions.

### 13.3.3.4 Differentiation and Growth

The next step in the regenerative process is the redifferentiation of cells into the components of the normal RNC within the regenerated area that now expands the injury gap and eventually the growth of the structure to reach the size of the adjacent noninjured area. At this stage, the connective tissue band that separates the ectoneural from the hyponeural component becomes visible within the regenerated area. During this stage, all cellular events that were documented return to basal (or close to basal) levels. Thus, both cell proliferation and apoptosis are reduced, as well as the infiltration of spherule-containing cells.

Finally, with a completely reconnected RNC, the final step of regeneration occurs as the subpopulations of neurons appear in the RNC as evidenced by their specific neurochemistry. Therefore, at the end, the new RNC is identical or, at least, very similar to the original RNC in terms of the neuronal subpopulations and radial glial numbers and organization, with no scar left at the transection site.

### 13.3.3.5 Cellular Mechanisms

*Neurogenesis* A key process that has been studied in the holothurian RNC regeneration model has been the process of neurogenesis. Particular effort has been focused on determining what cell type or types constitute the neuronal precursors and whether these cells are dividing. Dividing cells were documented by electron microscopy and by incorporation of BrdU (Mashanov et al. 2008; San Miguel-Ruiz et al. 2009). Moreover, co-localization of BrdU incorporation and neuronal markers expression was observed, providing strong evidence that neurons (at least a subpopulation) originate from dividing precursors. Further experiments suggest that the neuronal precursors are the dedifferentiated glial cells. Thus, the available data suggests that the radial glial cells dedifferentiate early in the regenerative process and migrate to the injury area as part of a tubular outgrowth. These dedifferentiated cells can divide either in transit or as they reach the regenerating structure. At some point, and under unknown signaling pathways, the dedifferentiated glia can differentiate into either radial glia or neurons. The mechanisms by which neurons differentiate into the many subpopulations are not known. However, an interesting finding by our group (San Miguel-Ruiz et al. 2009) is that at least a subpopulation of neuropeptide expressing neurons appears to originate from nondividing precursors.

*Role of glia* As has been described above, it becomes evident that glial cells play a pivotal role in RNC regeneration. Such role has been best described by Mashanov et al. (2013), where the use of two antibodies that label glial populations (RS—Reissner substance and ERG1—a monoclonal that labels echinoderm radial glia) provides a detailed description of the changes that the glial population undergoes following RNC transection. This includes the loss of basal processes, the degradation of intermediate filaments, and the acquisition of a flattened morphology that incorporate into a tubular outgrowth that bridges the injury gap between the two stumps. These cells maintain their ERG1 immunoreactivity but decrease the expression of RS, and in those cells at the tip of the tubular outgrowth, there is a complete loss of RS expression. Glial cells eventually restore the RNC epithelial organization and reexpress both ERG1 and RS immunoreactivity. The regenerative role of the glial cells depends not only on the dedifferentiation response but also in their proliferative capacity as it has been shown that they account for most of the cells that are undergoing cell division in the regenerating RNC (Mashanov et al. 2013). BrdU birth-dating experiments have shown that glial cells give rise both to the new glia and to the neurons in the regenerated area of the RNC. Among the roles that glial cells might be taking in the regenerative response is the creation of a permissive environment for axon growth, as the tubular outgrowth composed of dedifferentiated glia precedes and is closely associated with numerous neuronal fibers in the process of reconnecting the two RNC stumps.

### 13.3.3.6 Molecular Basis

Recent genomic studies have identified the gene expression profile important for the process of RNC regeneration (Mashanov et al. 2014b). This RNAseq project produced a reference library of ~70,000 contigs out of which ~24,000 had significant BLAST similarities; most of them with the sea urchin, *Strongylocentrotus purpuratus* (Echinoidea, Camarodonta), predicted protein sequences. As with the gene profiling studies of intestinal regeneration, thousands of genes were found to be differentially expressed between normal and regenerating animals. Not surprisingly, the most dominant categories of genes throughout the whole process of regeneration were those involved in developmental morphogenesis. It is important to note that within the GO gene categories that were most prominent for the different regenerative stages, the most drastic changes were observed in genes involved in the synthesis and organization of the extracellular matrix (ECM) components, ECM remodeling, and cell-cell and cell-ECM interaction. When analyzed at individual regeneration phases during the growth phase, those categories related to DNA synthesis and protein translation were overrepresented, in the reconnection phase and in the differentiation phase.

Rather than focusing on single genes, this study aimed to determine functional gene groups that might be associated with neural regeneration and neurogenesis. Some of the major findings were: (1) Many of the differentially expressed genes in early regenerative stages were genes associated with oncogenesis (WNT, WNT receptors, survivin). (2) At all regeneration stages, some of the most highly overexpressed genes were those associated with ECM components and ECM-cell interactions (collagen, MMPs, TIMPs, integrins, selectins). (3) Surprisingly, most genes associated with pluripotency were not differentially expressed (*Klf2*, *Sox2*, *Oct1*, *Lgr5*, *Bmi-1*, *Ephb2*, *Msi2*, *Sall4*, *Zic3*, *Lin28*) (Mashanov et al. 2015a, 2017a). Only *Myc* was found to be overexpressed at all regenerative stages. (4) Most neurogenesis-related genes were already expressed in the uninjured RNC and did not show an overexpression during regeneration (*Thbs4*, *Musashi*, *DCLK1*, *Meis2*, *Nurr1*, *NeuroD1*, *Rein*, *FoxJ1*, *Prox1*, *WNT3*, *NeuN*). Only *Sox11* and *BMP-7* were upregulated, while *Prom1*, *Isl1*, and *Lhx3* were downregulated. (5) Genes associated with the production of glutamate or with possible steps in glutamate excitotoxicity (*GCPII*, *Abat*, *Glur1*, *Glur4*) were downregulated during regeneration, suggesting that such downregulation might be important in the survival of neurons following injury.

### 13.3.3.7 Gene Expression Patterns

The temporal and spatial expression of gene products has also been studied during regeneration of the RNC. Immunohistochemical studies have shown that various populations of cell fibers regenerate across the injury gap. These include neuropeptide-containing neuronal fibers labeled by anti-galanin and anti-



GFSKLYFa (neuropeptides) or with the antibody RN1 (now known to recognize a STARD lipid-binding protein) (Rosado-Olivieri et al. 2017; San Miguel-Ruiz et al. 2009). Similarly, neuronal cell bodies labeled with an anti-Nurr1 antibody have been shown to appear in the regenerated RNC, possibly originating from dividing precursors (San Miguel-Ruiz et al. 2009).

To determine the role of the Yamanaka factors in inducing the dedifferentiation (and possible reprogramming) of the radial glia that was observed in the RNC, their expression was probed during regeneration using *in situ* regeneration. These genes were found to be expressed in the apical region of both ectoneural and hyponeural components of normal and regenerating animals as well as in the glial tubular structure that is formed during regeneration. However, when compared to their expression patterns did not vary greatly during regeneration nor when compared with normal non-transected RNCs.

### 13.3.3.8 Functional Studies

Two important functional studies have been performed using the transection model of RNC regeneration in *H. glaberrima*. The first was aimed at determining the role of cell division versus cell dedifferentiation/migration in the regeneration of the severed RNC (Mashanov et al. 2015b). The treatment of regenerating animals with a cell proliferation inhibitor significantly reduced cell division without any observable effect on RNC regeneration. The results suggest that the dedifferentiated cells that migrate into the injury site make a much higher contribution of the cellular elements that populate the regenerated nerve and that this mechanism can restore the injury site even when cell proliferation is reduced (Mashanov et al. 2015b, 2017b).

The second functional study addresses the role of *Myc* in RNC regeneration (Mashanov et al. 2015c). In this study *Myc* expression is downregulated by electroporation of *Myc* dsRNA. The results of the experiment are a decrease in radial glia dedifferentiation and in cell death. Therefore, the *Myc* expression that has been previously documented to take place in the regenerating RNC is involved in inducing the two main processes observed early in the regeneration process, cell apoptosis and cell dedifferentiation.

While the latter experiment provides some insight into the function of one pivotal transcription factor to the regeneration process, its main value lies elsewhere. The study provides the first time that RNA interference-mediated gene knockdown is used in adult echinoderms. Therefore, the development of this technique opens the door for other gene functional studies that hitherto could not be performed until this date.

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# Chapter 14

## Regeneration in Stellate Echinoderms: Crinoidea, Asteroidea and Ophiuroidea



**Yousra Ben Khadra, Michela Sugni, Cinzia Ferrario, Francesco Bonasoro, Paola Oliveri, Pedro Martinez, and Maria Daniela Candia Carnevali**

**Abstract** Reparative regeneration is defined as the replacement of lost adult body parts and is a phenomenon widespread yet highly variable among animals. This raises the question of which key cellular and molecular mechanisms have to be implemented in order to efficiently and correctly replace entire body parts in any animal. To address this question, different studies using an integrated cellular and functional genomic approach to study regeneration in stellate echinoderms (crinoids, asteroids and ophiuroids) had been carried out over the last few years. The phylum Echinodermata is recognized for the striking regeneration potential shown by the members of its different clades. Indeed, stellate echinoderms are considered among the most useful and tractable experimental models for carrying comprehensive studies focused on ecological, developmental and evolutionary aspects. Moreover, most of them are tractable in the laboratory and, thus, should allow us to understand

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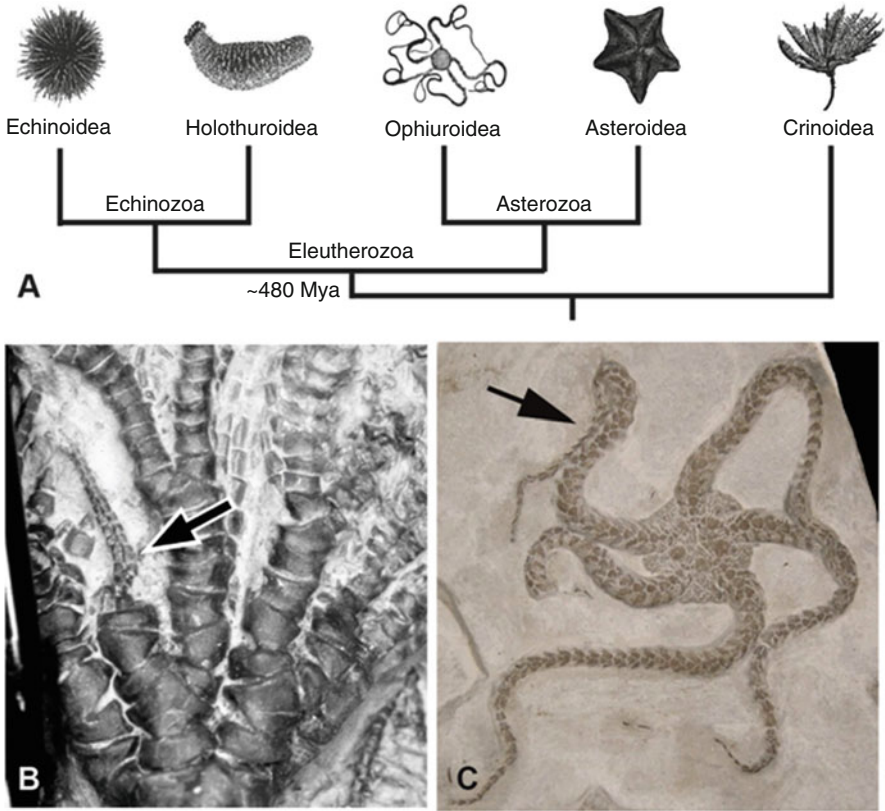
the underlying mechanisms, cellular and molecular, which are involved. Here, a comprehensive analysis of the cellular/histological components of the regenerative process in crinoids, asteroids and ophiuroids is described and compared. However, though this knowledge provided us with some clear insights into the global distribution of cell types at different times, it did not explain us how the recruited cells are specified (and from which precursors) over time and where are they located in the animal. The precise answer to these queries needs the incorporation of molecular approaches, both descriptive and functional. Yet, the molecular studies in stellate echinoderms are still limited to characterization of some gene families and protein factors involved in arm regeneration but, at present, have not shed light on most of the basic mechanisms. In this context, further studies are needed specifically to understand the role of regulatory factors and their spatio-temporal deployment in the growing arms. A focus on developing functional tools over the next few years should be of fundamental importance.

## 14.1 A Phylogenetic Perspective of Echinoderms

The phylum Echinodermata is divided in five extant classes: Crinoidea (sea lilies), Asterozoa (starfishes), Ophiurozoa (brittle stars), Holothurozoa (sea cucumbers) and Echinozoa (sea urchins). All classes are characterized by a rich fossil record (dating from the Cambrian Period), which, in principle, should help to identify the relationships between classes and species. However, this has not been the case and historically the classification of all classes has generated a heated debate. Recently, thanks to the introduction of large molecular datasets (mostly transcriptomic (Janies et al. 2016) and some genomic (Sodergren et al. 2006; Hall et al. 2017), the field has undergone a profound transformation and has allowed different authors to reach a new consensus. Molecular phylogenomic studies clearly establish the Echinodermata phylum within the Deuterostomia and a sister relationship with the Hemichordata (Ambulacraria = Echinodermata + Hemichordata; see Cannon et al. 2013). Within the Echinodermata, Crinoidea (the only extant class of the Pelmatozoa subphylum) is the sister group of the remaining four classes, which form the subphylum of Eleutherozoa. Most recent phylogenies (O'Hara et al. 2014; Telford et al. 2014; Reich et al. 2015) support the existence of a clade that includes Asterozoa + Ophiurozoa (Asterozoa) and a clade with Echinozoa + Holothurozoa (Echinozoa) (Fig. 14.1a). This arrangement implies the existence of a common ancestor for all stellate echinoderms. An alternative hypothesis, not as well supported, places Ophiurozoa as sister to Echinozoa (Echinozoa + Holothurozoa). This hypothesis, known as the Cryptosyringida (Pisani et al. 2012), would support a different interpretation of adult and larval morphological evolution but will not be considered any further in this chapter.

The detailed combination of large, carefully selected, molecular and morphological characteristics further allowed the inference of intraclass relationships that were, until recently, difficult to trace (O'Hara et al. 2014; Thuy and Stöhr 2016). These last





**Fig. 14.1** (a) Phylogenetic relationships of the five extant classes of echinoderms. Cladogram based on Telford et al. (2014) and evolutionary clock based on O'Hara et al. (2014). (b) Fossil of *Isocrinus oregonensis* (Oligocene) showing a regenerating arm (arrow); photo by J. Schneider (Oji 2015). (c) Upper Cretaceous (Cenomanian, ~95 million years old) fossil of *Ophiotitanos tenuis* from Southerham Grey Pit, Lewes, East Sussex, UK. Image courtesy of Tim Erwin, specimen collected and prepared by Robert Randall and kindly donated by the Firle Estate. Arrow indicates a small regenerating arm

investigations have led to specific propositions on the evolutionary changes associated with the diversification of each class and the reconstruction of ancestral states (Feuda and Smith 2015), thus allowing the recognition of unique character changes occurring within each class (for instance, characters linked to the regeneration process discussed in this chapter).

All extant classes of echinoderms (Candia Carnevali 2006) as well as some species of hemichordates (Luttrell et al. 2016) are able to efficiently regenerate adult body parts. The fossil record largely supports the ancient origin of echinoderm regenerative potential, identifying regenerative structures in crinoids, asteroids and ophiuroids dating back to the Palaeozoic Era (Fig. 14.1b, c; Oji 2001). However, the

extent and efficiency of regeneration can vary enormously among echinoderms, a property that is linked to the ecological pressure created by predation (Oji 2015).

Understanding the molecular underpinnings of ambulacrarian (or, specifically, echinoderm) regeneration will be critical for our understanding of the process in its varied presentations. Moreover, this knowledge should probe instrumental in deciphering how gene batteries and regulatory networks have changed over evolutionary time in order to meet the adaptation demands of each class (or constitutive clades). In this chapter we will review the studies undertaken to characterize cellular and molecular processes of regeneration in stellate echinoderms (Asterozoa + Crinoidea; Fig. 14.1a).

## 14.2 Echinoderm Regeneration: Not Only a Replacement

Regeneration is an intrinsically conservative post-embryonic developmental process providing not only an indispensable requisite for individual survival but also the necessary complement for the asexual reproduction programme. If a response to injury is evoked in all animals, the regeneration potential, i.e. the degree of morphological and functional recovery, actually varies significantly in different groups, in closely related species and even in different tissues and organs of the same animal (Dinsmore 2001; Brockes and Kumar 2008; Sánchez Alvarado and Tsonis 2006; King and Newmark 2012). The extent of the repair/regrowth is quite independent of the phylogenetic position of the group, depending exclusively on the individual histogenetic and morphogenetic potential that allows the expression of a new developmental programme or the re-expression of the old one in the species.

In living echinoderms, the regenerative potential is expressed to a maximum extent and the regeneration processes, well described in old and recent literature, are frequent at all stages of the life cycle (embryo, larva, adult) and largely employed to rebuild body parts and even complete individuals from fragments following mutilation processes of different types (Candia Carnevali 2006).

The extensive employment of regenerative potential throughout the phylum indicates that, in echinoderms, regeneration has the widest range of biological implications, increasing the chances of survival of the individual and, being the indispensable requisite for fissiparity, allowing the rapid colonization of new habitats through the production of multiple clones well adapted to local conditions. Hence, due to its evident adaptive and strategic significance/value, regeneration certainly contributes to the fitness of the species and to the success of echinoderms throughout the marine ecosystem.

Although in all echinoderm classes regenerative phenomena are extensively employed to reconstruct internal or external organs and structures (Candia Carnevali 2006), arm regeneration in the stellate echinoderms represents one of the most useful and tractable experimental models for carrying comprehensive studies focused on ecological, developmental and evolutionary aspects as well as on involved mechanisms, cellular and molecular (Candia Carnevali 2006; Dupont and Thorndyke 2007;

Franco et al. 2011a, b; Czarkwiani et al. 2016; Ben Khadra et al. 2017). In addition, in all the three groups the detached arm fragments (explants) can survive for a long time and undergo, as well, partial or total regeneration. In some asteroid species, a single arm can even regenerate a whole animal (Ben Khadra et al. 2017). The arm explants can therefore represent simplified and controlled *in vivo* regenerating systems, providing a valuable tool for testing mechanisms and processes (Candia Carnevali et al. 1998).

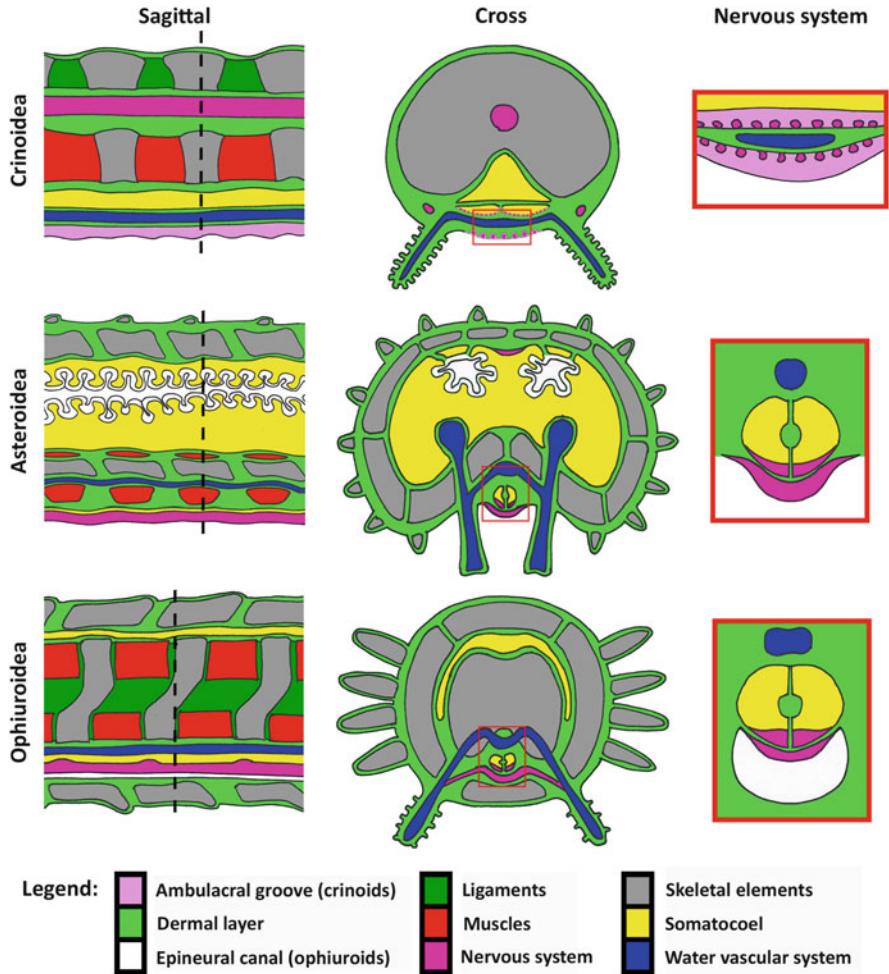
Although the ability to regenerate an arm occurs in all stellate echinoderms, differences in extent, timing, energetic costs and frequency of regeneration (Lawrence 2010) are clearly observed within and among classes. These differences can be related to the specificities of the anatomy, physiology and behaviour of the species. In general, the long, thin and fragile arms of crinoids (Mladenov 1983) and infaunal ophiuroids are frequently lost and usually regenerate more efficiently and rapidly than the larger asteroid arms (see next paragraph). For example, in natural populations of burrowing brittle stars (non-euryalid ophiuroids) up to 80–100% of individuals show signs of regeneration and have more than 70% of arms undergoing regeneration at one time (Wilkie 1978; Bourgoïn and Guillou 1994; Clements et al. 1994; Sköld and Rosenberg 1996; Soong et al. 1997; Yokoyama and Amaral 2010). Regardless of the species, arm loss can occur for different reasons, including predation, environmental perturbation or intraspecific fighting (Emson and Wilkie 1980; Ramsay et al. 2001).

In the different models explored so far, arms or explants, regeneration processes turned out to be an extremely complex developmental phenomenon that involves both repair and regrowth. They require not only new cell recruitment but also implementing detailed spatial information in order to properly specify the identity of the new tissues. Overall, the arm regeneration process tends to follow a common basic scheme, which is characteristic of the group and well understood in terms of costs, efficiency of regrowth and resource allocation. However, the same process can be, at the same time, plastic enough to achieve the same goal by changing the roles and contributions of the involved cells according to the individual situation (namely, the mutilation preconditions but also age, health and nutrients availability) or the environmental conditions (Candia Carnevali 2006). This means that each regeneration event is a very plastic phenomenon, which can be, and truly is, intrinsically different for each individual or species and can be adapted to the specific (environmental) requirements.

In the following paragraphs, an up-to-date overview of the phenomenon of arm regeneration in stellate echinoderms is provided, and this is done by briefly revisiting and comparing the most thoroughly explored models, emphasizing differences and similarities, true or apparent, in the involved mechanisms at the tissue, cellular and molecular level. The comparison of results derived from different experimental arm models in the same phylum, employing different approaches, can provide insights on the specificity of processes, and their underlying regulatory mechanisms, governing large-scale pattern formation in newly formed tissues. Due to the complexity of the regenerative phenomenon, it is obvious that comparing how different animal models achieve arm regeneration must be approached from multiple angles and requires the use of an integrate view.

### 14.3 Arm Regeneration: The Cellular and Tissue Perspective

The standard arm anatomy of crinoids, asteroids and ophiuroids has been reviewed by several classical works (Hyman 1955; Harrison and Chia 1994) and is here summarized in Fig. 14.2 for a generalized representative of each class. In this



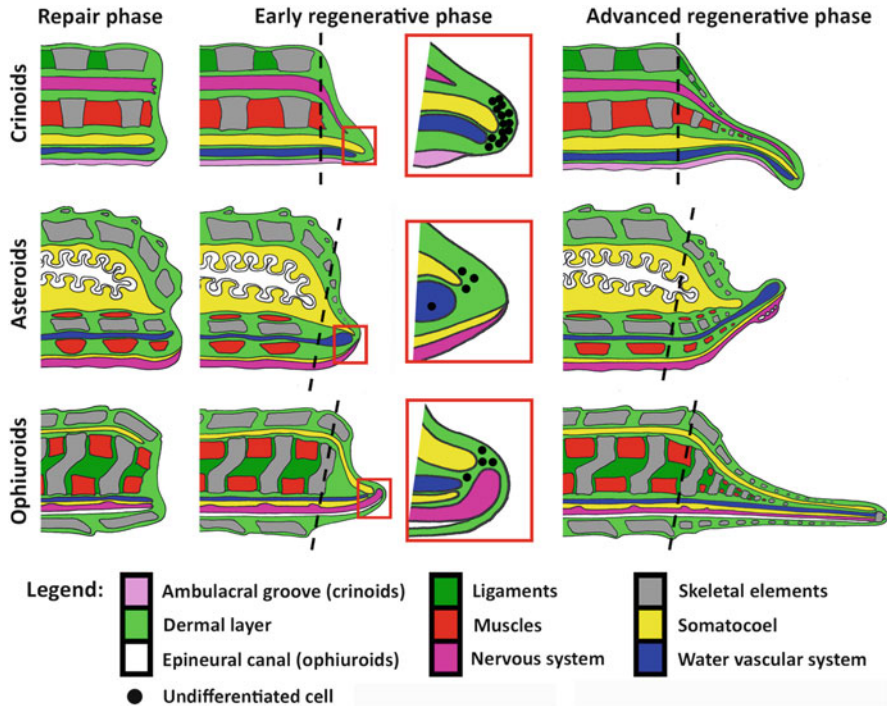
**Fig. 14.2** Representative schemes of sagittal and cross sections of the three stellate echinoderm arms. For simplicity sagittal sections recapitulate tissues present at different longitudinal planes and muscles are not shown in cross sections. Crinoid arm is on purpose shown with the oral and aboral sides inverted to make easier the comparison with that of asteroids and ophiuroids. Inserts on the right show the nervous system components in the three classes. For details see colour key at the bottom of the figure. Dotted lines in sagittal sections represent the level of the labelled cross sections. The pyloric caeca of asteroids are shown in white

paragraph, we will recall only some of the main anatomical and architectural differences that have to be kept in mind in order to understand the observed diversity in regenerative mechanisms.

In general, it must be recalled that crinoids, being the only extant members of the Pelmatozoa subphylum, have an arrangement of anatomical structures opposite to that shown by the members of the Eleutherozoa (i.e. ophiuroids and asteroids), meaning that the oral side (the ambulacral groove) faces the water column rather than the substratum (as the latter group). They have three distinct nervous sub-systems (ectoneural, hyponeural and entoneural), which differ in anatomical localization and specific functions (Cobb 1995). The most evident nerve structure of crinoid arm is the entoneural brachial nerve, which basically consists of a bundle of neurons and nerve processes running throughout the central part of the arm (Candia Carnevali et al. 1997). In both ophiuroids and asteroids, the main nervous structure is the radial nerve cord (RNC) that is mainly composed by a thick ectoneural neuroepithelium and a thin layer of hyponeural neurons and fibres (Hyman 1955). In all the three classes, the ectoneural system remarkably innervates both the outer (basiepithelial plexus of the epidermis) and the inner epithelia (basiepithelial plexus of the enteric epithelia), giving rise to a diffused neural network reaching the main body organs (Harrison and Chia 1994). Other evident inter-class differences are related to the diverse presence and arrangement of skeletal elements. Indeed, crinoids have very conspicuous columnar ossicles, which occupy most of the arm and are serially repeated along it; the “armoured” ophiuroids have five distinct prominent elements (two lateral, an oral and an aboral shields and an inner conspicuous vertebra), again serially repeated along the arm with little connective tissue. The starfishes contain, instead, a meshwork of several medium-sized ossicles immersed in abundant connective tissue giving to their body wall a “softer” consistency (Blowes et al. 2017).

### 14.3.1 Regenerative Phases

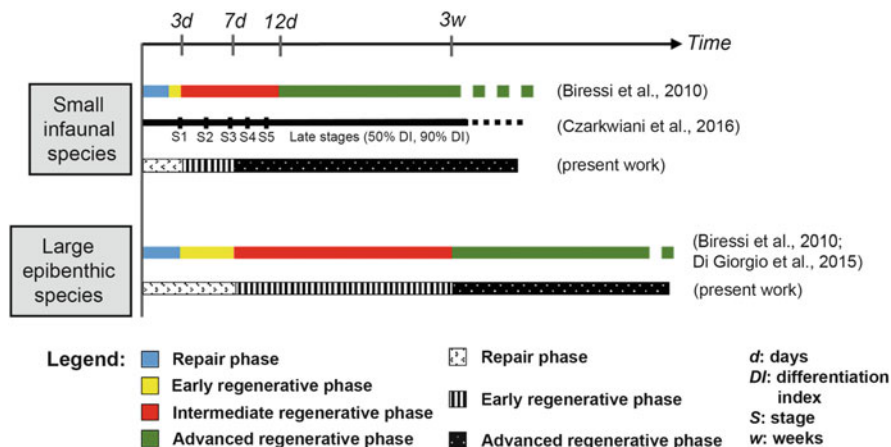
The main histological events of the regenerative processes have been established in a series of studies following pseudo-autotomic or traumatic amputations (Mladenov et al. 1989; Candia Carnevali et al. 1993, 1995a, b, 1997; Moss et al. 1998; Biressi et al. 2010; Ben Khadra et al. 2015a, b; Czarkwiani et al. 2016). In general, and regardless of the class, the overall process can be subdivided into three main phases: a **repair phase**, an **early regenerative phase** and an **advanced regenerative phase** (Fig. 14.3). These can differ in terms of timing and specific processes involved, both between classes and also within the same class. For example, within ophiuroids, small-sized and frequently regenerating species, such as *Amphiura filiformis*, usually regenerate faster than large epibenthic species, such as *Ophioderma longicaudum* or *Ophioplocus januarii* (Biressi et al. 2010; Di Giorgio et al. 2015; Czarkwiani et al. 2016). In ophiuroids, different staging systems have been used (Biressi et al. 2010; Di Giorgio et al. 2015; Czarkwiani et al. 2016) (Fig. 14.4). In order to provide a



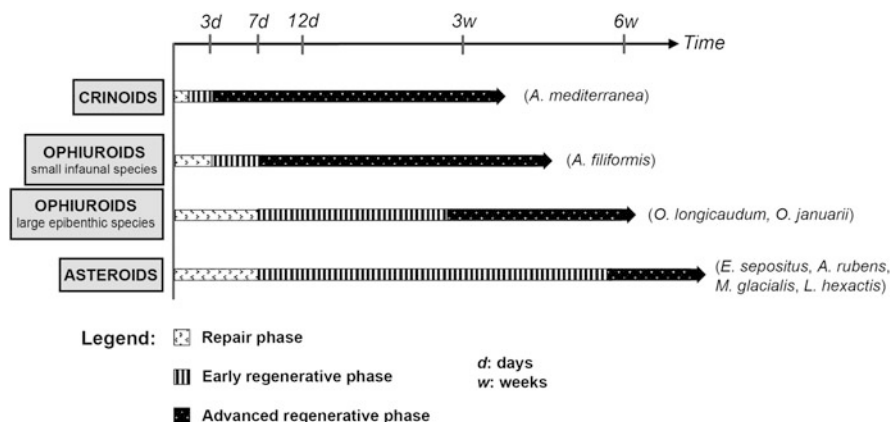
**Fig. 14.3** Schemes of the three regenerative phases of stellate echinoderms. Insets show the details of the regenerate tip during the early regenerative phase with a specific focus on undifferentiated cells (black circles) present in the regenerating area. For details see colour key at the bottom of the figure. Dotted lines represent the amputation planes. The pyloric caeca of asteroids are shown in white

generalized classification including all the different species studied so far and to allow an easier inter-class comparison with the other stellate echinoderms, we present here a reviewed staging system based on regenerative phases (Fig. 14.5).

Regardless of the class and species, during the **repair phase** emergency reactions and wound closure must occur. First events are addressed to seal the main coelomic cavities, stop fluid loss and avoid the entrance of pathogens. In all the three classes, this is ensured by very similar events, such as clotting phenomena by the free-wandering coelomocytes (which have also immune functions; Smith et al. 2010), rapid re-epithelialization by stretching and remodelling of the stump epidermal tissue and wound contraction/apical shrinkage to reduce the wound exposed surface (Candia Carnevali et al. 1993; Biressi et al. 2010; Ben Khadra et al. 2017; Ferrario et al. 2018). The latter event is particularly evident in asteroids, whereas it is inconspicuous in crinoids, due to the intrinsic differences in their arm anatomy (vast somatocoel and relatively “soft” body wall of starfishes compared to the highly calcified arms of crinoids; see Fig. 14.2). Therefore, instead of contracting the whole



**Fig. 14.4** Comparison of the regenerative staging systems in ophiuroids, showing the time differences between large epibenthic and small infaunal species as described in the literature and unified in the present work. For details see colour key at the bottom of the figure



**Fig. 14.5** Comparison of regenerative phases in the three classes of stellate echinoderms, using for ophiuroids the staging system proposed in Fig. 14.4. For details see colour key at the bottom of the figure

arm wall, in the “armoured” arms of crinoids and ophiuroids, muscle valves present along the main axial canals/vessels (particularly the radial water canal), ensure the sealing of the canal itself upon arm injury and immediately stop the loss of body fluid (Candia Carnevali et al. 1993; Fig. 2B in Czarkwiani et al. 2016). These valves are serially located along the arm, a property in line with the ability of these animals to perform autotomy at multiple (specific) arm levels and quite different to what occurs in starfishes, which have only one single autotomy plane at the base of the arm (Wilkie 2001).

Re-epithelialization is rapid in all the three stellate echinoderms (in relation to their regeneration speed, Fig. 14.3) as it is accomplished at the middle of the repair phase (Candia Carnevali et al. 1993; Ben Khadra et al. 2015a; Ferrario et al. 2018). It is typically due to a stretching of the wound edge epidermal cells rather than to their proliferation (Candia Carnevali et al. 1995a, b; Mladenov et al. 1989; Moss et al. 1998; Czarkwiani et al. 2016), representing a major difference between echinoderms and mammals and possibly accounting for the more efficient repair (and, therefore, regenerative) ability of the former group (Ferrario et al. 2018).

In some asteroid species, such as *Echinaster sepositus*, a transient syncytium of phagocytes ensures stump tissue protection and provides a support for the overlying migration of epidermal cells (Ben Khadra et al. 2015a). A similar syncytial tissue has never been described in either crinoids or ophiuroids, although functionally comparable cell accumulations can be present (Ferrario et al. 2018). In large epibenthic ophiuroids, during the final step of the repair phase, a thick cicatricial layer is visible (Biressi et al. 2010; Di Giorgio et al. 2015); this is a tissue composed of a heterogeneous population of cells, including phagocytes, coelom-derived elements and apparently undifferentiated cells, immersed in a dense collagenous matrix. In many aspects, this layer histologically resembles the oedematous area (or granulation tissue) described in starfishes (Ben Khadra et al. 2015a, b, 2017) and, therefore, can be considered functionally and morphologically homologous. In starfishes and large ophiuroids, this structure possibly constitutes a “filling tissue” necessary in those large-sized arms where the amputation plane following the trauma is concave and the first signs of regeneration appear after long time (Biressi et al. 2010; Di Giorgio et al. 2015). As in mammals (Pastar et al. 2014), this is a temporary tissue whose progressive maturation eventually leads to the formation of the scaffold for the subsequent regenerative growth. This event is generally not necessary for the thin arms of small brittle stars and crinoids, which complete the repair phase (and also the whole regenerative process) in just a few days (Fig. 14.5). Exceptions to this condition are those species living in extreme environmental conditions, such as the small Antarctic brittle stars *Ophiura crassa* and *Ophionotus victoriae*, where the repair phase is extremely delayed and the first signs of regrowth are visible only after several months (Clark et al. 2007; Clark and Souster 2012). This delay, however, might be related to a generalized reduced metabolic rate due to cold waters (Hughes et al. 2011) or to different energy allocation (Lawrence 2010).

When the injury is completely healed, the **early regenerative phase** starts. In all the three classes of echinoderms, these are the first genuine signs of cell differentiation, which will create the basis for the subsequent tissue regrowth. This includes the prominent event of axes determination (oral-aboral and proximal-distal). For those models where an epimorphic regenerative mechanism is employed, this phase is characterized by the appearance of a blastemal bud, a pool of undifferentiated cells which will, eventually, give rise to all the new arm structures (see next paragraph). A common feature shared by the three models is the concomitant regrowth of the main axial structures, which define the polarity along these axes: the coelomic cavities (both the perivisceral coelom and the radial water canal) and the nervous structures (the brachial nerve in crinoids and the RNC in ophiuroids and asteroids) with their



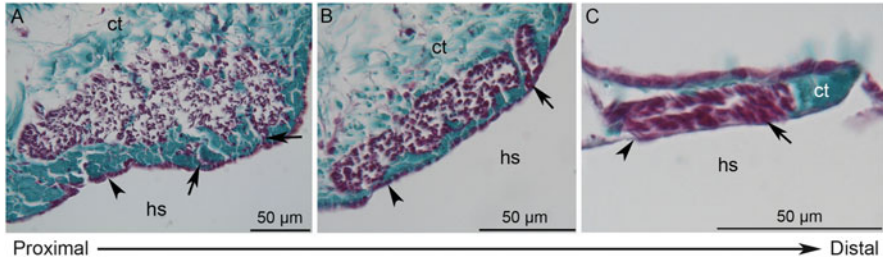
associated sinuses. The regenerating tip of the coelomic cavities appears generally hypertrophic, probably as a result of a local and intense fluid secretion by the coelomic epithelia (Candia Carnevali et al. 1993; Biressi et al. 2010; Guatelli 2017); this might have a functional consequence, leading to the generation of a hydrostatic pressure that allows the regrowth of the canals within the regenerate and also provides the turgidity and support to the regenerate itself, at least until the skeletal and muscle elements are completely developed. Therefore, the use of the coelomic cavities as physical drivers of the regeneration process is a common principle used in stellate echinoderms that should not be ignored. Neural structures are also among the first tissues to be regenerated, and this is a feature that underlines their fundamental role in echinoderm regeneration (see below). In the RNC of both ophiuroids and asteroids, the cell body layer of the neuroepithelium is first restored, and it is only later that the neuropile, containing the nervous processes, becomes visible (Ben Khadra et al. 2015a, b; Czarkwiani et al. 2016). In crinoids this differential aspect is less evident, possibly due to the different anatomical organization of the brachial nerve (see above).

In asteroids and ophiuroids, the first signs of skeletogenesis also appear during this phase; this occurs particularly early in the former class, and it might be related to the precocious development of the terminal ossicle, according to the so-called distalization-intercalary mode of regeneration (see below) (Czarkwiani et al. 2016; Ben Khadra et al. 2015b, 2017).

A fundamental aspect of the early regenerative phase that needs to be emphasized is the progressive repopulation of the developing dermal (or “mesenchyme”) layer, located between the outgrowths of the axial structures and the regenerated epidermis, by relatively undifferentiated cells (Fig. 14.3). This is markedly evident in crinoids where this process ultimately gives rise to a typical blastema (see next paragraph) (Candia Carnevali and Bonasoro 2001). In starfishes, cell migration is largely occurring by the mobilization of different cytotypes from different areas, such as coelomic cavities and hyponeural sinus, all converging towards the regenerating area (Mladenov et al. 1989; Ben Khadra et al. 2015b).

Besides the onset of differentiation processes, during the early regenerative phase also dedifferentiation phenomena become clearly detectable, particularly in the muscular tissues including also those far from the amputation plane and not directly affected by the trauma (Candia Carnevali and Bonasoro 2001; Biressi et al. 2010; Ben Khadra et al. 2017). Whether dedifferentiation is only a source of materials or also of cells remains an open question. Nevertheless, the concomitant occurrence of massive tissue (muscle) remodelling and tissue/structure regrowth (= increased metabolic needs) supports the idea that the remodelled tissue is acting as a source of energy/materials.

The **advanced regenerative phase** is characterized by the actual morphogenesis and final differentiation of all tissues, following a proximal-distal direction of regrowth, a process involving tube feet, neural structures (i.e. the optic cushion in starfishes) and skeletal elements. Czarkwiani et al. (2016) noted that in *A. filiformis*, the external shields (lateral, oral and aboral) differentiate earlier than the inner vertebrae suggesting a different regulatory developmental mechanism. Muscle



**Fig. 14.6** Paraffin sagittal section of *E. sepositus* arm-tip regeneration showing the proximal-distal gradient (a-c) of muscle (lower transverse ambulacral muscle) differentiation during the advanced regenerative phase. Muscle cells (arrows) originate from cells of the coelomic epithelium (arrowheads) lining the hyponeural sinus. Abbreviations: *ct* connective tissue; *hs* hyponeural sinus

bundles are generally the last tissues to be differentiated in line with their late ontogenetic origin. They originate from the coelomic wall by myocyte ingression in the connective tissue situated below and migrate to their definitive site (Fig. 14.6; Rieger and Lombardi 1987; García-Arrarás and Dolmatov 2010).

For all echinoderm classes at the end of this stage, the new miniaturized regenerate resembles the non-regenerating arm, in both morphology and functionality (Candia Carnevali and Bonasoro 2001; Biressi et al. 2010; Ben Khadra et al. 2015b). In the starfish *E. sepositus* two tissues, namely, pyloric caeca and papulae, show no signs of differentiation until 4 months postamputation, possibly because that they are not fundamental for the small regenerate's immediate survival (Ben Khadra et al. 2015b). The regenerate, as the normal non-regenerating arm, will continue grow to throughout the animal's life.

Regeneration rate can markedly vary among the three classes and also within members of the same class. In general, crinoids and small ophiuroids display fast arm regeneration, whereas the big epibenthic ophiuroids and asteroids normally require longer periods for their complete arm regrowth. This might reflect size-related aspects or species/class-specific mechanisms of regeneration.

According to Dupont and Thorndyke (2006), in ophiuroids, depending on the amount of lost arm segments, the animal will invest more in the number of regenerated segments (and, therefore, in the overall regenerated length) or in the rapid differentiation of the regenerated segments. In other words, arms amputated more proximally (closer to the disc) will have longer but less differentiated regenerates, whereas those amputated more distally will rapidly differentiate the new segments but have shorter regenerates. Similarly, in asteroids, a proximally amputated arm regenerates a higher number of segments than a more distally amputated one over the same time period (Sugni, Ferrario, Bonasoro and Candia Carnevali personal observations).

### 14.3.2 *Blastema or Not Blastema?*

Sánchez Alvarado and Tsonis (2006) defined the regenerative blastema formed after the amputation as a structure “made of a superficial sheet of cells of epithelial origin covering the full extent of the bud, with an underlying localized mass of undifferentiated cells of mesenchymal origin”. These latter are typically characterized by a high proliferative activity and will later differentiate in all the specific lineages necessary to rebuild the lost body part. According to the classical definition by Morgan (1901), the regenerative mechanism is *epimorphic* when a localized blastema is formed, whereas it is *morphallactic* if no evident blastema is present and the cells involved in the regrowth come from the stump tissue after dedifferentiation, transdifferentiation and redifferentiation as well as migration of mature cells without intense and local proliferative activity (Candia Carnevali 2006).

In this sense, arm regeneration in crinoids is a typical blastemal regeneration process in which new structures develop from a mass of pluripotent actively proliferating cells, which are responsible for both repair (Candia Carnevali et al. 1995a, b) and tissue regeneration (Candia Carnevali et al. 1997). The blastema becomes clearly evident during the early regenerative phase (about 3 days post-amputation) as a pigment-less bud protruding from the amputation plane (inserts in Fig. 14.3). The blastema is mostly composed of morphologically undifferentiated cells, such as amoebocytes and coelomocytes. The former cells are normally located in the cortex of the brachial nerve, where they are in a “quiescent state” and can therefore be considered as a stem cell reservoir. Following the trauma they become active and migrate along the nerve towards the amputation plane (Candia Carnevali et al. 1995a, b, 1997). The latter cells are morphologically very similar to the free-wandering cells of the coelomic fluid and are thought to derive from the coelomic epithelium, which they presumably cross to reach the underlying “mesenchymal” tissue that composes the blastema.

Starfish regeneration has been historically considered a “typical” morphallactic process, since a well-defined and localized blastema, *sensu stricto*, is never formed during the process, and cell proliferation is widespread in different tissues, including those distant from the regenerating area (Bonasoro et al. 1998; Moss et al. 1998; Hernroth et al. 2010).

It is important to point out in this context that ophiuroid regeneration was traditionally considered an epimorphic blastemal process (Dupont and Thorndyke 2006; Di Giorgio et al. 2015). However, a detailed re-examination of the available data and a deep inter-class comparison suggest that a true localized blastema (as that of crinoids) is never formed in these animals (Biressi et al. 2010; Czarkwiani et al. 2016) and that the regenerative mechanism is much more similar to that of asteroids than that of crinoids. As discussed above, the pre-blastemal area mentioned by Biressi et al. (2010) is similar to the oedematous area or granulation tissue of asteroids. Moreover, the true primordial regenerative bud (often improperly referred as the blastema) mainly contains the outgrowths of the coelomic cavities and RNC, which, although displaying a rather undifferentiated cell morphology and an intense

proliferative activity at their distal-most end, always maintain their own histological (epithelial) individuality, being clearly delimited by their respective basal lamina (Czarkwiani et al. 2016; Piovani 2017; Ferrario et al. 2018). In both the Eleutherozoan classes, the area between the wound epithelium and the outgrowths of the axial structures, which in crinoids corresponds to the area of blastema formation, is never filled by a conspicuous localized mass of undifferentiated cells. What we detect is, instead, a zone of relatively loose mesenchyme in which, however, scattered and scarcely differentiated cells can be found immersed in a loose collagenous matrix (Fig. 14.3). This represents the so-called blastema-like structure described by Ben Khadra et al. (2017) in asteroids and the pools of mesenchymal/progenitor cells surrounding the tip of the coelomic canals and radial nerve cord mentioned by Biressi et al. (2010) and Piovani (2017) in ophiuroids. Whether this is truly homologous to the crinoid blastema is an interesting issue to consider. Noteworthy, in all the three classes, most of the cells of this temporary tissue likely have, at least partially, a coelomic origin (Candia Carnevali et al. 1997; Ben Khadra et al. 2017; Piovani 2017). In crinoids also the amoebocytes migrating from the brachial nerve contribute to the pool of blastemal cells (Candia Carnevali and Bonasoro 2001). This might be related to the different organization of their nervous system, which is formed by bundles of fibres, perykaria and relatively “free” satellite cells (the amoebocytes) not constrained by a basal membrane, which is present in the RNC of the Eleutherozoans (Heinzeller and Welsch 1994).

All in all, while the classification as blastemal/non-blastemal regeneration can still be valid, the limitation of using the mutually “exclusive” terminology epimorphosis and morphallaxis is becoming more and more evident in echinoderms (Candia Carnevali 2006) and, in general, animal regeneration (Agata et al. 2007). The coexistence of the two mechanisms, together with the difficulties in the true understanding of the origin of cell composing the regenerate, makes the use of this classification often ambiguous and inappropriate and, therefore, should be used with caution (Agata et al. 2007).

### ***14.3.3 Regeneration-Competent Cells***

Different types of cells are involved in echinoderm arm regeneration, some being specific of a class, others having inter-class roles. In crinoids the main players are migratory, morphologically undifferentiated, cells, such as amoebocytes and coelomocytes. These are hypothesized as pluripotent stem cells. Recent *in vitro* and *in vivo* studies allowed to identify the different progenitor cell types and to show their involvement in migration, proliferation and dedifferentiation processes (Di Benedetto et al. 2014). In particular, *in vitro* results have confirmed the undifferentiated morphology of amoebocytes and their amoeboid activity. Indeed, as previously mentioned, during arm regeneration *in vivo*, these cells migrate along the brachial nerve towards the amputation site and undergo an extensive local proliferation in the developing blastema. Previous studies suggested that migratory amoebocytes ultimately produce

both blastemal and blastema-derived differentiated cells. In contrast, the coelomocytes are supposed to give rise to all the differentiated elements related to coelomic tissues, including peritoneocytes, myocytes and free coelomocytes, although a contribution to tissue regrowth of extra-coelomic compartments (i.e. skeletal tissue) cannot be excluded. Indeed, this would be consistent with previous studies (Czarkwiani et al. 2016; Piovani 2017), which suggested that sclerocytes responsible for skeleton regeneration might originate from coelomic epithelium-derived progenitor cells.

In starfishes, cell recruitment has been traditionally considered to rely on stump cell dedifferentiation/transdifferentiation rather than on activation of stem cell stocks (Ben Khadra et al. 2017). However, Hernroth et al. (2010) recently proposed that cells of mixed origin might be recruited from distant sources of stem/progenitor pools rather than distant differentiated elements. This hypothesis was suggested based on the analyses of cell aging parameters (lipofuscin content, telomerase activity, etc.), which indicated how the cells composing the regenerate display “youth features” (typical of stem cells). Nevertheless, as suggested by the authors themselves, these characteristics might also be the result of a secondary rejuvenation process of already differentiated cells.

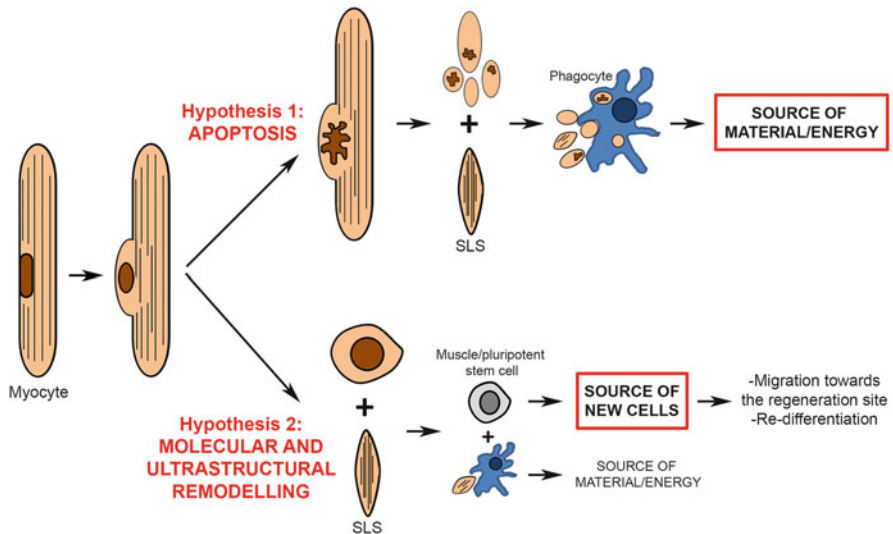
Besides pluripotent progenitor cells, in all the three classes of echinoderms, well-differentiated cytotypes also actively participate in the different phases of the regenerative process. These include phagocytes, specialized coelomocytes, granulocytes and pools of dedifferentiated cells, particularly myocytes (Candia Carnevali and Bonasoro 2001; Candia Carnevali 2006; Parma et al. 2006; Candia Carnevali and Burighel 2010; Di Benedetto et al. 2014). Phagocytes are the functional homologues of the vertebrate macrophages (Ottaviani 2011): indeed, they are recruited and actively involved, particularly in the repair phase, when removal of cell debris and pathogens is necessary (Candia Carnevali et al. 1993; Biressi et al. 2010; Ben Khadra et al. 2017; Ferrario et al. 2018). As previously mentioned, in starfishes, these phagocytes can also form a transient syncytial structure covering the wound (Ben Khadra et al. 2017). Besides these specialized cells, the phagocytic activity is, however, a relatively common ability of all echinoderm cells, including epithelial cells (Sugni, Ferrario, Bonasoro and Candia Carnevali personal observation).

Because the “coelomocytes” include a very heterogeneous population of cytotypes (Smith et al. 2010), there is often a misunderstanding in their description and the roles they play. Coelomocytes *sensu stricto* should be referred to as the cells freely wandering in the coelomic fluid. As previously said, in crinoids these are morphologically undifferentiated cells that migrate and ultimately give rise to the blastema. In starfishes (as in other echinoderms, such as echinoids and holothuroids), these elements mostly include highly specialized and morphologically differentiated cells that are involved in clot formation, encapsulation and phagocytic activities as well as in the production of antibacterial compounds (Pinsino et al. 2007; Smith et al. 2010). The undifferentiated cells occasionally observed within the coelomocyte population are considered to be a progenitor source of these highly differentiated cytotypes (Gorshkov et al. 2009; Guatelli 2017). Therefore, in starfishes the *sensu stricto* coelomocytes are mainly involved in the reparative events and in the first immune response following the injury (Pinsino et al. 2007; Gorshkov et al. 2009;

Holm et al. 2008; Ramírez-Gómez and García-Arrarás 2010; Sharlaimova et al. 2010; Sharlaimova and Petukhova 2012). Coelothelium-derived cells, instead, likely contribute to new tissue regrowth (see Sect. 14.3.5).

Other types of migratory cells involved in regeneration include the granulocytes or “wanderzellen” (Reichensperger 1912) of crinoids. These are randomly scattered in all tissues, although particularly localized around the brachial nerve and in the regenerating tissues during the first regenerative phases. It has been hypothesized that these cells could be a source of putative growth factors even if, so far, they have not been identified (Thorndyke and Candia Carnevali 2001).

A remarkable common feature of echinoderm arm regeneration (and generally of all echinoderm regenerative processes) is the “recycling” and plasticity of adult stump cells. This occurs particularly in muscle tissues (muscle bundles as well as coelom-associated myocytes), and, to a minor extent, in other mesodermal cell types (i.e. skeleton, ligaments, etc.; Candia Carnevali et al. 1993; Candia Carnevali and Bonasoro 2001; Candia Carnevali 2006; Czarkwiani et al. 2016; Ben Khadra et al. 2017). From a histological point of view, this is evidenced by myocytes losing their typical fusiform shape and showing signs of disorganization in their contractile apparatus, which is progressively compacted. At present it is still unclear the real fate of these remodelled myocytes and their physiological significance for the regenerative process. Two hypotheses can be considered (Fig. 14.7): Hypothesis



**Fig. 14.7** Schematic representation of the two hypotheses myocytes fate. Hypothesis 1: myocytes undergo apoptosis; therefore, both the apoptotic nucleus (with associated reduced cytoplasm) and the produced SLS are phagocytized and eventually used as source of energy/material for regeneration. Hypothesis 2: SLS are expelled from the cell, phagocytized and eventually used as source of energy/material for regeneration, whereas the remaining nucleus (with associated reduced cytoplasm) becomes a “pluripotent cell” and can therefore migrate and redifferentiate contributing to regeneration. SLS spindle-like structures

(1) myocytes undergo nuclei degeneration (apoptosis) and the remaining spindle-like structures (SLS; García-Arrarás and Dolmatov 2010) are, therefore, used just as a source of material/energy for the regenerative process (Ben Khadra et al. 2017); Hypothesis (2) they undergo a true dedifferentiation process by extrusion of their contractile apparatus and production of characteristic SLS (eventually used as source of energy/material), while the remaining cell body becomes regeneration-competent pluripotent cells. Nevertheless, it is interesting to stress that in the remodelling muscles no conspicuous signs of apoptosis (i.e. nuclei with abnormally condensed chromatin) have generally been noticed at the transmission electron microscope (TEM) level (Sugni, Ferrario, Bonasoro and Candia Carnevali personal observations).

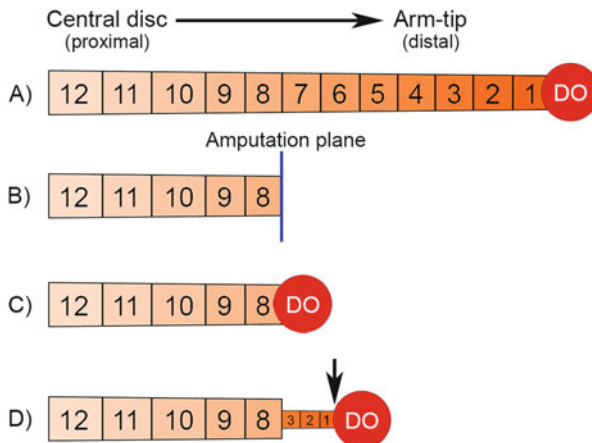
In the present chapter, the remodelling phenomena of injured tissues (i.e. directly involved in the amputation), which normally undergo histolytic processes, are not considered.

In regeneration muscle remodelling is remarkably relevant in asteroids (Ben Khadra et al. 2017). By contrast, in ophiuroids and crinoids, this phenomenon is generally of minor importance, and usually only a limited cell turnover can be observed at the periphery of the muscle bundles (Candia Carnevali and Bonasoro 2001; Biressi et al. 2010). In crinoids, however, myocyte remodelling remarkably increases under stress conditions, such as following exposure to contaminants or in arm explants (Bonasoro et al. 1998; Candia Carnevali et al. 2001a, b, 2003; Sugni et al. 2007). In all the three echinoderm classes, muscle remodelling often occurs *via* a more or less evident distal-proximal gradient (i.e. muscle bundles or tube feet muscle layers closer to the amputation plane are the most remodelled; Ben Khadra et al. 2015a). This fact might indicate the presence of a gradient of triggering molecules “released” at the wound site (such as inflammatory-like molecules) that diffuse along the arm.

The phenomena of tissue rearrangement can involve also the extracellular matrix and other cytotypes, i.e. fibroblasts, scleroblasts, cells of the coelomic epithelium or, in asteroid arm explants, even cells of the digestive apparatus (i.e. pyloric caeca) (Ben Khadra et al. 2015a, 2017), thus highlighting the plasticity of echinoderm tissues and the ability of these animals to use different sources of materials or cells according to the specific conditions and needs. However, as for myocytes, the final fate of these dedifferentiating cells remains unclear. The absence of specific cell tracking systems makes impossible to definitively demonstrate if they are actually employed as a secondary indirect source of raw materials for the production of new cells or as primary direct sources of undifferentiated/pluripotent cells (or perhaps, a combination of both). Regardless of this conundrum, the uncommon plasticity and “recycling” ability of echinoderm adult cells are certainly one of the “secrets” of their amazing regenerative abilities with the possible existence of “cell rejuvenation processes” in these animals opening fascinating perspectives for translation to the fields of ageing and human regeneration.

### 14.3.4 Distalization-Intercalary Regeneration

To overcome the ambiguity and difficulties of classifying a regenerative process as epimorphic or morphallactic, in 2003 Agata and co-workers (Agata et al. 2003, 2007) proposed a different but unifying principle to approach regeneration in different animal models, such as planarians, cnidarians (*Hydra*) and amphibian urodeles. This view was articulated as the distalization-intercalary regeneration model. According to this model, the most distal structure is formed first, and this acts as a signalling centre for organizing and inducing the intercalation of new elements between the stump and the distal element itself. This model has been recently successfully applied also to echinoderm arm regeneration (Fig. 14.8; Hotchkiss 2009; Ben Khadra et al. 2015b, 2017; Czarkwiani et al. 2016), although differences in the modelled structures are obvious in the three classes. For example, in asteroids, the distal element is the terminal tube foot with its associated ossicle, which are highly differentiated structures. In crinoids this is actually the blastema itself, which is an undifferentiated tissue, as it is the case of planarians (Agata et al. 2003). Ophiuroids represent a peculiar situation since the terminal structures (i.e. terminal tube foot and associated ossicle) are morphologically well recognizable only at late stages of regeneration, but once differentiated they likely act as the



**Fig. 14.8** Schematic representation of distalization-intercalary regeneration model. *Numbers* refers to both position and differentiation level of the segments: the higher, the more differentiated (and thus older). (A) Each arm is normally provided with a terminal structure (the distal organizer, DO), which can be either a well-differentiated (terminal tube foot/ossicle in asteroids) or an undifferentiated (blastema in crinoids) tissue; a proximal-distal morphogenetic potential gradient is present along the whole arm, the segment closest to the central disc (most proximal) being the older/more differentiated, the more distal the younger/less differentiated. (B) Arm amputation abruptly interrupts the gradient. (C) The first structure to be regenerated and differentiated is the terminal element (DO). (D) New segments are progressively intercalated between the DO and the stump, following a proximal-distal gradient until the original full structure (and gradient) is re-established. The distal regrowth area is indicated by an *arrow*



asteroid's counterpart (Czarkwiani et al. 2016). In both classes the actual growth zone is therefore located proximal to the terminal structure (Ben Khadra et al. 2015b; Czarkwiani et al. 2016).

An alternative and unifying hypothesis is that the true distal organizer in all three echinoderm classes is the anatomical region (not specifically definable) where the tips of the axial structures (i.e. coelomic cavities and nerve) meet, which is actually the growth proper zone. This might be the real key area and a structure controlling the whole arm regeneration process, where the new structures originate.

Noteworthy, in the animal models used by Agata et al. (2003), the distal element is usually a transient structure that disappears once regeneration is accomplished. Differently, in echinoderms, the distal organizer is a permanent structure that remains active throughout life, in agreement with the continuous arm growth. In this sense echinoderm regeneration can be seen as a regulative phenomenon, which reproduces, in accelerated form, the usually slow but continuous growth of the arms.

Some exceptions to this model can be found in specific conditions, as in crinoid unconventional amputations (i.e. not on the natural autotomy site; Candia Carnevali and Bonasoro 1995) or in asteroid arm explants (Ben Khadra et al. 2017). In the former, a delayed repair phase, due to an amputation far from the natural arm autotomic plane or due to a basal arm mutilation and subsequent exceptional extent of the wound, may produce abnormal regenerates. In this case, only the distal part is well differentiated, whereas the intermediate part presents evident anomalies (Candia Carnevali and Bonasoro 1995). In asteroid double arm explants (i.e. the intermediate part of an arm originated from a proximal and a distal cut), the distal side regenerates following the distalization-intercalary model (although much delayed if compared to normal arm-tip regeneration). In contrast, at the proximal side only distalization is observed, i.e. only the distal elements, the terminal tube foot and ossicle, is regenerated, but no new segment is intercalated between the latter and the stump (Valoti et al. 2016).

### ***14.3.5 Coelom and Nervous Tissue as the Key Players of Echinoderm Regeneration***

#### **14.3.5.1 The Coelomic Epithelium as Organogenetic Tissue**

The coelomic epithelium (also referred to as “peritoneum”) is considered a unique tissue for its histogenetic potential (Rieger and Lombardi 1987). In vertebrate embryogenesis this is a highly active cell layer, which is locally able to supply mesenchymal cells that contribute to the mesodermal elements of many organs, such as the heart, liver, lungs, gonads and more, and provide essential signals necessary for the development of other cells, tissues and organs (Ariza et al. 2016). From a cellular point of view, cell recruitment occurs by epithelial-mesenchymal transition (EMT). During this process, the epithelial cells lose their polarity as well as their cell junctions and migrate into the underlying connective tissue (mesenchyme) to become mesenchymal cells, i.e. “free” undifferentiated or multipotent cells. The EMT is a key event during both

echinoderm embryo development (Wu et al. 2007) and adult regeneration (Ben Khadra et al. 2017; Guatelli 2017). Indeed, the coelomic epithelium has been proposed to act as an “ancient multifunctional organogenetic tissue” also in the regenerative development of different echinoderm classes (Dolmatov 1993; Candia Carnevali et al. 1995a, b; García-Arrarás et al. 2011). A significant role of the coelomic structures was originally suggested by pilot studies carried out during the last century on ophiuroids (Dawydoff 1901). In recent times this idea was re-proposed for all the stellate echinoderms (Candia Carnevali and Bonasoro 2001; Bannister et al. 2008; Biressi et al. 2010; Ben Khadra et al. 2017; Piovani 2017). Most of these studies indicate that in echinoderms, the coelom is a fundamental cell supplier during regeneration (see Sect. 14.3.3) with the somatocoel being apparently the primary source of cells in all the three classes (Candia Carnevali et al. 1993; Biressi et al. 2010; Ben Khadra et al. 2017), although a contribution of the radial water canal has also been suggested (Bannister et al. 2008). Although cell tracking experiment is still necessary, in both asteroids (Ben Khadra et al. 2017; Guatelli 2017) and ophiuroids (Piovani 2017), EMT has been proposed as a specific mechanism recruiting cells from the coelomic epithelium to the surrounding mesenchymal tissue, similar to what has been described in holothurian visceral regeneration (García-Arrarás et al. 2011) and vertebrate embryogenesis (Ariza et al. 2016). Thus, it is reasonable to consider the adult echinoderm coelomic epithelium as an extremely plastic tissue, which is highly specialized and differentiated in normal physiological conditions but that can be turned into a “multipotent cell supplier” upon the reception of specific signals and under stress conditions, such as those generated by traumatic amputation.

#### 14.3.5.2 Nervous System as Coordinator

The key role of the nervous system during the regenerative process is well documented in all three echinoderm classes (Huet 1975; Huet and Franquinet 1981; Walsh et al. 1986; Moss and Thorndyke 1994; Moss et al. 1998; Candia Carnevali et al. 2001c; Thorndyke and Candia Carnevali 2001; Sugni et al. 2010) and other animal models (Dinsmore and Mescher 1998; Tanaka and Ferretti 2010; Kumar and Brockes 2012). In echinoderms, the nervous system acts as a promoter/coordinator of the overall regenerative process, mainly contributing to the release of regulatory factors. These factors have been identified by both qualitative and quantitative methods (immunocytochemistry and histofluorescence, biochemical assays and molecular techniques), which have revealed the presence of at least three different classes of regulatory molecules involved to some extent in regeneration: neurotransmitters, particularly monoamines, such as dopamine and serotonin; neuropeptides, such as substance P, SALMF amide 1 (S1), SALMF amide 2 (S2); or nerve-derived growth factors, such as TGF- $\beta$  and related peptides (BMP), NGF and FGF-2 (Candia Carnevali et al. 1996; Thorndyke and Candia Carnevali 2001; Patruno et al. 2002, 2003).

In asteroids, the “exposed” anatomical position of the RNC, which is facing the external environment (Fig. 14.2; Smith 1937), allows to perform ablation

experiments, i.e. partial removal of the nerve cord. When this has been done during arm-tip regeneration experiments, the regrowth process of the arm-tip has been markedly impaired, although not completely prevented (Moss et al. 1998). In crinoids, the “silencing” of nervous activity by pharmacological treatment causes a decrease in the proliferative activity of the regenerate, which in turn ultimately results in a reduced regrowth (Sugni et al. 2010). Eventhough the specific molecular pathways involved are still unknown, these different evidences underline the shared nerve dependence of arm regeneration in the different echinoderm classes.

It is important to recall that the nervous system includes not only the anatomically well-defined component of the brachial nerve but also the diffuse conspicuous components of the basiepithelial plexuses, and the subepithelial plexus associated to the coelom (Harrison and Chia 1994). This means that the basic “pilot role” played by the nervous system in regeneration would really imply a significant contribution from the coelomic plexus. In other words, the prompt and effective contribution of coelomic cells that can be observed in all the echinoderm regeneration models studied so far (see above), in terms of migration, proliferation and differentiation, could be a phenomenon regulated by the local release of neural factors from the different nerve components.

In crinoids the nervous system seems also to exert a specific direct induction, as far as the organization/differentiation of other tissues is concerned, an effect that is particularly evident in the muscle-skeletal components (Candia Carnevali et al. 1989, 1999).

In summary, in the paragraphs above we discussed how stellate echinoderms display an extraordinary variety of regenerative mechanisms, in terms of cells and tissues involved. This diversity (Table 14.1) can be ascribed to diverse factors, including evolutionary, ecological and individual (physiological) aspects, and thus they can provide a valuable perspective on how the same process (regeneration) can be performed by different routes.

**Table 14.1** Summary table of the main differences among the stellate echinoderm regenerative process described in the previous paragraphs, focusing on a cell/tissue perspective

Event/mechanism/process	Crinoids	Ophiuroids	Asteroids
Transient phagocyte syncytium			x
Granulation tissue-like formation		x	x
Presence of a true localized blastema	x		
Terminal tube foot and ossicle as distal structures		x	x
Differential regeneration of nervous system components		x	x
Maintained regeneration polarity in arm explants	x	x	
Stem cell recruitment	Massive	Limited	Limited
Adult cell dedifferentiation	Reduced	Massive	Massive
Prevalent regenerative mechanism	Epimorphosis	Morphallaxis	Morphallaxis

## 14.4 Arm Regeneration: The Molecular Perspective

Though the basic morphological aspects of echinoderm regeneration are relatively well known (see the above sections), the paucity of data on the regulatory mechanisms involved in this developmental process is hampering our progress, underlying the necessity of improving our analysis of the molecular aspects of the process and the incorporation of new technologies that allow us to interfere with it. In the next sections, we describe the knowledge we have gathered over the last years on this subject.

### 14.4.1 Crinoids

In spite of the striking regenerative potential that crinoids display, most of the analyses on this process have been done at the cellular level, and the molecular studies are still limited. The first molecular study on crinoid regeneration was carried out in 1996 and aimed at investigating the potential role of the nervous system in regeneration by testing the specific involvement of neural factors (the monoamine neurotransmitters dopamine and serotonin) in arm regeneration of *Antedon mediterranea* (Candia Carnevali et al. 1996). The distribution pattern of these molecules at standard regenerative stages was compared with that of normal non-regenerating arms. Immunocytochemical (ICC) examination showed that both dopamine and serotonin dramatically change in both their distribution and concentration during the repair and regenerative processes, indicating that they could be important neural (perhaps growth-promoting) factors used in crinoid arm regeneration (Candia Carnevali et al. 1996). Other than that, the control and the initiation of the regenerative response are not mediated only by neurotransmitters. Other different regulatory molecules are probably involved. So far, only one family of factors native to crinoids has been identified in regenerating tissues: the members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) group of signalling molecules. Secreted proteins of the TGF- $\beta$  superfamily are dimeric cytokines involved in fundamental aspects of wound healing and regeneration in vertebrates (Lévesque et al. 2007). The expression of TGF- $\beta$ -like molecule(s), detected using an anti-human TGF- $\beta$ 1 antibody, is significantly upregulated at the level of the regenerating brachial nerve in the apical blastema of *A. mediterranea* (Patruno et al. 2002). In the early stages of regeneration, localization of TGF- $\beta$ 1 is detected in several migratory amoebocytes close to the amputation surface, whereas the TGF- $\beta$  type II receptor is detected in migrating amoebocytes in the brachial nerve (Patruno et al. 2002). These data indicate that a TGF- $\beta$ -like molecule (or molecules) is normally present in the adult nervous tissues of *A. mediterranea* and is significantly upregulated during regeneration. Later on, Patruno et al. (2003) reported the sequence of another identified native member of TGF- $\beta$  superfamily from *Antedon bifida*, belonging to the bone morphogenetic proteins (BMPs) subfamily (named *Anbmp2/4*). BMPs are a group of multifunctional growth factors involved in embryonic and regenerative development (Chen et al. 2004). They demonstrated that *Anbmp2/4* is actively involved in the early stages of blastemal

regeneration, at a time when fundamental patterns are being established. The authors speculated that *Anbmp2/4* could be involved in the specification of migratory stem cells derived from the coelomic epithelium (Patruno et al. 2003).

To check the origin of stem cells, different “stemness” (or stem-related) genes, such as *vasa* and *piwi* (*unpublished*), plus some regulatory genes involved in anterior-posterior patterning of embryos, for example, *otx* and *pax6*, were identified in *Oxycomanthus japonicus* embryos and adult tissues (Kondo and Akasaka 2010). The detailed analysis of these genes during regeneration may clarify the identity and fate of stem cells in these animals. Moreover, and in order to promote studies of crinoids at the molecular level, this research group constructed cDNA libraries of embryos and adult tissues from *O. japonicas* cultures, which bring the system to the state of becoming a “model” for studying regeneration in crinoids, as it is in the previously described *A. mediterranea*.

Unfortunately, to date, only these above-mentioned molecules have been investigated in crinoid regeneration. This fact stresses the urgency of performing molecular studies on the regulatory mechanisms that control regenerative processes in this class of echinoderms.

#### 14.4.2 Asteroids

Although different studies have been carried out with the aim of determining the detailed morphological aspects of asteroid regeneration (reviewed in Ben Khadra et al. 2017), there is still, as stated, a considerable gap in the knowledge of the molecular aspects regulating the process. Some studies were conducted attempting the identification and cloning of some genes involved in regeneration. These molecules were mainly identified in the context of asteroid larval regeneration. The first characterized molecule was the so-called asteroid regeneration-associated protease (*srap*) in the asteroid *Luidia foliolata* during its larval regeneration (Vickery et al. 2001). Following a similar approach, Oulhen et al. (2016) identified a *srap* gene and two other genes, *vasa* and *vitellogenin* relatives, involved in the regeneration of *Patiria miniata* bipinnaria larvae. These approaches did not involve an exhaustive characterization of the gene function; however, the authors speculated that the *srap* gene might have a role in wound repair and apoptosis.

Some other studies aimed at the characterization of a small set of regulatory molecules/genes involved in adult asteroid regeneration. These studies characterized some members of families with known roles in regulating key aspects of embryonic and post-embryonic development, such as *homeobox*-containing and *wingless* genes. Ben Khadra et al. (2014) used a PCR screening protocol to identify different *Hox* and *ParaHox* genes putatively involved in arm regeneration of *E. sepositus* and *A. rubens*. Based on comparisons with vertebrate and *Strongylocentrotus purpuratus* *Hox/Hox*-related fragments and with the use of phylogenetic analysis, eight distinct *Hox* genes expressed in regenerating *E. sepositus* and six in regenerating *A. rubens* were characterized, in addition to four putative homologues of the vertebrate genes

*Xlox* in *E. sepositus*, *Mox* in *A. rubens* and *Gbx* in both species. All *Hox* fragments showed the clearest affinity with deuterostome genes (the so-called anterior, medial and posterior groups) (Ben Khadra et al. 2014). Among all the characterized *Hox* and *ParaHox* genes identified by Ben Khadra et al. (2014), only the expression of *Hox1* has been analysed during asteroid regeneration by using a species-specific antibody. Thorndyke et al. (2001) showed that *Hox1* is expressed at low levels in normal radial nerve cord of *A. rubens*, and then it is upregulated during its regeneration. The authors speculated that the expression of *Hox1* seen in whole-mount ICC could interfere with the dedifferentiation and/or cell proliferation process during regeneration (a hypothesis never formally investigated).

Ben Khadra and co-workers (*unpublished*) characterized members of the *wingless/Wnt* family genes present in regenerating tissue of *E. sepositus*. *Wnts* are a family of secreted glycoproteins with significant roles in many biological processes. Beside the crucial role of *Wnt* genes during early embryonic developmental processes, it has been demonstrated that *Wnt* signalling is also required for adult tissue regeneration in different species (Wodarz and Nusse 1998; Niehrs 2001; Veeman et al. 2003). Ben Khadra and co-workers identified specifically two *Wnt* genes expressed in the *E. sepositus* regenerating arms, the orthologous of *Wnt1* and *Wnt4* genes, named here, respectively, *EsWnt1* and *EsWnt4*. The expression profiles of both genes at different stages of this starfish arm regeneration were studied by RT-PCR. The expression profiles of *EsWnt1* and *EsWnt4* genes showed a variation in different stages of regeneration, with the highest expression detected at 24 and at 72 hours post-amputation. These findings suggest that these two genes are probably involved in the wound healing process. In fact, it is known that the activation of the *Wnt* signalling pathway is an early response to trauma in different species (Whyte et al. 2012). In starfishes this initial phase is followed by a late expression at 4 and 7 weeks post-amputation where the expression of *EsWnt1* and *EsWnt4* coincides with the morphogenesis of some missing tissues, such as skeletal elements and muscles. These preliminary investigations need further experiments to validate the specific roles played by each gene in specific processes of *E. sepositus* regeneration.

In a recent study (Ferrario et al. 2018), the expression patterns of two genes were described during the repair phase of the asteroid *E. sepositus*. A fibrinogen-like gene (*Ese-fib-like*) was detected in the regenerating epidermis and coelomic epithelium, suggesting an involvement of these tissues in the post-traumatic inflammatory response. Indeed, fibrinogen is a well-known molecule involved in vertebrate haemostasis following injury (Laurens et al. 2006). The expression of a prolyl 4-hydroxylase gene (*Ese-p4h*), a key enzyme for collagen biosynthesis, in the regenerating epidermis suggested that this tissue is involved in collagen deposition during the repair phase, when the oedematous area is formed (Ben Khadra et al. 2017).

An alternative study of the regenerative process has been done in asteroids using proteomic tools. By means of these technologies, *Wnt* (and other) proteins belonging to this signalling pathway were identified in the regenerates. This finding is in line with the suggestion that *Wnt* signalling controls stem cell operation and provides the positional information necessary for shaping the regenerating tissues (Clevers et al. 2014). Proteins of this pathway have been clearly identified in the regenerating RNC

of the starfish *M. glacialis* (Franco et al. 2011a). A preliminary study performed on this species showed significant differences between the coelomocytes' proteomes in regenerating animals and those of the wild type animals (*unpublished data*). This finding suggests that the coelomocytes are key participants of the regenerative process in line with what has been observed at the cellular level. Indeed, the proteome characterization of coelomocytes during regeneration in starfishes showed the up- and downregulation of the heat shock proteins (Hsp), which are key regulators of cell homeostasis. In a different species, *A. rubens*, an increase in Hsp72 levels was observed in coelomocytes after 24 hours post-amputation (Holm et al. 2008) and in the RNC until 6 weeks post-amputation (when compared to the control, non-amputated, specimens, which showed no changes of Hsp70 levels (Patruno et al. 2001)). Several other proteins are specifically involved in neurogenesis during regeneration. For instance, a total of 528 proteins were detected having significant variations in their expression during starfish neural regeneration in vivo using difference gel electrophoresis (2D-DIGE) (Franco et al. 2014). The authors suggested that these molecules might modulate the regenerative pathways leading to successful nervous system regeneration in the starfish (reviewed in Ben Khadra et al. 2017). It is important that proteomic characterization of the RNC of an asteroid model revealed a close similarity between echinoderm and mammal central nervous system physiology, in particular their synaptic communication components (Franco et al. 2011a). A more extensive analysis is needed to understand the basic mechanisms that regulate the regeneration of asteroid tissues and how these different proteins act during this process. The future use of similar technologies in members of other asteroid clades should provide us with insights on the commonalities and differences between the regenerative processes in the Asteroidea and beyond.

In parallel to the mentioned proteomic studies, other global (high-throughput) approaches, using the analysis of transcriptomes and/or genomes, are starting to be used. These approaches give us an unprecedented view at biological processes, such as regeneration, and how their regulation is effected at the transcriptional level in other asteroids (Gabre et al. 2015; Semmens et al. 2016; Hall et al. 2017).

Even though starfish regeneration started to attract researchers' attention long time ago, there is still a big gap which needs to be urgently filled in order to understand how the independently gathered cellular and the molecular data fit together.

### 14.4.3 Ophiuroids

Among the stellate classes, the Ophiuroidea are the best studied at molecular level for their ecological and regenerative importance. In recent years, with the rising of easy and low-cost next-generation sequencing (NGS) approaches, there has been an emergence of various molecular resources and genome projects (summarized in Table 14.2) that greatly helped and will continue to help filling the gap of molecular mechanisms underlying echinoderm regeneration. Several studies have been done

**Table 14.2** Ophiuroidea genomic and transcriptomic resources publicly available

Species	Genome (G)/ transcriptome (T)	NCBI	URL
<i>Ophiothrix spiculata</i>	G	PRJNA182997	<a href="http://www.echinobase.org/Echinobase/OsAbout">http://www.echinobase.org/Echinobase/OsAbout</a>
<i>Astrophyton muricatum</i>	Adult body T	PRJNA299886	<a href="http://echinodb.uncc.edu/">http://echinodb.uncc.edu/</a>
<i>Ophioderma brevispina</i>	Adult body T	PRJNA299887	<a href="http://echinodb.uncc.edu/">http://echinodb.uncc.edu/</a>
<i>Amphiura filiformis</i>	Embryo and adult T	PRJNA349786; PRJNA256912; PRJNA256029; PRJNA244369; PRJNA192896	<a href="http://www.echinonet.eu/blast/">http://www.echinonet.eu/blast/</a>
<i>Ophionereis fasciata</i>	G	GCA_900067615.1	<a href="http://ryanlab.whitney.ufl.edu/genomes/Ofas/">http://ryanlab.whitney.ufl.edu/genomes/Ofas/</a>
<i>Ophionotus victoriae</i>	Adult body T	PRJNA273557; PRJNA387478	British Antarctic Survey
<i>Ophioderma longicaudum</i>	Adult body T	PRJNA360063	University of Basel
<i>Ophiopsila arana</i>	Adult body T	PRJNA266533	University of Mons
<i>Ophiolimna perfida</i>	Adult body transcriptome	SRX1625120	Museum Victoria
<i>Ophiocoma wendtii</i>	Adult body transcriptome	SRX1625119 & PRJNA299897	Museum Victoria
<i>Ophioleuce brevispinum</i>	Adult body transcriptome	SRX1625118	Museum Victoria
<i>Ophiomusium lymani</i>	Adult body transcriptome	SRX1625117	Museum Victoria
<i>Ophioceres bispinosus</i>	Adult body transcriptome	SRX1625116	Museum Victoria
<i>Amphiophiura laudata</i>	Adult body transcriptome	SRX1625115	Museum Victoria
<i>Ophiactis resiliens</i>	Adult body transcriptome	SRX1625114	Museum Victoria
<i>Ophiophragmus wurdemanii</i>	Adult body transcriptome	SRX1625113	Museum Victoria
<i>Ophiothrix caespitosa</i>	Adult body transcriptome	SRX1625112	Museum Victoria
<i>Ophiopeza cylindrica</i>	Adult body transcriptome	SRX1625111	Museum Victoria
<i>Ophiophrura liodisca</i>	Adult body transcriptome	SRX1625110	Museum Victoria
<i>Ophiophycis johni</i>	Adult body transcriptome	SRX1625109	Museum Victoria

(continued)



**Table 14.2** (continued)

Species	Genome (G)/ transcriptome (T)	NCBI	URL
<i>Ophiologimus prolifer</i>	Adult body transcriptome	SRX1625108	Museum Victoria
<i>Gorgonocephalus pustulatum</i>	Adult body transcriptome	SRX1625107	Museum Victoria
<i>Ophiolepis impressa</i>	Adult body transcriptome	SRX1625106	Museum Victoria
<i>Clarkcoma canaliculata</i>	Adult body transcriptome	SRX1625105	Museum Victoria
<i>Ophiura sp. MVF193435</i>	Adult body transcriptome	SRX1625104	Museum Victoria
<i>Bathypectinura heros</i>	Adult body transcriptome	SRX1625103	Museum Victoria
<i>Ophiernus vallincola</i>	Adult body transcriptome	SRX1625102	Museum Victoria
<i>Ophiotreta eximia</i>	Adult body transcriptome	SRX1625101	Museum Victoria
<i>Ophiomoeris obstricta</i>	Adult body transcriptome	SRX1625100	Museum Victoria
<i>Asteroschema bidwillae</i>	Adult body transcriptome	SRX1625099	Museum Victoria
<i>Ophiomyxa australis</i>	Adult body transcriptome	SRX1625098	Museum Victoria
<i>Asteronyx loveni</i>	Adult body transcriptome	SRX1625097	Museum Victoria
<i>Ophionereis schayeri</i>	Adult body transcriptome	SRX1625096	Museum Victoria
<i>Amphiura constricta</i>	Adult body transcriptome	SRX1625095	Museum Victoria
<i>Ophiactis abyssicola</i>	Adult body transcriptome	SRX1625094	Museum Victoria
<i>Leptosynapta tenuis</i>	Adult body transcriptome	SRX1625093	Museum Victoria
<i>Ophiothrix angulata</i>	Adult body transcriptome	SRX1625092	Museum Victoria
<i>Aporometra wilsoni</i>	Adult body transcriptome	SRX1625091	Museum Victoria
<i>Luidia senegalensis</i>	Adult body transcriptome	SRX1625090	Museum Victoria

on single-gene expression and large gene expression datasets of ophiuroids, including transcriptomic and proteomic approaches; however, here we will focus only on those relevant to the regenerative processes.

Genes encoding two proteins of the family of bone morphogenetic factors (BMPs) have been isolated and studied in the context of *A. filiformis* arm

regeneration. *AfiUni*, closely related to the sea urchin *Univin* gene, and *AfiBmp2/4* have their expression detectable only in advanced regenerative phase (3 and 2 weeks post-amputation, respectively) in cells with a migratory morphology within the radial water canal (RWC) of the regenerates (Bannister et al. 2005, 2008). The authors have interpreted this as a supporting evidence for the RWC as being an important source of cells in *A. filiformis* regeneration. Furthermore, *AfiUni* expression is localized in two regions of the regenerates: a proximal region and the most distal tip. This led the authors to suggest a dual role of *AfiUni*: in segmentation of the regenerating arms and the subsequent growth and patterning of the individual segments. Noteworthy, this expression pattern perfectly fits with the distalization-intercalary model described above, where the most distal tip and the proximal segments are those with a higher differentiation level (Czarkwiani et al. 2016). The *AfiBmp2/4* late expression is in contrast with the early expression observed in crinoids (see above), thus suggesting a large plasticity of the regulatory programme of echinoderm regeneration.

Czarkwiani et al. (2013, 2016) conducted studies on skeletal regeneration and identified dynamic patterns of expression of genes encoding transcription factors (*AfiAlx1*, *AfiEts1/2*, *AfiFoxB* and *AfiGataC*) and terminal differentiation genes (*AfiP19*, *AfiP58b*, *AfiClectin* and *Afiacoll*). Strong upregulation of RNA levels of the transcription factor genes at early stage of regeneration, just before the skeletal primordia appears, has been shown by quantitative analysis. In situ hybridizations revealed expression of these genes in discrete inner structures of the early regenerates, with exception of *AfiFoxB*, which was expressed in the epidermal layer. These data identify the presence of different cell types organized in various structures already in early regenerative stages of *A. filiformis*, further supporting the absence of a “true” blastema in this species. Later in advanced phases of regeneration the same transcription factor genes mark distinct mineralized tissues in proximal position: *AfiAlx1* is specifically expressed in spines and tube feet, *AfiEts1/2* is highly transcribed in cells that coincide with the shape and position of the vertebrae, and *AfiGataC* is primarily localized in the lateral shields. Interestingly, all these genes are co-expressed in an area just under the distal tip of the late regenerates (50% regeneration) that corresponds to the highly proliferative zone that adds new segments to the regenerating arm (see Fig. 14.8; Czarkwiani et al. 2016). In contrast, the differentiation genes (*AfiP19*, *AfiP58b*, *AfiClectin*) are all identified in the dermal layer of regenerates in the advanced regenerative phase (stage 3–5 in Czarkwiani et al. 2016) and later in various skeletal elements.

An alternative strategy to characterize regulatory factors involved in regeneration was the use of classical PCR screens. Using the similar approach that was used in asteroids, eight fragments encoding *homeobox* transcription factors (five *Hox* and three *Hox*-related family) have been isolated from cDNA of regenerating tips of *A. filiformis* arms (Ben Khadra et al. 2014). They correspond to the anterior, medial and posterior groups of *Hox* genes, plus some *ParaHox* genes and a *Rough* orthologue. The presence of a complex set of regulatory genes of the body plan in the regenerating arm of *A. filiformis* suggests their role in echinoderm regenerative

processes, although more needs to be done to better characterize their expression domains and, thus, their precise roles.

A “blind” approach has also been used in the context of ophiuroid regeneration, and this implies the characterization of transcriptomes. Recently, ophiuroids’ molecular studies have been extended to large gene expression dataset with the aim to identify the whole complement of genes differentially regulated in different phases of the regenerative process and to understand the molecular basis of ophiuroid regeneration. An initial set of studies using a 9216 cDNA clones array showed that roughly 50% of the genes have a differential expression sometime during the *A. filiformis* regenerative process (Burns et al. 2011). Interestingly, in the early phase of regeneration a large set of genes associated with cell proliferation has been detected as well as the absence of TGF- $\beta$  family genes, whereas in advanced phases a clear upregulation of *BMP-1*, consistent with previous studies, has been reported. Furthermore, to address the molecular regulation of explant polarity, using a similar experimental set-up, genes differentially expressed in various body parts of 7 days post-amputation explants have been identified and compared with normal regenerating arms (Burns et al. 2012). Of particular interest are the signalling genes of the *Notch*- and *Frizzled*-related families, which are differentially upregulated in the distal part of the explant, suggesting involvement of *Wnts* and *Notch* signalling in establishing the intrinsic polarity of the explant. These genes may have a role also in regulating the regenerative proximal-distal axis. Furthermore, a very similar molecular make-up of the explant and normal regenerative part has been established, making the *A. filiformis* explants a very powerful tool for dissecting the molecular mechanisms underpinning echinoderm regeneration.

A combined proteomic and transcriptomic study on wound healing phases (1, 2 and 3 days post-amputation) of *A. filiformis* regeneration identified several hundred genes up- or downregulated in injured arms, among them a sharp downregulation of the signalling gene *TGF- $\beta$ 2* and the muscle gene myosin heavy chain. At the protein expression level, the biggest difference was seen in the regulation of the translation and integrin-mediated adhesion (Purushothaman et al. 2015). These data provide a molecular support to the observed remodelling of muscle cells and the cell migratory events described in the early phases of regeneration.

The molecular studies in *A. filiformis* provide a strong basis to understand the genetic control of Ophiuroidea regeneration; however, many questions remain open on the specific role of regulatory genes as well as signalling molecules involved in the explant polarity, in the initiation and progression of the regenerative process and in the specification of various cell types. Finally, the molecular studies should be coupled with cellular data to have a complete understanding of the origin of cells in the regenerate and the morphogenesis of the newly formed tissues to ensure a complete re-establishment of a functional arm.

## 14.5 Conclusions

Like in many other organisms, in echinoderms the ability to regenerate ultimately depends on the capacity to access a source of stem cells and/or to reprogramme differentiated cells (Galliot and Ghila 2010). Because the arm regenerative phenomena extensively involve the key contribution of progenitor cells present in the tissues, the obvious crucial and still unanswered issues pertaining to the regeneration research (Dunn et al. 2013) are (1) stemness properties of responsible cells, in terms of origin and derivation (stem cells or reprogrammed cells), (2) activities (proliferation and/or migration) and (3) plasticity and differentiation potential (final cellular phenotypes). All these cellular mechanisms are mediated by specific genetic pathway(s). However, very little is known about the origin/fate of cells contributing to the regenerated tissues, or the molecular regulators of the process, for almost any echinoderm, this being a paradox for a phylum well known for its striking regenerative abilities. Thus, the need of filling the gap of knowledge in our understanding of regeneration in stellate echinoderms and, by extension, in all echinoderms is more and more evident. Such future projects will build on the thorough knowledge of the arrangement of cells in the regenerating arms, but it will take a completely different set of approaches (i.e. electroporation, gene mapping, CRISPR-Cas9, cell tracking, knock-out/knock-in experiments) to understand how these cells are specified during development and how their developmental programmes are regulated by genes or gene batteries.

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# Chapter 15

## Solitary Ascidians as Model Organisms in Regenerative Biology Studies



Tal Gordon and Noa Shenkar

**Abstract** Regeneration, the process of replacing lost or damaged body parts, has long captured human imagination and is a key feature among all animal phyla. Due to their close phylogenetic relationship to vertebrates and their high regenerative abilities, ascidians (Chordata, Ascidiacea) are often used as models to shed light on the cellular and genetic process involved in tissue regeneration. Surprisingly, ascidian regeneration studies are based on only a few model species. In this chapter, we point out the important potential of solitary ascidians in regenerative and stem cell studies. We review recent studies of regeneration among solitary ascidians and discuss the cellular mechanism of tissue regeneration and the possible involvement of circulating cells in these processes. New data regarding the relationship between age and regeneration abilities of the solitary ascidian *Polycarpa mytiligera* (Stolidobranchia, Styelidae) are presented. The unique regeneration abilities found in *P. mytiligera* following evisceration of its digestive system and following amputation of its neural complex and siphon-associated structures and nerves imply on its potential to serve as a novel model system for understanding tissue regeneration.

**Keywords** Regeneration · Ascidians · Evisceration · Neural complex · *Polycarpa mytiligera*

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## 15.1 Introduction

The ability to regenerate lost or damaged body parts has long captured human imagination. While a variety of different organisms are capable of regenerating organs and even an entire body, mammals possess limited regeneration abilities (Poss 2010; Giangrande and Licciano 2014). Our desire to understand and eventually control and manipulate regeneration process had led scientists from around the world to study regeneration in a variety of model systems (Sanchez Alvarado and Tsonis 2006; Gurley and Sanchez-Alvarado 2008). We find evidence for regeneration studies as early as the eighteenth century, when Abraham Trembley first discovered the ability of the hydra to completely regenerate its head and foot after their amputation (Lenhoff and Lenhoff 1986). Today we know that regeneration is widely distributed among the animal kingdom and includes very diverse organisms and distant evolutionary taxa (Bely and Nyberg 2010). The ability to regenerate all body parts (whole-body regeneration) is mostly studied in basal metazoan such as the diploblast hydra, which is able to completely regenerate from isolated fragment of epithelial cells (Noda 1971; Gierer et al. 1972; Technau et al. 2000), and in the triploblastic freshwater planarians, which can regenerate all body parts due to pluripotent stem cells distributed throughout their body (Agata and Watanabe 1999). Among annelids, some species successfully regenerate heads and tails, while other groups, such as leeches, show no such abilities (Bely 2006). In the chordates, some amphibians such as the newts and salamanders are able to regenerate part of their body such as limbs, tail, and jaws (Goss 1969; Tsonis 2000), while birds and mammals appear to have lost the ability to regenerate nearly all structures (Carlson 2007).

Despite the extensive regeneration studies done in the past 200 years and the possible implications of a breakthrough in this field in terms of regenerative medicine and stem cell biology, there is still a big gap in knowledge regarding the cellular and molecular mechanisms involved in this phenomenon (Sanchez Alvarado 2000). One possible explanation is the limited number of model organisms and restricted experimental approach used in regeneration studies (Sanchez Alvarado 2000; Gurley and Sanchez Alvarado 2008; Grillo et al. 2016; Reddien and Tanaka 2016). As Sanchez-Alvarado and Tsonis (2006) pointed out, significant progress was achieved in regenerative biology by using new model organisms and the application of genetic tools in traditional models of regeneration. The evolutionary basis of regeneration is also yet to be understood (Goss 1992; Sanchez Alvarado 2000; Brockes et al. 2001), and the question as why some animals regenerate and others do not remains unsolved.

Being part of the chordates, ascidians comprise the closest living relatives of vertebrates (Delsuc et al. 2006, 2008). They include colonial species that reproduce both sexually and asexually and solitary species known to reproduce only sexually. Ascidians have powerful regeneration abilities. Colonial species are able to regenerate a whole zooid (an individual unit in the colony) even from small fragments or blood cells (Cohen and Berrill 1936; Voskoboynik et al. 2007; Kawamura et al.

2008; Brown et al. 2009). Solitary ascidians can regenerate their neural complex, peripheral structures such as the siphons (Hirschler 1914; Dahlberg et al. 2009; Jeffery 2015a, b) and internal organs such as the digestive system (Selys-Longchamps 1915; Shenkar and Gordon 2015). Solitary ascidians also show a negative correlation between regeneration rate and the animal age, as regeneration abilities decline in old individuals (Jeffery 2012; Dahlberg et al. 2009).

With their high regenerative abilities, variable lifestyle (solitary and colonial), and close phylogenetic relationship with the vertebrates, ascidians could serve as a useful and interesting model system for regenerative, aging, and evolutionary studies (Dahlberg et al. 2009; Jeffery 2015a, b, c).

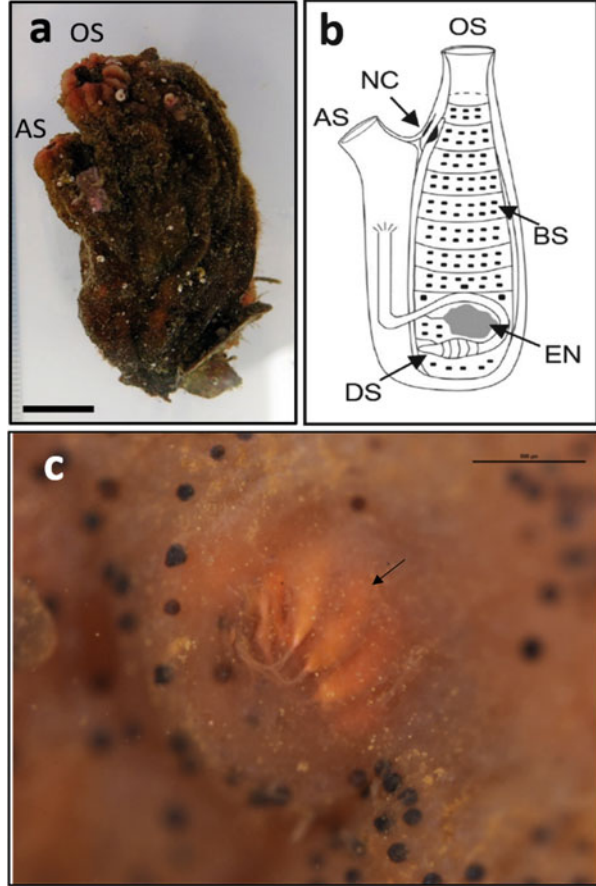
Here, we review recent studies of regeneration among solitary ascidians. Due to its extraordinary regenerative properties (Gordon 2016; Gordon and Shenkar unpublished), we use the solitary species *Polycarpa mytiligera* as a model and present new data regarding the relationship between these species regeneration abilities and age.

### 15.1.1 Background

The class Ascidiacea belongs in the phylum Chordata and subphylum Tunicata. The name “tunicate,” first set by Lamarck in 1816, originates from the polysaccharide-cellulose tunic that forms the flexible exoskeleton of these marine invertebrates (Lambert 2005). Ascidiaceans, solitary and colonial, comprise approximately 3000 species found in all marine habitats (Monniot et al. 1991; Shenkar and Swalla 2011). Adult ascidiaceans show little resemblance to typical chordates, while the tadpole-like larvae bear the four fundamental characteristics of the phylum: a dorsal tubular nerve cord, notochord, rudimentary pharyngeal gill slits, and post-anal tail. Another important character is the presence of the endostyle, an elongate tube-shaped organ, running along the ventral side of the branchial sac and evolving as the thyroid gland in vertebrates (Fujita and Nanba 1971). During the metamorphosis stage, the larvae lose all of these features except for the endostyle and the gill slit rudiments in the pharynx (Millar 1971), which become functional and develop to form the branchial sac. All ascidiaceans, colonial and solitary, share a similar basic body plan. The adult body consists of a hollow sac open to the outer environment through two siphons (Fig. 15.1a, b). The branchial sac is used to capture food particles (Pestarino et al. 1988) and for respiration (Fisher 1976). Food particles are later transported to the stomach for digestion (Monniot et al. 1991). The viscera include the stomach, intestine, and heart (Fig. 15.1a). The neural complex (NC), which consists of a single ganglion (brain) with an associated glandular organ (neural gland), lies in the apex between the two siphons (Mackie and Burighel 2005; Dahlberg et al. 2009) (Fig. 15.1a).

Ascidians have the most powerful capacities for regeneration within the chordate phylum (Berrill 1951). These regeneration abilities appear only at the adult stage, following metamorphosis, due to the mosaic development of ascidian embryos

**Fig. 15.1** *Polycarpa mytiligera*. (a) A single individual showing the oral siphon (OS) and atrial siphon (AS). (b) A scheme showing the oral siphon (OS), atrial siphon (AS), neural complex (NC), endocarp (EN), and digestive system (DS) (Modified from Mackie and Burighel (2005), © 2008 Canadian Science Publishing. Reproduced with permission). (c) A gonad embedded in the body wall, indicated by arrow. Scale bars, (a) 1 cm and (c) 1000  $\mu$ m



(Sato 1994). Colonial ascidians received most attention by the scientific community due to their remarkable regenerative plasticity. Their regeneration properties and modes of asexual reproduction have been well studied in a variety of species (Kawamura et al. 2008; Tiozzo et al. 2008; Voskoboinik et al. 2008; Brown et al. 2009). Conversely, solitary ascidian regenerative abilities have only been studied so far in a few species (Medina et al. 2015; Jeffery 2015a). Recent studies revealed unique regenerative capabilities in *P. mytiligera* (Gordon 2016; Gordon and Shenkar unpublished) further highlighting the potential in using solitary species as new model systems in the field of regeneration biology.

### 15.1.2 *Polycarpa mytiligera*

The genus *Polycarpa* (order, Stolidobranchia; family, Styelidae) includes 139 solitary species (Shenkar et al. 2017). The principal characteristics of *Polycarpa* are the shape and multiplicity of the gonads (named “polycarps” as they are short with very short ducts) (Fig. 15.1c). Most often the gonads are either arranged in rows or randomly scattered over the body wall. They may be entirely embedded in the body wall or projecting partly or completely into the peribranchial cavity (Monniot et al. 1991). Some species of the genus *Polycarpa* are oviparous, while others are viviparous (Millar 1962; Chen and Dai 1998). In some species, the larval phase is skipped altogether, and development is direct (Kott 1985). *Polycarpa* can be found either as isolated individuals or in large populations of tightly packed bodies (Monniot et al. 1991).

The solitary ascidian *P. mytiligera* (Fig. 15.1a) is one of the most common solitary ascidians in the Gulf of Eilat, Red Sea (Koplovitz and Shenkar 2014). Our recent studies showed that this species has extraordinary regenerative abilities with regard to peripheral organs and neural complex (Gordon 2016; Gordon and Shenkar unpublished). *P. mytiligera* also possesses a unique ability to regenerate internal organs following evisceration (Selys-Longchamps 1915; Shenkar and Gordon 2015), a feature that has not been documented to date in any other ascidian genus.

## 15.2 Solitary Ascidians as Model System for Regenerative Studies

Among solitary ascidians, *Ciona intestinalis* (Phlebobranchia, Cionidae) is commonly used as a model system for regeneration studies (Jeffery 2015a). The ability of this species to regenerate removed distal body structures, such as siphons, sensory organ, and neural complex, had been well studied throughout the past century (reviewed by Jeffery 2015a). More recently, a variety of molecular and genetic tools are being developed for studying its regenerative capacity (Hamada et al. 2015; Jeffery 2015b, c; Yoshida et al. 2017).

A fundamental question in regenerative studies is the identification of the cellular origins of the renewed tissues (Poss 2010; Tanaka and Reddien 2011). The cellular source for regenerating tissue could involve pluripotent stem cells (i.e., cells that are capable of differentiation into diverse cell types) as in the case of the planaria (Wagner et al. 2011) or tissue-specific stem cells (i.e., cells that may generate different cell types depending on their tissue of origin) as found in the axolotl limb regeneration (Bryder et al. 2006; Kragl et al. 2009). Another option is the dedifferentiation of fully differentiated cells within the local tissue into a blastema (i.e., a mass of undifferentiated progenitor cells). Blastema formation was documented in vertebrate limb regeneration (Brockes and Kumar 2005; McCusker et al. 2015). Finally, cells involved in the regeneration may originate from transdifferentiation

(i.e., transformation of defined cells into another cell type) of differentiated cells into the missing cells of the damaged tissue. In the newts, for example, the lens regeneration was shown to involve transdifferentiation, as the new lens formed from the dorsal pigmented iris (Eguchi et al. 1974).

Jeffery (2015c) reviewed several possible origins for adult stem cells in *C. intestinalis* and included the following sources: (1) stomach and intestinal epithelium, (2) basal stalk or stolon (the structure that attaches the body to the substrate), (3) lymph nodes in the transverse vessels of several structures, and (4) siphon walls.

To determine the cellular source of the regenerating organs in *C. intestinalis*, several methods were applied, including labeling with a cell proliferation marker (EdU) and labeling with pluripotent stem cell markers alkaline phosphatase and PIWI (an Argonaute protein family expressed in germ cells and stem cells of diverse organisms) (Cox et al. 1998; Cox et al. 2000; Auger et al. 2010; Jeffery 2015b, c). These studies suggested that the source of progenitor cells involved in *C. intestinalis* oral siphon regeneration varies according to the amputation treatment. A siphon tip regeneration was found to involve precursors located in the siphon itself, resulting in a short-distance regeneration process, whereas regeneration of a larger part of the siphon, close to its base, involved both short- and long-distance processes, including adult stem cell located in lymph nodes lining the transverse vessels of the branchial sac (Auger et al. 2010; Jeffery 2015b, c). These cells initiated proliferation in response to injuries and invaded the wounded areas, which was followed by the formation of sensory organs and muscle fibers in the regenerated siphon (Jeffery 2015b).

*C. intestinalis* was also suggested as a model for central nervous system regeneration. The live imaging and use of transgenic animals to track nerve regeneration gave a new insight into *C. intestinalis* neural complex regeneration (Dahlberg et al. 2009). It was suggested that a blastema, formed at the edge of the regenerating nerve ending following amputation, is the source of the cells involved in regeneration of the neural complex. However, the cellular source of the blastema remains unknown (Dahlberg et al. 2009).

The solitary ascidian *Styela plicata* (Stolidobranchia, Styelidae) was also used for studying neural complex regeneration. Medina et al. (2015) developed a new method for studying ascidian's regeneration, using niacinamide antagonist 3-acetylpyridine (3AP). This neurotoxin was used to investigate the degeneration and regeneration of the cerebral ganglion complex. The 3AP produced lesions in *S. plicata* neural complex and caused reduction of glial cells and neurons. This method resulted in a faster regeneration process compared to that observed in *C. intestinalis* when total ablation of the neural complex was performed (Dahlberg et al. 2009). During *S. plicata* regeneration process, recruitment of blood stem cells to the lesion site was observed, implying a possible stem cell source (Medina et al. 2015).

Recent studies revealed that the solitary ascidian *Polycarpa mytiligera* has high regeneration abilities in comparison to other species (Gordon 2016; Gordon and Shenkar unpublished). This species is able to regenerate peripheral organs, including the siphons and neural complex. In addition, it survives and regenerates massive



amputation of over 50% of its body resulting in a complete regeneration of both amputated parts (anterior and posterior) into two separated complete individuals. The ability of this species to regenerate the posterior part of its body from a residual anterior part is exceptional among solitary ascidians. *C. intestinalis*, the most studied solitary ascidian for regeneration studies (Dahlberg et al. 2009; Jeffery 2015a), was only able to regenerate an amputated anterior part from a posterior part but unable to regenerate a posterior part (Mingazzini 1891; Hirschler 1914; Jeffery 2015a, b).

*C. intestinalis* regeneration of the anterior parts from a small posterior part occurred only when it contained a piece of the branchial sac or the epicardium, a cavity containing blood cells (Hirschler 1914; Jeffery 2015a). Furthermore, a recent study on *C. intestinalis* has shown that when the animals were trisected by two cuts along the anterior-posterior axis, producing anterior, middle, and posterior portions, the middle part, which contains the branchial sac complex, was able to regenerate anterior organs but unable to regenerate a complete posterior portion containing the digestive system (Jeffery 2015b). It is possible that the unique ability of *P. mytiligera* to regenerate eviscerated internal organs (Selys-Longchamps 1915; Shenkar and Gordon 2015) holds the key to its ability to regenerate posterior organs from an anterior section of the body.

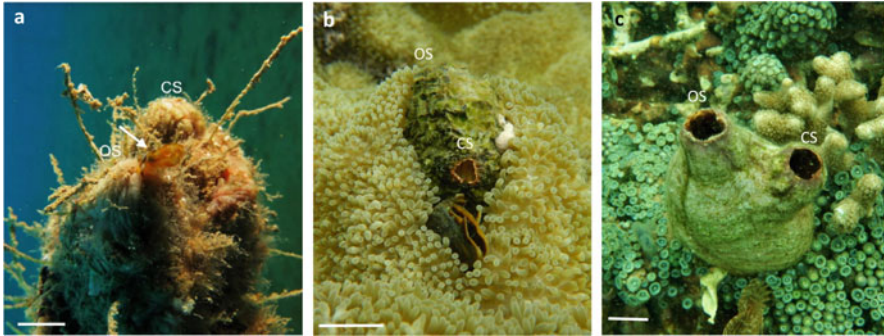
*P. mytiligera*'s branchial sac entirely overlays the internal organs, as in all members of the Stolidobranch order, in contrast to the *C. intestinalis* (Phlebobranch) in which the internal organs lie beneath the branchial sac. It is therefore possible that the cells participating in the regeneration process in *P. mytiligera* might have originated from the branchial sac and endostyle remains found in both parts of the ascidian's amputated body, allowing it to regenerate anterior as well as posterior parts.

Previous studies indicated that circulating cells are also involved in budding and regeneration of colonial species (Freeman 1964; Sugino et al. 2007; Brown et al. 2009; Kawamura and Sunanaga 2010; Gutierrez and Brown 2017). Brown et al. (2009) proposed that multiple stem cell types circulating within *Botrylloides violaceus* vasculature systems were involved in whole-body regeneration. Voskoboynik et al. (2008) identified the endostyle as a stem cell niche for adult stem cells participating in regeneration. These cells can migrate out of the endostyle and contribute to developing tissues within the colony.

As shown above, the source of cells can vary from one model species to another. The involvement of circulating cell in the regeneration of colonial and solitary ascidians implies that a population of stem cell within the circulating system may contribute to the regeneration of different organs and tissues.

### 15.3 Regeneration of Internal Organ

Evisceration, which could be considered as a variation of autotomy, is the ejection of internal organs commonly known among holothurians (Mashanov and García-Arrarás 2011). These echinoderms regularly discard all or most of their internal



**Fig. 15.2** *Polycarpa* species. (a) *P. mytiligera* eviscerate in the field, arrow pointing to the gut expelled through the oral siphon. From Shenkar and Gordon (2015). (b) *P. insulsa*. (c) *P. spongiabilis*. Scale bar, 1 cm. OS oral siphon, CS cloacal siphon

organs in response to an external provocation (Emson and Wilkie 1980) or on a seasonal basis (Swan 1961). The eviscerated organs are then completely regenerated. Studies investigating the nature of the cells recruited to repair injury showed that the regeneration of the gut lumen involved epithelial morphogenesis either by the expansion of the stump epithelium or by transdifferentiation of the mesothelium (García-Arrarás et al. 1998; Shukalyuk and Dolmatov 2001). Mashanov et al. (2005) showed that in the holothurian *Eupentacta fraudatrix*, the regeneration of the digestive track involved transdifferentiation of epithelial cells.

Among ascidians, evisceration of internal organs and their complete regeneration from the atrial epithelium were first described in the genus *Polycarpa* (Selys-Longchamps 1915). This phenomenon was only anecdotally mentioned by taxonomists in the following decades and was not fully investigated (Tokiooka 1970; Kott 1985; Monniot et al. 1991). A recent study (Shenkar and Gordon 2015) has shown that *P. mytiligera* has the ability to eviscerate and regenerate internal organs as a result of mechanical pressure (Fig. 15.2a). Following evisceration, the digestive system was completely regenerated within 12 days and the injured branchial sac in 19 days. The ability to eviscerate was reported exclusively in *Polycarpa* and has not been documented in any other ascidian genera. However, not all of *Polycarpa* species are able to perform evisceration. Evisceration was described in only 10 *Polycarpa* species out of 139 known species (Shenkar and Gordon 2015; Shenkar et al. 2017). Attempt to induce evisceration by applying mechanical pressure according to previous study (Shenkar and Gordon 2015) was performed on over 20 individuals of *P. spongiabilis* and *P. insulsa* (Fig. 15.2b, c), all of which failed (Gordon T. personal observation).

The cellular processes involved in *P. mytiligera* regeneration of internal organs are yet to be studied in depth. However, the regeneration of the digestive system is well studied in colonial species. In the colonial ascidian *Polyandrocarpa misakiensis*, regeneration of the gut was found to involve transdifferentiation of somatic cells (Kaneko et al. 2010). *P. misakiensis* was able to regenerate the

esophagus, stomach, and intestine following their amputation, by transdifferentiation of the atrial epithelium near the cut surface. The same study also found that retinoic acid is required for the regeneration of the gut as it acts as a trigger for the transdifferentiation of the atrial epithelium into the gut (Kaneko et al. 2010).

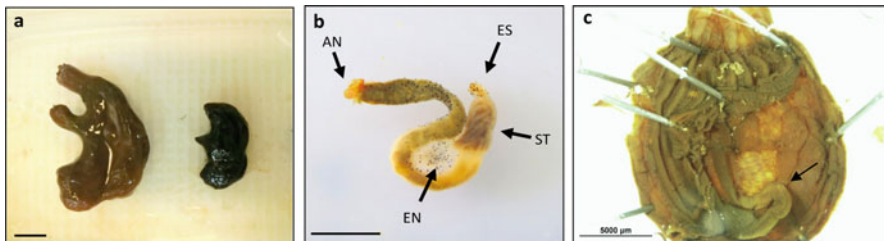
### 15.3.1 Regeneration and Aging

Regenerative capacity changes during development and through the organism life stages. In zebrafish, for example, a decline in telomerase expression, telomere length, and regeneration capacity was observed in adult animals (Anchelin et al. 2011). In *Caenorhabditis elegans*, axon regeneration also declines during adulthood (Wu et al. 2007).

A similar correlation between age and the rate of regeneration was found in ascidians (Dahlberg et al. 2009; Jeffery 2012). In *C. intestinalis*, the regeneration of the neural complex and the oral siphon was affected by aging. *C. intestinalis* individuals presented different regeneration capacities at different life stages (Auger et al. 2010; Jeffery 2012, 2014, 2015b). During the larval stage, the developed larvae lacked the ability to regenerate due to the highly determinative development (Jeffery 2001). Following metamorphosis, individuals showed high regenerative abilities (Carnevali and Burighel 2010), while adult animals, close to the end of their life span, showed a significant decline in regenerative capacities (Jeffery 2012).

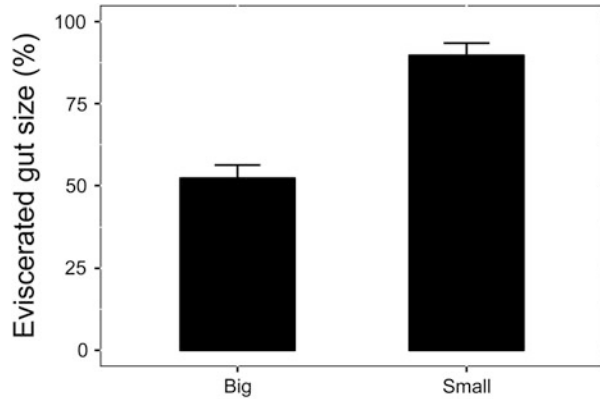
Following the documentation of evisceration in *P. mytiligera*, the relationship between age and the ability to eviscerate and regenerate internal organs was investigated in individuals of two size classes: Class 1, tagged as “Big” (3–5 cm), and Class 2, tagged as “Small” (1–2 cm) (Fig. 15.3a). Body length was used as an estimation for age (Dahlberg et al. 2009; Jeffery 2012).

These experiments showed that small individuals eviscerated a larger portion of their digestive tract (Fig. 15.3b, c) in comparison to big individuals (Fig. 15.4). In

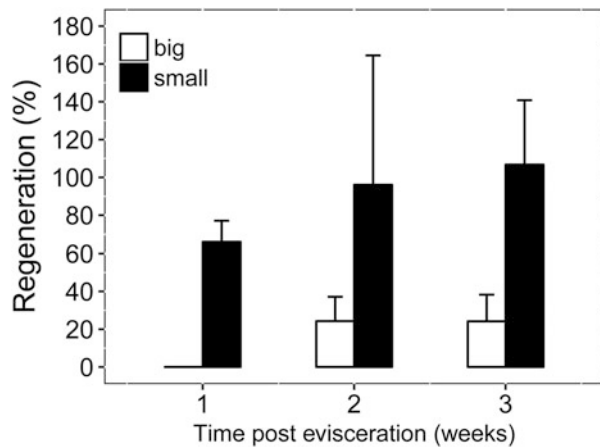


**Fig. 15.3** *P. mytiligera*. (a) Example of the two studied size classes, “Big” (3–5 cm) on the left side and “Small” ( $\leq 3$  cm) on the right. Scale bar, 1 cm, tunic removed. (b) Discarded digestive tract. Arrows showing the anus (AN), endocarp (EN), stomach (ST), and esophagus (ES). Scale bar, 0.5 cm. (c) Dissected specimen with regenerated digestive tract (arrow). Scale bar, 0.5 cm

**Fig. 15.4** Average size ( $\pm$ SE) of eviscerated guts (percentage) according to size groups: Big ( $>3$  cm,  $n = 22$ ) and Small ( $<3$  cm,  $n = 17$ )



**Fig. 15.5** Average proportion ( $\pm$ SE) of regenerated gut in relation to the eviscerated gut (percentage) of following 3-week intervals, post-evisceration in Big ( $>3$  cm,  $n = 7$ , white bars) and Small ( $<3$  cm,  $n = 9$ , black bars) individuals of *P. mytiligera*



addition, larger individuals regenerated slower than smaller individuals. While small individuals almost completely regenerated their ruptured branchial sac and most of their digestive system after a period of 1 week, the larger individuals only started to regenerate their wounded organs in the second week (Fig. 15.5).

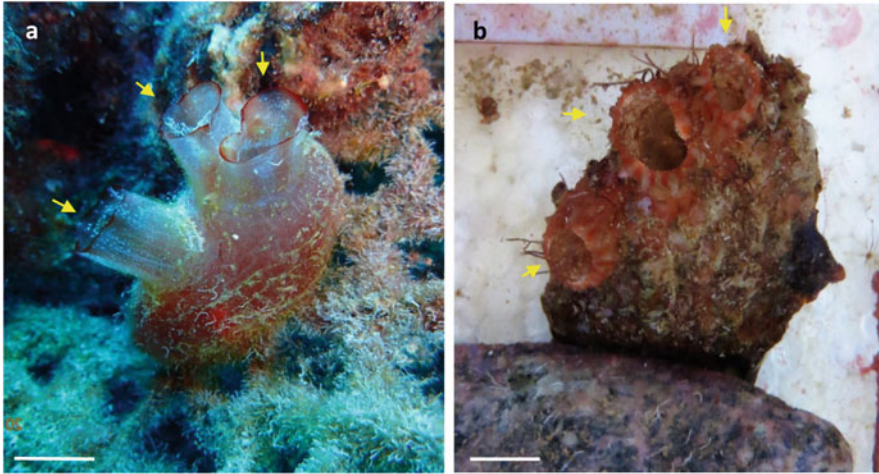
Studies on the effect of repeated amputation treatments on regeneration capacity were performed in invertebrate and vertebrate model systems. A study on caudal fin regeneration found that the regenerative abilities were not affected by recurrent fin amputations in zebrafish (Azevedo et al. 2011). Repeated amputation and regeneration of the flat worm *Macrostomum lignano* showed no effect on its regenerative abilities or on its stem cell pool (Egger et al. 2006). A long-term lens regeneration project showed that neither time nor repetition had effect on the regeneration efficiency of newt (Eguchi et al. 2011). In *P. mytiligera*, only small individuals were able to undergo a second and third evisceration. However, while dissecting the big individuals, it seemed that they had started the evisceration process but did not complete it by ejecting the digestive system. Small individuals that were able to

perform a second evisceration after a 3-week period were able to go through the third evisceration as well.

## 15.4 Ecological Significance of Regeneration

As mentioned earlier, the regeneration is highly variable among the different phyla. One interesting and frequently asked question in regeneration studies refers to the mechanism involved in the maintenance and loss of regeneration capacity (Goss 1992; Bely 2010; Bely and Nyberg 2010; Giangrande and Licciano 2014). Although no single answer had been widely accepted, several authors proposed that regeneration is an ancestral trait that has been maintained along evolution due to selection (Goss 1992; Bely 2010; Bely and Nyberg 2010) or phylogenetic inertia (Bely and Nyberg 2010). Animals that undergo asexual reproduction usually possess high regenerative ability, such as whole-body regeneration, in comparison to species that reproduce only sexually (Technau et al. 2000; Bely 2006; Voskoboynik et al. 2007; Brown et al. 2009). Sánchez Alvarado (2000) suggested that the regenerative cascade of events following injury might have originally developed as a reproductive strategy, later used as a survival mechanism. Ascidian regenerative abilities are highly advanced among asexually reproducing colonial species. Solitary ascidians reproduce only sexually and yet can regenerate peripheral structure, internal organs, and the neural complex. Why and how are these abilities maintained? One of the hypotheses suggested that the answer might relate to the ecological advantage of regeneration (Bely and Nyberg 2010). As tunicates are preyed by a variety of predators (Gulliksen and Skjæveland 1973; Dalby 1989; Pérez-Portela and Turon 2007), it is possible that sublethal predation is responsible for maintaining of regeneration (Lindsay 2010). *P. mytiligera* is among the most common species in the Gulf of Eilat (Koplovitz and Shenkar 2014), and yet no evidence for injured individuals has been documented in nature. However, solitary ascidians, including *P. mytiligera*, were occasionally found in the wild to have three siphons, usually one oral and two atrial (Fig. 15.6a). As suggested for the solitary ascidians *Styela clava* (Stolidobranchia, Styliidae) from Vancouver Island, siphon duplication in stolidobranch ascidians is most likely a result of regeneration following injury (Epelbaum et al. 2009). In addition, an experimental amputation of *P. mytiligera* siphons and neural complex resulted in the regeneration of three siphons (Fig. 15.6b). Similar results were obtained in *C. intestinalis* when individuals showed the regeneration of a new siphon as the result of an injury to the existing siphon (Mingazzini 1891; Von Haffner 1933; Jeffery 2015a). The presence of three individual siphons in nature could imply a predation activity, which might be responsible for the maintaining of these organisms' regeneration abilities. The ability to eviscerate and regenerate internal organs in *P. mytiligera* was also suggested to be a survival strategy against predation (Shenkar and Gordon 2015).

Another hypothesis for the maintaining of regeneration in *P. mytiligera* could be its phylogenetic position. According to Bely and Nyberg (2010), some organisms'



**Fig. 15.6** A three-siphoned ascidians. **(a)** *Herdmania momus* on artificial substrate, Red Sea. **(b)** *Polycarpa mytiligera* following neural complex amputation treatment. Arrows indicate the siphons. Scale bar, 1 cm. Photo: **(a)** H. Dror and **(b)** T. Gordon

regenerative abilities are ancestral trait that bear no advantage today and are only maintained due to historical reasons. It is therefore possible that the close phylogenetic position of *Polycarpa* to colonial species among the Styliidae (Pérez-Portela et al. 2009; Shenkar et al. 2016) is responsible for *Polycarpa* advanced regeneration abilities.

## 15.5 Summary and Future Directions

The development and increasing accessibility of new genetic tools had led scientists to new insights into the pathways involved in regeneration (Chen and Poss 2017). The use of transgenic animals, genome-wide profiling methods, and new genome-editing technologies has uncovered factors and pathways involved in tissue regeneration (Dahlberg et al. 2009; Kragl et al. 2009; Fei et al. 2014). Studies on the cellular sources for regeneration, at cell-level resolution, are now being applied to model systems (Kragl et al. 2009; Tanaka 2016). There is great importance in applying those methods to a variety of model organisms in addition to the traditional and well-studied systems (Grillo et al. 2016). In this chapter, we have reviewed the regeneration abilities of solitary species focusing on the unique abilities of the solitary species *P. mytiligera*. This solitary ascidian was found to possess high regeneration capabilities of peripheral and internal organs (Shenkar and Gordon 2015; Gordon 2016; Gordon and Shenkar unpublished). Age was found to affect this species ability to eviscerate and regenerate internal organs, as older individuals eviscerated smaller portion of their digestive tract, regenerated slower, and lost

their ability for multiple evisceration acts. As the need for new model system rises, *P. mytiligera* has promising potential to serve as an important model system for regenerative studies and stem cell biology as well as studies in the field of aging and regeneration.

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# Chapter 16

## Whole-Body Regeneration in the Colonial Tunicate *Botrylloides leachii*



Simon Blanchoud, Buki Rinkevich, and Megan J. Wilson

**Abstract** The colonial marine invertebrate *Botrylloides leachii* belongs to the Tunicata subphylum, the closest invertebrate relatives to the vertebrate group and the only known class of chordates that can undergo whole-body regeneration (WBR). This dramatic developmental process allows a minute isolated fragment of *B. leachii*'s vascular system, or a colony excised of all adults, to restore a functional animal in as little as 10 days. In addition to this exceptional regenerative capacity, *B. leachii* can reproduce both sexually, through a tadpole larval stage, and asexually, through palleal budding. Thus, three alternative developmental strategies lead to the establishment of filter-feeding adults. Consequently, *B. leachii* is particularly well suited for comparative studies on regeneration and should provide novel insights into regenerative processes in chordates.

Here, after a short introduction on regeneration, we overview the biology of *B. leachii* as well as the current state of knowledge on WBR in this species and in related species of tunicates. Finally, we highlight the possible future directions that research might take in the study of WBR, including thoughts on technological approaches that appear most promising in this context. Overall, we provide a synthesis of the current knowledge on WBR in *B. leachii* to support research in this chordate species.

**Keywords** Whole-body regeneration · *Botrylloides leachii* · Chordate · Tunicate · Ascidian

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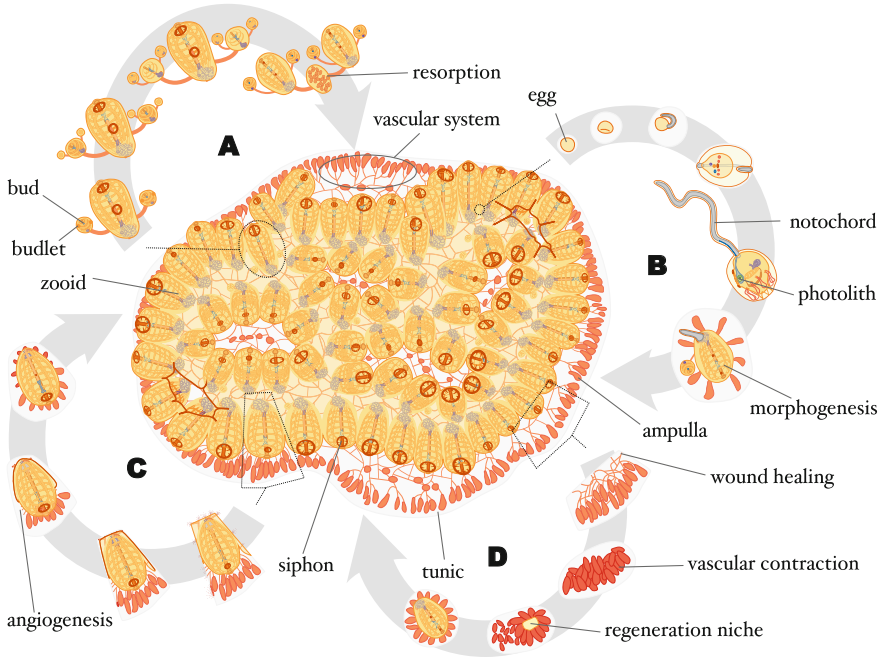
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## 16.1 Introduction

Tunicates are marine invertebrates considered to be the closest living invertebrate relatives to the vertebrate subphylum (Delsuc et al. 2006). Despite a drastically different body plan during their adult stage, the tunicate larvae possess typical chordate features, including a pharynx with gill slits, a notochord, a dorsal nerve cord and muscle segments (Millar 1971; Satoh 2003, 2011). Thus, the study of these animals may offer a unique evolutionary perspective into a wide range of biological processes conserved within the phylum Chordata. Colonial tunicates are also established models for other important biological processes including allrecognition, chimerism, ageing and angiogenesis (Rinkevich and Weissman 1987; Saito et al. 1994; Paz and Rinkevich 2002; Kürn et al. 2011; Gasparini et al. 2014; Voskoboynik and Weissman 2015; Kassmer et al. 2016). Of particular interest, these modular organisms are the only known chordates that can undergo whole-body regeneration (WBR), whereby a fully functional adult is restored from a minute portion of their vascular system containing as little as 100–200 circulating blood cells in a short period of about 10 days (Rinkevich et al. 1995, 2007b; Voskoboynik et al. 2007; Brown et al. 2009).

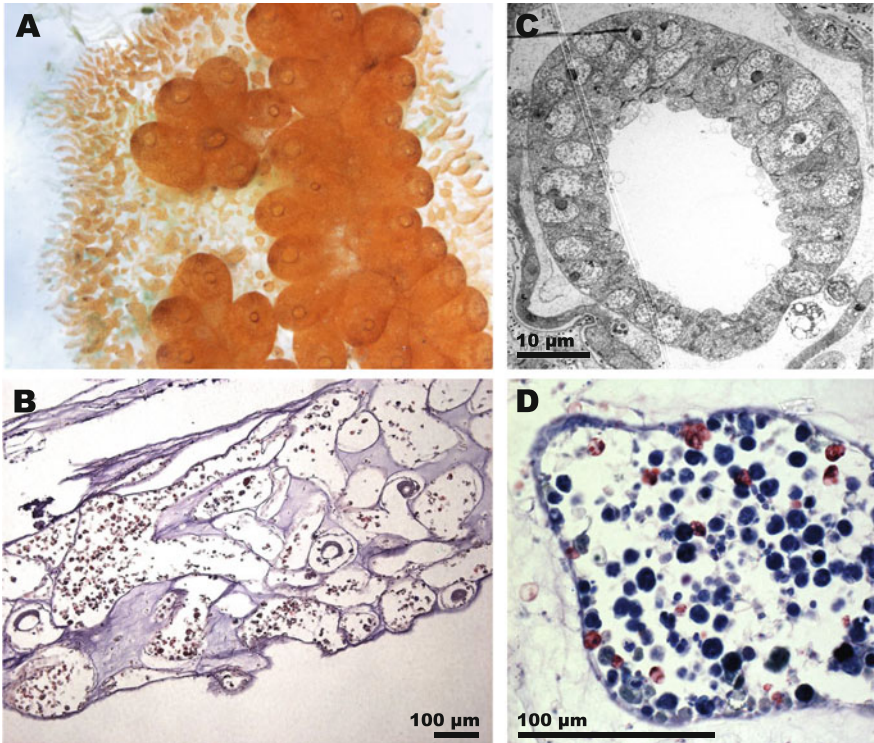
Multicellular animals capable of varying degrees of regeneration are distributed widely throughout the Metazoa, suggesting that this biological phenomenon has an ancestral origin (Sánchez Alvarado and Tsonis 2006; Bely and Nyberg 2010). However, regeneration ability inversely correlates with the tissue complexity of an organism (Sánchez Alvarado 2000). Among vertebrates, most species only present a limited regeneration capacity with few mammals, teleost fishes and urodele amphibians that can regenerate either body parts or organs following injury (Roy and Gatien 2008; Poss 2010; Seifert et al. 2012; Jaźwińska and Sallin 2016). Although the study of these organisms has brought an extensive knowledge on patterning and cellular programmes required for regeneration of severely injured organs, no vertebrate has yet been shown to undergo WBR. Therefore, deciphering the mechanisms underlying regeneration in the closely related Tunicata subphylum might provide new insights into shared principles of cellular plasticity in adult chordates. Consequently, colonial ascidians have emerged as model systems for the study of WBR and other regenerative processes in chordates (Tiozzo et al. 2008a; Kürn et al. 2011; Voskoboynik and Weissman 2015; Kassmer et al. 2016). Here we focus on WBR in *Botrylloides leachii* (class Ascidiacea, order Stolidobranchia, family Styelidae), a colonial sedentary marine chordate, first identified 200 years ago along the British coasts and since found worldwide (Savigny 1816; Michaelsen and Stephenson 1934; Brewin 1946; Brunetti 1976; Myers 1990; Tamilselvi and Sivakumar 2011).



**Fig. 16.1** *Botrylloides leachii* schematic physiology. Top view of a stereotypical colony composed of 72 zooids. (a) Blastogenic cycle of an adult zooid with two daughter buds and four budlets. (b) Viviparous sexual reproduction in *B. leachii* takes place inside brood pouches; larvae are released and undergo metamorphosis thereby initiating a new colony. (c) Healing of an injured adult zooid. (d) *B. leachii* WBR is initiated from an isolated fragment of vascular tissue

## 16.2 The Biology of *Botrylloides leachii*

*B. leachii* is a sessile suspension-feeding colonial ascidian that can commonly consist of hundreds of 2–3-mm-long adults, termed zooids, organized in a series of ladder-like parallel rows, known as systems (Figs. 16.1 and 16.2a; Savigny 1816). The colony remains attached to its substrate, typically on seagrass or rocks in the shallow subtidal zone, through a semi-transparent gelatinous matrix called tunic (Savigny 1816; Berrill 1947; Rinkevich et al. 1993). *B. leachii* zooids feed by filtering food particles from seawater using a thin sheet of mucus that covers their pharyngeal baskets (Millar 1971; Sawada et al. 2001). This mucus is produced by the endostyle, spread over the gill slits of the basket by ciliary movements and collected by the dorsal lamina, which eventually propels it into the stomach. Seawater is drawn through the oral siphons of the zooids, pushed through the gill slits by the contraction of the animal's body and finally expelled, along with any waste, through an excurrent siphon shared by the zooids within a system (Millar 1971; Sawada et al. 2001).



**Fig. 16.2** *B. leachii* WBR morphology. (a) Top view of a portion of a *Botrylloides leachii* colony settled on a glass slide. Zooids are arranged in ladder-like structures. Peripheral and central ampullae are clearly visible in the tunic matrix as they harbour orange pigment cells. The inhalant siphons of the zooids are open and delineated by darker pigment cells. (b) Four regeneration niches at different developmental stages colonize the contracted vascular system at WBR stage 3 (H&E stain). (c) Scanning electron microscope image of the first vesicular stage of WBR. (d) Macrophage-like cells (dark blue) compose the majority of the haemocytes in the vasculature at WBR stage 3 (Giemsa stain)

While each zooid has an independent heart and an open circulatory system, the entire colony shares a common vasculature that is embedded in the tunic and closed at its periphery by terminal ampullae, the contractile blind ends of marginal vessels (Fig. 16.1; Mukai et al. 1978). Originating from the ectoderm, this vasculature has been shown to resemble that of vertebrates and even respond to human factors that stimulate angiogenesis such as *vascular endothelial growth factor* (Gasparini et al. 2008, 2014; Tiozzo et al. 2008b). *B. leachii* blood (haemolymph) is mostly colourless and composed of nine cell types with functions incipient to that of vertebrate blood cells, which can be classified into five cell lineages (Cima et al. 2002; Blanchoud et al. 2017): undifferentiated cells (haemoblasts), immunocytes (hyaline amebocytes, macrophage-like cells, granular amebocytes, morula cells), mast cell-like cells (granular cells), transport cells (compartment amebocytes,

compartment cells) and storage cells (pigment cells, nephrocytes). It appears that there may be multiple niche sites for blood cell production, including cell clusters located near the endostyle (Voskoboynik et al. 2008; Brown et al. 2009; Rinkevich et al. 2013; Lauzon et al. 2013).

*B. leachii* zooids are viviparous hermaphrodites and can reproduce both sexually, to colonize new locations through a tadpole larval stage, and asexually, to expand the colony. This latter reproduction, also known as palleal budding or blastogenesis, is a cyclic phenomenon that starts through the consecutive budding and invagination of the outer epithelial mantle of a zooid (Fig. 16.1a; Berrill 1947). Typically, budding occurs on both sides of each adult within the colony and therefore produces two offspring per zooid. Each colony harbours at any time three generations of colonial modules: the zooids, the primary buds and the budlets (secondary buds, located on the primary bud). Blastogenesis can be classified into several developmental stages, culminating by the 'takeover' phase (lasting about 12 h) where all functional zooids in a colony are simultaneously resorbed by cellular waves of apoptosis and phagocytosis (Manni et al. 2007; Rinkevich et al. 2013; Gutierrez and Brown 2017). The primary palleal buds then mature into functional filter-feeding zooids to replace the previous generation, while budlets develop into primary buds that will themselves carry a new generation of budlets. The duration between two consecutive cycles of takeover depends on water temperatures but typically takes about a week at 18–20 °C (Berrill 1947).

In a sexually mature *Botrylloides* colony, which occurs upon favourable environmental conditions (Brunetti 1976; Rinkevich et al. 1993), male and female gonads are situated side by side in every single zooid (Berrill 1947; Millar 1971; Mukai 1977; Mukai et al. 1987). Both gonads require multiple blastogenic cycles to reach maturity (Kawamura et al. 2011). Ovulation takes place following the opening of the oral siphons during the takeover phase of blastogenesis (Zaniolo et al. 1994). Autogamy is avoided under natural conditions by a temporal delay between this release of the eggs and the discharge of sperm, thus providing time for external fertilization (Mukai et al. 1987). Fertilized eggs develop viviparously within brood pouches located inside the tunic in between zooids (Mukai 1977). The embryo then follows a stereotypical ascidian process of embryonic development (Mukai et al. 1987; Brown and Swalla 2012) whereby a motile larva is produced within a week and released to the environment. The tadpole eventually settles on an appropriate substrate and undergoes metamorphosis to give rise to a young adult (called an oozoid) that in turn will produce daughter zooids through blastogenesis (Fig. 16.1b; Berrill 1947).

To resist to adverse environmental conditions, colonial ascidians have rapid healing capacity in response to local injuries (Fig. 16.1c). For instance, bleeding from the vasculature stops in a few seconds from haemolymph aggregation and vessel contractions after lesion (Blanchoud et al. 2017); adults can heal in as little as 1 day from a localized surgical incision performed to access germ cells (Milkman 1967); and restoration of ablated vessels requires less than 2 days to complete (Tiozzo et al. 2008b). Albeit incompletely studied, *B. leachii* healing displays genomic markers similar to those observed in vertebrate wound healing and involves

an initial cellular response through the infiltration of cytotoxic cells followed by clearing of the debris by macrophage-like cells (Zondag et al. 2016; Blanchoud et al. 2017). On top of this healing mechanism, *B. leachii* colonies can undergo WBR from a minute fragment of their vascular tissue to compensate for the loss of all their zooids (Fig. 16.1d; Rinkevich et al. 1995).

### 16.3 Current State of Research on WBR in *B. leachii*

WBR in *Botrylloides leachii* was first observed by Rinkevich et al. who were also the first to coin the term WBR to this type of regeneration in colonial ascidians (Rinkevich et al. 1995). Using light microscopy and histological sections, Rinkevich and colleagues described the sequence of events leading to the regeneration of a fully function zooid from an isolated fragment of the vascular system. Importantly, they observed that WBR will only be triggered following the loss of all zooids from the colony and may be started from as little as a single ampulla containing as few as 100–200 blood cells in just 10 days (Rinkevich et al. 1995). One single zooid per colonial fragment will invariably regenerate despite the transient appearance of multiple regeneration niches, suggesting the existence of an uncharacterized competition mechanism. WBR has since then been first classified into three distinctive phases (dynamic reorganization in vessel architecture, initial stages of regeneration, the race for predominance; Rinkevich et al. 2007b) that were then further refined into the following six stages (Zondag et al. 2016):

#### **Stage 0, Injury of the Colony [0 Hour Post-ablation (hpa)]**

After the initial ablation, haemolymph loss is stopped in less than 30 s by a combination of tissue contraction and haemocyte aggregation. Flow will then restart after a pause shorter than 10 min, driven by spatially and temporally synchronized ampullar contractions (Rinkevich et al. 1995; Blanchoud et al. 2017).

#### **Stage 1, Healing of the Injury [15 hpa]**

Retinoic acid (RA)-expressing macrophage-like cells are observed adjacent to aggregates of small cytotoxic (morula) immune cells (Rinkevich et al. 2007b). RA signalling is essential for WBR as both its chemical and molecular disruptions inhibit regeneration (Rinkevich et al. 2007b). Morula cells massively infiltrate into the tunic matrix and an increase in the populations of haemoblasts takes place (Blanchoud et al. 2017). Differential gene expression analysis of the transcriptome shows upregulation of genes involved in cell-cell and cell-matrix adhesion, apoptotic and metabolic and cellular processes, as well as of developmental signalling pathways including WNT, TGF- $\beta$  and Notch (Zondag et al. 2016). These biological processes are suggestive of a global switch in gene expression for the activation of healing that includes RA signalling.



**Stage 2, Remodelling of the Vascular System [24 hpa]**

Vascular remodelling starts with a change in shape of the terminal ampullae, the contraction of the isolated vascular system and the creation of novel tunic vessels (Rinkevich et al. 1995, 2007b; Blanchoud et al. 2017). This remodelling is blocked by RA inhibition (Rinkevich et al. 2007b). Giant cells can be observed throughout the vascular system, and retinoic acid receptor (RAR) expression starts (Rinkevich et al. 2007b). So-called dormant cells, which line the internal epithelium of the blood vessels, express the evolutionarily conserved stemness marker *Piwi* and the cell proliferation indicator *proliferating cell nuclear antigen* (*PCNA*) (Rinkevich et al. 2010). Inhibition of *Piwi*, both chemically and using gene knockdown by RNA interference, prevents WBR but not the remodelling of the vascular tissue (Rinkevich et al. 2010, 2013). Macrophage-like cell conglomerates express a *trypsin-like serine protease* (*TrSP*) (Rinkevich et al. 2007a).

**Stage 3, Condensation of the Ampullae [72 hpa]**

The fully contracted vascular system is colonized by various regeneration niches, defined as spherical aggregates of cells within the vascular lumen (Fig. 16.2b; Rinkevich et al. 1995, 2007b; Blanchoud et al. 2017). These blastula-like structures vary in size (10–95  $\mu\text{m}$  in diameter), cell numbers (20–200) and sphericity (Fig. 16.2c; Rinkevich et al. 2007b). *Piwi*<sup>+</sup> cell numbers increase within lumens of blood vessels as regeneration proceeds, colonizing all regeneration niches and expressing PCNA (Rinkevich et al. 2010). Chemically blocking cell cycle entry using mitomycin C restricts the *Piwi*<sup>+</sup> to the internal epithelium, preventing their expansion in the vessel lumen (Rinkevich et al. 2010). *RAR* gene expression specifically demarcates all these regeneration sites (Rinkevich et al. 2007b). Additional supplementation of RA to regenerating fragments results in an accelerated regeneration and the restoration of multiple functional zooids (Rinkevich et al. 2007b). Analysis of differentially expressed sequence tags (ESTs) identifies an increase of immune-related transcripts, including proteases such as *TrSP* that is expressed in the regeneration niches (Rinkevich et al. 2007a). This EST screen also highlighted genes belonging to major signalling pathways, including Notch/Delta, JAK/STAT, protein kinases, nuclear receptors, Ras oncogene family members, G-protein-coupled receptor (GPCR) and transforming growth factor beta (TGF- $\beta$ ) (Rinkevich et al. 2008). Representative transcripts of these signalling pathways are expressed specifically during the early stages of regeneration in the conglomerates of round haemocytes within regeneration niches (Rinkevich et al. 2008). A large increase in phagocytic cells is also observed at this stage (Fig. 16.2d; Blanchoud et al. 2017).

**Stage 4, Establishment and Development of Regeneration Niches [144 hpa]**

*Piwi*<sup>+</sup> cell numbers consistently increase within the lumens of blood vessels where they proliferate until their later differentiation (Rinkevich et al. 2010). Regeneration niches develop by invagination of their vesicles' wall, which will develop into the various chambers and organs of the zooid (Rinkevich et al. 2007b). Representative transcripts of the major signalling pathways present during stage 3 disappear from developing buds, becoming restricted to incipient niches (Rinkevich et al. 2008).

Transcriptome analysis highlights the upregulation of pathways required for protein synthesis, biogenesis and the organization of cellular components (Zondag et al. 2016). Transport, mast cell-like and storage cells are at their lowest during this stage, while macrophage-like cells remain at a higher proportion than in uninjured colonies (Blanchoud et al. 2017).

### **Stage 5, Fully Regenerated Zooid [240 hpa]**

A single fully functional zooid is regenerated, irrespective of the original size and content of the isolated vascular system (Rinkevich et al. 1995, 2007b). This zooid is larger and rounder than usual, displaying the pallear buds expected from an uninjured adult but without any visible gametes (Rinkevich et al. 1995, 2007b; Blanchoud et al. 2017). Regular blastogenic cycles will ultimately result in generations of zooids that display sexual activities (B. Rinkevich, unpublished data).

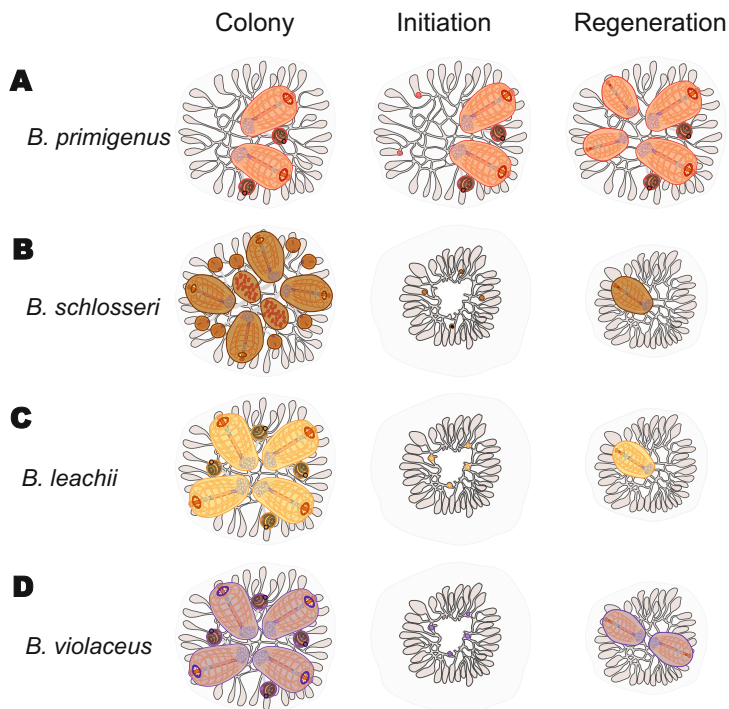
Overall, WBR in *B. leachii* has unique regeneration characteristics when compared to other representative models (Rinkevich et al. 2007b). These are highlighted by the presence of multiple initiation sites in regeneration niches, a systemic induction through retinoic acid, the participation of circulating haemocytes that restore both germ line and soma as well as the intra-organismic competition between regeneration niches that lead to a single survivor.

## **16.4 Inferences from WBR and Asexual Reproduction in Other Colonial Ascidi**

Although tunicates have been shown to undergo faster genomic evolution than vertebrates (Berna and Alvarez-Valin 2014), they have also been shown to share numerous conserved biological mechanisms. Consequently, data from other tunicates, and from related colonial ascidians in particular, could be helpful to draw pertinent inferences to design novel experiments for studying the mechanisms underlying WBR in *B. leachii*.

### **16.4.1 Differences in WBR Ability Between Colonial Ascidi**

Despite their similarities in morphology and life cycle, colonial ascidians present variations in their regenerative capacity, even within the genus *Botrylloides* (Fig. 16.3; Rinkevich et al. 1993). Indeed, *Botrylloides lenis*, *Botryllus delicatus* and *Botryllus primigenus* (Fig. 16.3a) are documented botryllid ascidians that undergo spontaneous vascular budding, whereby buds develop from their vascular epithelium, at the morphological level similarly to WBR in *B. leachii* (Oka and Watanabe 1959; Saito and Watanabe 1985; Okuyama and Saito 2001). In *Symplesma brakenhielmi*, an unsynchronized blastogenic species, vascular budding



**Fig. 16.3** Schematic illustration of the variability of WBR in colonial ascidians. (a) *Botryllus primigenus*. Vascular budding occurs spontaneously at the base of multiple actively growing terminal ampullae, at the end of developmental stage B, leading to the maturation of potentially multiple zooids (Oka and Watanabe 1957). (b) *Botryllus schlosseri*. Upon ablation of all zooids at developmental stage D (takeover), multiple regeneration niches are initiated at the base of terminal ampullae, leading to the maturation of one single zooid (Voskoboynik et al. 2007). (c) *Botrylloides leachii*. Upon ablation of all zooids, multiple regeneration niches are initiated throughout the vascular system, leading to the maturation of one single zooid (Rinkevich et al. 1995). (d) *Botrylloides violaceus*. Upon ablation of all zooids, multiple regeneration niches are initiated throughout the vascular system, leading to the maturation of potentially multiple zooids (Oka and Watanabe 1959)

is the main source of asexual development (Gutierrez and Brown 2017), similarly to *Clavelina lepadiformis* where isolated fragments of stolon typically regenerate functional adults within 8 days (Berrill and Cohen 1936).

By contrast, in *B. leachii*, *Botrylloides violaceus* and *Botryllus schlosseri*, WBR occurs only if no bud, no zooid and even no fragment of zooid remain present in the tissue (Fig. 16.3b–d; Rinkevich et al. 1995; Voskoboynik et al. 2007; Brown et al. 2009). For the successful regeneration of *B. schlosseri* colonies, the vascular system must be intact with an active blood flow, and the zooids must be ablated during the takeover stage of blastogenesis (Fig. 16.3b; Voskoboynik et al. 2007). In addition, *B. schlosseri* WBR goes through several generations of morphologically abnormal zooids before forming a normal colony (Voskoboynik et al. 2007), whereas, in

*B. leachii*, one morphologically normal zooid directly forms at the completion of WBR (Fig. 16.3c; Rinkevich et al. 1995; Zondag et al. 2016; Blanchoud et al. 2017). Likewise, *B. violaceus* WBR can be initiated at any point during its blastogenic cycle but leads in this species to the restoration of multiple zooids (Fig. 16.3d; Oka and Watanabe 1959).

### 16.4.2 Insights from Asexual Reproduction

Asexual reproduction and regeneration capacity have been shown to correlate (Rychel and Swalla 2009), and even to be convergent processes in tunicates (Kawamura et al. 2008), thus raising the possibility that both mechanisms could have a similar cellular origin. Compound ascidians present a wide variety of forms of asexual reproduction (palleal, vascular, stolonial, pyloric and strobilation budding; Brown and Swalla 2012) and various degrees of regenerative capacities (Sect. 16.4.1). Furthermore, early buds express a range of precursor lineage markers such as *Piwi*, *Pl10*, *Raldh*, *Vasa* and *SoxB1*, providing additional evidence that they have a progenitor cell identity (Rosner et al. 2009, 2013; Brown et al. 2009; Rosner and Rinkevich 2011). It is believed that, at least in botryllid ascidians, primordial germ cells develop during late embryogenesis (Rosner et al. 2013).

In *Perophora viridis*, budding of irradiated colonies could be rescued by the injection of a subpopulation of healthy blood cells physiologically similar to haemoblasts (Freeman 1964). Similarly, in *Botryllus schlosseri*, injection of previously enriched circulating stemlike cells can infiltrate both the germ line and the soma of host colonies (Laird et al. 2005). Interestingly, injection of immobile cells located around the endostyle can also induce long-term infiltration of the host *B. schlosseri* colony (Voskoboynik et al. 2008). However, single-cell injections have shown that these cells are unable to colonize both lineages, suggesting the existence of two distinct stem populations (Laird et al. 2005), a conclusion supported by studies on chimeric organisms (Stoner et al. 1999). The stemness of these cell lineages is supported by the observed high telomerase activity during bud development (Laird and Weissman 2004). Alternatively in *Polyandrocarpa misakiensis*, asexual budding is based on transdifferentiation of atrial epithelium cells (Kawamura and Fujiwara 1994, 1995; Kawamura et al. 2018). Similarly, in the asexual reproduction of *Polycitor proliferus* through strobilation, differentiated epicardium is the origin of the multipotent stem cells (Kawamura et al. 2008). Altogether, these results suggest the existence of a several multipotent stem cells that are involved in asexual reproduction in colonial ascidians.

### 16.4.3 *The Origin of the Progenitor Cells Driving WBR*

In botryllid ascidians, two populations of circulatory adult pluripotent stem cells have been identified (germ line and somatic stem cells; Stoner et al. 1999; Laird et al. 2005; Voskoboynik et al. 2008; Rosner et al. 2009). However, they are not the only cellular players as WBR in *B. leachii* requires the activation of dormant *Piwi*+ adult stem cells that line the internal vasculature epithelium of blood vessels, but not that of circulatory haemoblasts (Rinkevich et al. 2010). Nevertheless, the origin of these stemlike cells remains to be determined, as they could not be identified in uninjured colonies (Rinkevich et al. 2010). Therefore, an early role for circulatory cells in WBR cannot be totally rejected.

In the closely related species *B. violaceus*, *PCNA*+/*Piwi*+ circulatory haemocytes surround the site where WBR takes place and their number slowly decreases as regeneration of the adult occurs, suggesting that they are the main population of progenitor cells for WBR in this species (Brown et al. 2009). In addition, in the more distant *S. brakenhielmi*, circulating undifferentiated cells expressing the mitotic marker phospho-histone H3 are involved in a vascular budding process that also takes place after ablation of the adult zooids (Gutierrez and Brown 2017). By contrast, the cells providing the potential for WBR in *B. schlosseri* have been shown to be fixed to the vascular wall (Milkman 1967), and no *Piwi*+ cells are present in regeneration niches during *B. primigenus* WBR (Sunanaga et al. 2010).

One potential explanation for the observed developmental emancipation during WBR is the absence of colonial control. Indeed, experimental manipulations in which buds and zooids were surgically removed and placed under in vitro conditions, away from any discrete colonial regulatory cues, induced the resurgence of developmental processes otherwise masked by undetermined colonial controls (Rabinowitz and Rinkevich 2003, 2004, 2011; Rabinowitz et al. 2009). For instance, excised senescent zooids stopped their degeneration, extended their lifespan from a few hours to 1 month and produced de novo epithelial monolayers (Rabinowitz and Rinkevich 2004). In *S. brakenhielmi*, a potentially weaker colonial control, as suggested by the absence of developmental synchronization, might permit the observed spontaneous occurrence of WBR.

Consequently, WBR in colonial ascidians appears to utilize a variety of progenitor cells and developmental processes. Identifying the origins of multipotent and stemlike cells and regulatory mechanisms is of particular interest to further understand how WBR is activated.

### 16.4.4 *The Role of Immune-Associated Cells During WBR*

Gene expression for components of the complement system (*C3b*, *C-type lectin*) is upregulated during WBR (Rinkevich et al. 2007a; Zondag et al. 2016), potentially

for their known roles in immune cells recruitment and phagocytosis stimulation (Murphy 2012). Macrophages and the complement system function together in other biological systems to promote cell proliferation and tissue remodelling (Ohashi et al. 1999; Davidson and Swalla 2002; Voskoboynik et al. 2004; Roberts et al. 2007), two processes important for successful WBR. In addition, a dramatic increase in the fraction of circulating macrophage-like cells during WBR in *B. leachii* suggests an important role for these immune cells during regeneration (Fig. 16.2d; Blanchoud et al. 2017). Interestingly, macrophage-like cells are also involved during the reactivation phase after aestivation in *B. leachii* (Burighel et al. 1976), as well as during the takeover stage of blastogenesis in *B. schlosseri* where they are implicated in the degeneration of the apoptotic organs by coordinating death and clearance signals (Lauzon et al. 2002, 2013; Voskoboynik et al. 2004; Franchi et al. 2016). Macrophage-induced phagocytosis generates an oxidative stress that should lead to cell lethality when performed at the scale observed during takeover, but which is instead balanced by the coordinated expression of antioxidant genes (Franchi et al. 2017). Moreover, the ablation of resorbing zooids during takeover prevents the full-sized development of the next generation, while ablation of all but one daughter bud leads to its hyperplasia (Lauzon et al. 2002).

Altogether, these results suggest an essential and regulated involvement of macrophage-like cells in WBR. Some of the above results also suggest that macrophage-like cells could be a general mechanism for removing cellular debris and potentially providing a source of nutrients for developing buds (Lauzon et al. 2002, 2013). In addition, this model would explain the resorption of the unsuccessful regeneration niches during the growth expansion phase of the successful bud to provide it with essential nutrients (Rinkevich et al. 1995; Blanchoud et al. 2017).

#### **16.4.5 Genes Involved in WBR Have Multiple Roles in Colonial Ascidian**

Several studies focused on gene expression characteristic to WBR, yet many of these genes also play roles in other developmental processes of tunicates. For example, the identification of genes involved with *B. leachii* WBR (Rinkevich et al. 2007a) and both budding in *P. misakiensis* and blastogenesis in *B. schlosseri* (Oren et al. 2007; B. Rinkevich, unpublished data) highlighted a large variety of genes from protein families such as serine and trypsin proteases, serine and trypsin inhibitors, coagulation factors, lectins, cathepsins and metalloendopeptidases as well as genes involved in signal transduction pathways (Rosner et al. 2014). However, this wide panel of molecular functions suggests that most of these genes are not specific markers of WBR but are rather co-opted from other developmental processes.

An example of such diversification of function is the *von Willebrand factor* (VWF), which is expressed in *B. leachii* WBR (Rinkevich et al. 2007a), in *B. schlosseri* allrecognition and blastogenesis (Oren et al. 2007; B. Rinkevich,

unpublished data), in *Boltenia villosa* metamorphosis (Davidson and Swalla 2002; Roberts et al. 2007), in *Ciona robusta* metamorphosis (Nakayama et al. 2002; Chambon et al. 2007) and in *P. misakiensis* budding (Kawamura et al. 1998, 2006). A second instance of multiple roles for WBR genes is that of the DEAD-box RNA helicase *Pl10*, which is expressed in *B. leachii* WBR by macrophage-like cell (Rinkevich et al. 2010), in *B. schlosseri* during blastogenesis and embryogenesis (Rosner et al. 2006; Voskoboynik et al. 2008) as well as in more distant species such as in the neoblasts of the planarian *Dugesia japonica* and the *Hydra magnipapillata* multipotent interstitial stem cells (Rosner and Rinkevich 2007).

Overall, these genomic analyses suggest a large-scale co-option of developmental genes during WBR and highlight the current lack of markers specific to WBR in colonial ascidians.

## 16.5 Future Directions

While a wide range of multicellular organisms possesses powerful regeneration capacities (e.g. members of the phyla Porifera, Cnidaria, Platyhelminthes, Echinodermata and Bryozoa; Bely and Nyberg 2010), WBR in colonial ascidians is a unique phenomenon in the Chordata that can provide a special phylogenetic perspective into this process. In addition, the presence of other colonial ascidians with a limited or even an absence of regenerative capacity is particularly well suited for comparative studies (Tiozzo et al. 2008a). Consequently, *B. leachii* is an appealing system for the study of chordate regeneration. Yet, when compared to other models of regeneration, relatively little is known about the nature of this process.

To understand WBR in *B. leachii*, researchers need to understand its major components. Any regenerative process requires at least the integration of (a) cellular building blocks, including but not restricted to stem cells; (b) a sufficient supply of nutrient and oxygen, typically in the form of a vascular network; (c) the deposition of an extracellular matrix to create an adequate developmental environment; and (d) correct positional information to dictate morphogenesis (Rinkevich and Rinkevich 2013). Having all above in mind, we foresee three major steps in the future of this research:

1. Identification of the cellular origin of WBR. Although essential stemlike cells have been identified in the lining of the vascular epithelium, the origin of these cells remains unknown. Identifying this origin will be of major importance for understanding how their stemlike state is achieved and for the potential reactivation of similar processes in vertebrates. It is also important to identify the roles of circulating cells in the formation of regeneration niches and of developing zooid (Rinkevich et al. 1995, 2007b).
2. Characterization of WBR at the morphological level. WBR in botryllid ascidians differs at several morphological levels from the regeneration regularly studied in vertebrate model organisms. One example is the appearance of multiple

regeneration niches, whereas vertebrate regeneration typically originates from a single blastema. Understanding the morphogenesis of the developing regeneration niches is ancillary to identifying common regenerative strategies and to establishing a unified theory for metazoan regeneration (Rinkevich and Rinkevich 2013).

3. Characterization of WBR at the genetic level. Dissection of the molecular and genomic components involved during WBR through perturbation analyses is key to deciphering the mechanisms underlying chordate WBR. Indeed, cross-phyla studies have indicated that the phenomenon of regeneration, while variable at most levels of biological design, portrays extensive conservation of developmental signalling pathways (Sánchez Alvarado and Tsonis 2006).

The common dogma supports the notion that regeneration is a primaevial attribute of metazoans (Brockes et al. 2001; Bely and Nyberg 2010; Slack 2017). However, it remains to be determined whether regeneration has emerged along a unified continuum (Rinkevich and Rinkevich 2013) or independently as multiphyletic convergent responses to a variety of biological and environmental settings. Irrespective of the nature of this origin, the high degree of variation observed in model organisms and the assortment of cellular sources involved in regeneration attest that it is a highly complex biological phenomenon (Tsonis 2000; Tiozzo et al. 2008a; Rychel and Swalla 2009; Bely and Nyberg 2010; Galliot 2013; Jaźwińska and Sallin 2016). Consequently, obtaining a more complete picture of WBR in *B. leachii* will likely be challenging. Fortunately, future research will greatly benefit from existing studies in related species, novel multidisciplinary approaches as well as from advances in technology. Cell labelling and live-cell tracking of regenerating tissue fragments will be relevant to understand cell migrations and lineages. The precision of these labelling techniques will further benefit from the development of molecular markers specific to *B. leachii* cell fates and tissues. Single-cell RNA sequencing, proteomics and genomics will provide the unbiased identification of spatial and temporal variations at the expression, transcription and protein level throughout regeneration. In addition, genome sequence will provide an essential basis for the development of transgenic lines and gene-editing platforms such as the CRISPR/Cas9 gene technology, which has been successfully used in a wide range of animal systems including the tunicate *Ciona robusta* (Stolfi et al. 2014). Finally, establishment of inbred colonies, similar to those founded in *Botryllus schlosseri* (Rinkevich and Shapira 1998), will reduce inter-colony variability and provide a reference strain for future studies.

Overall, this will improve our understanding of regeneration strategies and will reveal the nature of regeneration processes that vary between closely related taxa.

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**Part IV**  
**Biomolecules, Secretion, Symbionts**  
**and Feeding**

# Chapter 17

## Beach to Bench to Bedside: Marine Invertebrate Biochemical Adaptations and Their Applications in Biotechnology and Biomedicine



**Aida Verdes and Mandë Holford**

**Abstract** The ocean covers more than 70% of the surface of the planet and harbors very diverse ecosystems ranging from tropical coral reefs to the deepest ocean trenches, with some of the most extreme conditions of pressure, temperature, and light. Organisms living in these environments have been subjected to strong selective pressures through millions of years of evolution, resulting in a plethora of remarkable adaptations that serve a variety of vital functions. Some of these adaptations, including venomous secretions and light-emitting compounds or ink, represent biochemical innovations in which marine invertebrates have developed novel and unique bioactive compounds with enormous potential for basic and applied research. Marine biotechnology, defined as the application of science and technology to marine organisms for the production of knowledge, goods, and services, can harness the enormous possibilities of these unique bioactive compounds acting as a bridge between biological knowledge and applications. This chapter highlights some

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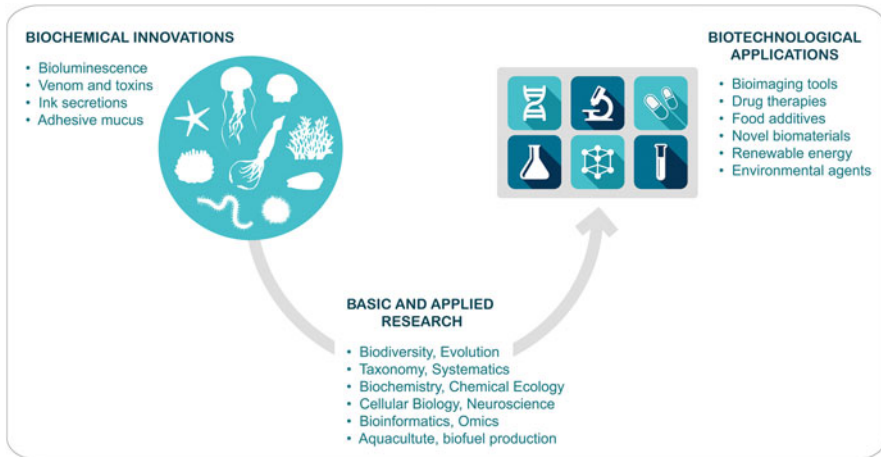
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**Fig. 17.1** Marine biotechnology workflow. Marine (blue) biotechnology relies on unique bioactive products derived from marine invertebrate biochemical innovations. These products and the animals that produce them are investigated using a biotechnological toolbox that applies basic and applied research methods such as biodiversity assessments and aquaculture production to develop a wide range of biotechnological applications

of the most exceptional biochemical adaptations found specifically in marine invertebrates and describes the biotechnological and biomedical applications derived from them to improve the quality of human life.

## 17.1 Marine Biotechnology and the Ocean as a Source of Chemical Diversity

Life on earth began in the ocean billions of years ago, and most organisms that exist today on land, or in freshwater and ocean ecosystems, originated in the sea. The ocean covers more than 70% of the Earth's surface and has high phylogenetic diversity, as it is home to 34 of the 36 known animal phyla that currently inhabit the planet. Because of its immense breadth and high degree of biodiversity, the ocean is arguably the ecosystem with the greatest potential for biodiscovery, and it is therefore a very promising source of innovation (Evans-Illidge et al. 2013). Marine biotechnology—broadly defined as the application of science and technology to marine organisms for the production of knowledge, goods, and services—can harness the enormous possibilities of marine biological resources, acting as a bridge between biological knowledge and applications to improve the quality of human life (Querellou 2010) (Fig. 17.1). However, despite the great potential of marine ecosystems to provide solutions to address global challenges, such as food scarcity, sustainable energy, and environmental and human health, the ocean remains largely unexplored (Trincon et al. 2015). In fact, we know more about the moon's surface than we know about the depths of our oceans. But this can change. We are at a











confluence in time where advances in the last 40 years have spearheaded technological breakthroughs above and below the seas. These innovations enabled new approaches for collecting marine organisms, using methods like remotely operated underwater vehicles (ROVs) and gliders, and for leveraging the twentieth-century revolution in molecular biology to decipher the genetic and chemical composition of marine organisms in a relatively fast and cost-effective manner.

The marine biotechnology enterprise (blue biotechnology) has grown exponentially in the last few decades, and with less than 5% of the vast oceanic environment explored, it has already delivered an array of innovations such as new medicines, chemicals, nanomaterials, nutritional supplements, bioenergy resources, and strategies for the sustainable use and management of the world oceans (e.g., Hannon et al. 2010; Livett et al. 2004; Schmidtko et al. 2010). Starting in the 1980s, there has been a significant increase in the number of marine compounds discovered each year, with annual numbers peaking at 400–500 novel compounds (Greco and Cinquegrani 2016). The biomedical industry has particularly benefited from the discovery of novel marine chemicals, with nine therapies derived from marine organisms currently approved for treating disease and disorders, namely, Adcertis®, Carragelose®, Cytosar-U®, Halaven®, Lovaza®, Retrovir®, Prialt®, Vira-A®, and Yondelis® (Arrieta et al. 2010) (Table 17.1). The applications of these compounds are as diverse as the organisms from which they were discovered. For example, Prialt® is a pain therapy developed from the venom of the marine snail *Conus magus*, while Adcertis® is an antibody-drug conjugate derived from the sea hare *Dolabella auricularia* that treats Hodgkin lymphoma (Hart 2015; Miljanich 2004). Halaven® is a breast cancer therapy derived from the sponge *Halichondria okadai*, while Retrovir®, also derived from a marine sponge, is an antiretroviral medication used to prevent and treat HIV/AIDS (Chiba and Tagami 2011; Rachlis 1990). As these successful cases illustrate, the great diversity of chemical compounds found in the ocean is a highly valuable but yet untapped resource for the discovery of novel bioactive agents with unique structures and diverse biological activities that can greatly improve human life (Evans-Illidge et al. 2013).

Marine habitats range from tropical coral reefs to ocean trenches and include ecosystems with the most extreme conditions of pressure, temperature, and light. As a consequence, marine organisms have evolved a plethora of remarkable adaptations that serve a variety of vital functions to survive in different habitats. For example, toxic secretions such as those produced by venomous marine organisms are in some cases used as a defensive mechanism against predators and in others as a weapon to subdue prey (Casewell et al. 2013; Verdes et al. 2018). Like venom, several other adaptations developed by marine organisms, such as bioluminescence and ink, represent biochemical innovations where the animals have attained novel and unique bioactive molecules that often show considerably greater potency than their terrestrial counterparts (Schroeder 2015; Trincone et al. 2015). Marine organisms have perfected these biochemical adaptations through millions of years of evolution, providing an immense reservoir of bioactive compounds with enormous potential for both basic and applied research (Schroeder 2015; Trincone et al. 2015) (Fig. 17.1). In this chapter, we provide an overview of some of the exceptional

**Table 17.1** Therapeutics derived from marine organisms. Examples of some successful, currently approved therapies derived from bioactive compounds isolated from marine organisms

Marine organism	Drug	Treatment	Company	References
 Sea hare <i>Dolabella auricularia</i>	Adcetris	Hodgkin lymphoma	Seattle Genetics (Bothell, WA, USA)	Hart (2015)
 Seaweed <i>Rhodophyceae</i>	Carragelose	Antiviral respiratory diseases	Marinomed (Vienna, Austria)	Li et al. (2014)
 Sponge <i>Tectiethya crypta</i>	Cytosar-U (Ara-C)	Leukemia	Bedford Laboratories (Ohio, USA)	Sullivan (1982)
	Vira-A	Antiviral herpes simplex	King Pharmaceuticals (NJ, USA)	Privat de Garilhe and de Rudder (1964)
 Sponge <i>Halichodria okadai</i>	Halaven	Breast cancer	Eisai (Tokyo, Japan)	Chiba and Tagami (2011)
 Fish	Lovaza	Hypertriglyceridemia	GlaxoSmith-Kline (Brentford, UK)	Halade et al. (2010)
 Marine snail <i>Conus magus</i>	Prialt	Chronic pain	Perrigo (Dublin, Ireland)	Miljanich (2004)
 Sea sponge	Retrovir	HIV/AIDS	ViiV Healthcare (NC, USA)	Rachlis (1990)
 Tunicate <i>Ecteinascidia turbinata</i>	Yondelis	Liposarcoma and leiomyosarcoma	PharmaMar (Madrid, Spain)	Erba et al. (2004)

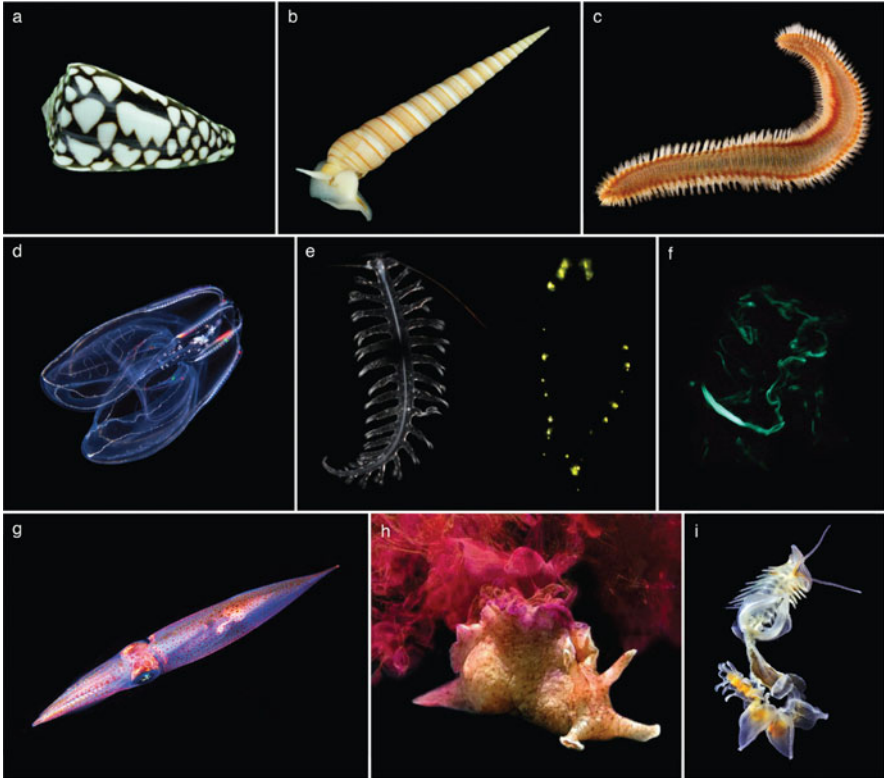
biochemical innovations found in marine organisms, specifically marine invertebrates, and the biotechnological and biomedical applications derived from them to advance and improve human life.

## 17.2 Biochemical Innovations of Marine Invertebrates

To survive in the varied ocean ecosystems they occupy, marine invertebrates have evolved an array of biochemical innovations that allow them to adapt and thrive in often extreme environments. These biochemical innovations, which range from secretions of mucus and toxins to light-producing molecules, play fundamental roles in marine ecological interactions, acting as pheromones, feeding deterrents, mediators of spatial competition, site recognition cues, antifouling agents, UV sunscreens, and facilitating reproduction (Harper et al. 2001). In a journey from the shallow intertidal to the abyssal depths, marine invertebrates display effortless biochemical innovations that humans have labored to reproduce in laboratories. In the following paragraphs, we describe a few examples of biochemical wonders produced by marine invertebrates, including toxins, ink secretions, adhesive gels, and light-producing compounds that have been translated to advance the blue biotechnology enterprise.

### 17.2.1 Marine Invertebrate Toxins

Many marine organisms defend themselves from predators by using toxic substances, including harmful secondary metabolites, poisonous molecules, and venomous secretions. These types of chemical defenses are particularly common among sessile and soft-bodied invertebrates such as sponges, corals, and ascidians that often dominate subtidal habitats with intense rates of predation (Lindquist 2002). For instance, venoms, which are generally defined as toxic secretions produced by one animal and delivered to another animal through the infliction of a wound, have evolved independently many times throughout the Metazoa (Casewell et al. 2013; Fry et al. 2009). Animal venoms are composed of a mixture of bioactive toxins and represent one of the most complex biochemical natural secretions known to date (Norton and Olivera 2006; Vonk et al. 2013). Venomous marine invertebrates are found in many phyla, from cnidarians such as sea anemones and jellyfish, which are the oldest venomous sea creatures recorded (Macek 1992; Ponce et al. 2016), to echinoderms such as starfish and sea urchins (Lee et al. 2015; Nakagawa et al. 1991); mollusks, such as cone snails and octopuses (Gorson et al. 2015; Olivera and Teichert 2007); annelids, including fireworms and bloodworms (Verdes et al. 2018; von Reumont et al. 2014a, b); and arrow worms (Thuesen et al. 1988) (Fig. 17.2a–c).



**Fig. 17.2** Biochemical innovations of marine invertebrates. Venomous conoidean snails **(a)** *Conus marmoreus* and **(b)** *Terebra tricolor*; **(c)** venomous fireworm *Eurythoe complanata*; **(d)** bioluminescent comb jelly *Bolinopsis infundibulum*; bioluminescent annelids **(e)** *Tomopteris helgolandica* under natural light (left) and in the dark after induced bioluminescence (right); and **(f)** *Odontosyllis enopla* bioluminescent display; inking mollusks **(g)** squid *Loligo vulgaris* and **(h)** sea hare *Aplysia californica*; **(i)** mucus-producing annelid *Chaetopterus variopedatus*. Images courtesy of Yves Terryn **(b)**, Denis Riek **(c)**, Alexander Semenov **(d, g, i)**, Anaïd Gouveneaux **(e)**, and John Sparks **(f)**

A particularly prolific group in terms of venom is the predatory marine neogastropods of the Conoidea superfamily that includes cone snails (Conidae), auger snails (Terebridae), and turrids (Turridae) (Fig. 17.2a–b). The Conoidea represents one of the most diverse groups of venomous marine organisms with numerous lineages characterized by a venom apparatus used for predation (Gonzales and Saloma 2014; Gorson et al. 2015; Gorson and Holford 2016; Holford et al. 2009; Puillandre et al. 2014). The genus *Conus* is the most comprehensively studied and includes species that produce complex venoms with hundreds of unique peptide toxins, referred to as conotoxins (Kaas et al. 2012; Puillandre et al. 2012). While cone snail venom has been studied for more than 40 years, less than 2% of their venom peptides have been characterized to identify their functional molecular

targets. Similarly, beyond cone snails, the venoms of terebrids and turrids are largely unknown. Recent efforts have begun to characterize the venom peptides of terebrids and turrids relying largely on advances in next-generation sequencing and other analytic techniques such as proteomics (Gonzales and Saloma 2014; Gorson et al. 2015). In fact, small, harder to collect, and often neglected venomous marine invertebrates are now being investigated in record numbers, thanks to what is sometimes referred to as the “rise of the omics” (Gorson and Holford 2016; von Reumont et al. 2014a, b). Given that venomous conoidean snails can conservatively produce between 50 and 200 peptides in their toxic arsenal and there are more than 10,000 species of conoideans, there is an amazing repertoire of venom peptides to be discovered. Whether in cnidarians, echinoderms, or mollusks, the venom peptides produced by these organisms are extremely potent, fast acting, and very specific, targeting hematic and neurotic pathways (Fry et al. 2009; Verdes et al. 2018).

### 17.2.2 *Marine Mollusk Ink Secretions*

Some mollusks, including sea hares (*Aplysia*) and coleoid cephalopods (octopuses, squid, and cuttlefishes), produce an ink secretion that functions as a chemical antipredator defense (Fig. 17.2g–h). Sea hares, in particular, have risen to scientific acclaim for their use in neuroscience to determine the mechanisms of learning and memory. These herbivorous animals, when threatened, release toxins sequestered from their diet of red algae (Kicklighter et al. 2007; Paul and Pennings 1991). Both sea hares and cephalopods secrete their chemically laden ink from two distinct glands. In the case of the sea hare, the products of the ink gland and the opaline gland are released into the mantle cavity and then pumped through the siphon toward the predator (Love-Chezem et al. 2013; Prince 2007). Opaline is a viscous substance that contains a high concentration of free amino acids and sticks to the chemosensory appendages of the predator, inactivating them and thus influencing its capacity to detect prey (Kicklighter et al. 2007; Love-Chezem et al. 2013). In addition to acting through sensory inactivation, ink secretion of sea hares can also act as an unpalatable repellent and as a decoy that misdirects and confuses the predator (Nolen et al. 1995; Nusbaum and Derby 2010). Cephalopod ink functions as antipredatory visual stimuli, acting either as a smoke screen or as a distracting decoy and possibly also disrupting the predator’s chemical sensors (Caldwell 2005). It is composed of a black ink containing melanin produced by the ink gland and a viscous mucous produced by the funnel organ (Derby 2014). Ink secretions of both sea hares and cephalopods represent a successful biochemical innovation that has enabled the organisms to survive and thrive.

### 17.2.3 *Viscoelastic Adhesive Gels*

A variety of marine invertebrates produce viscoelastic adhesive gels such as mucus secretions that consist of a network of polysaccharides and proteins entangled to form a gel with more than 95% of water content (Stabili et al. 2015). These mucous secretions are essential for the survival of many marine invertebrates as they are used for a variety of functions including protection against pathogens and parasites, to coat vulnerable organs, reduce drag forces, prevent sedimentation, enhance adhesion, limit water loss, and aid in locomotion and feeding (Smith 2002; Stabili et al. 2015; Weigand et al. 2017).

Sessile marine invertebrates, in particular, which are permanently attached to the sea floor with limited mobility, are more vulnerable to predation and use mucus secretions as an antipredator mechanism. In addition to mechanical protection, the mucus of many of these species contains toxic compounds that make the animal poisonous or distasteful (Iori et al. 2014). In many cases, the mucus also serves as an immune response system, producing a considerable amount of defensive compounds such as bioactive antimicrobials, toxins, and cytolytic molecules (Derby 2007; Iori et al. 2014; Stabili et al. 2015).

Numerous filter-feeding sessile organisms, such as the marine annelid *Chaetopterus* (Fig. 17.2i), use mucus to trap and filter food, to deter prey, and to gather sand particles to build protective tubes (Weigand et al. 2017). Other marine invertebrates, most notably mollusks, use mucus as an adhesive to attach to the substratum during locomotion. Some of these mollusks such as limpets and mussels are well-known for the extraordinary adhesive power of their mucus secretions (Smith 2002; Stewart et al. 2011).

### 17.2.4 *Light-Producing Compounds*

Bioluminescence, the ability to produce light by living organisms, is another outstanding biochemical innovation that has independently evolved in many lineages across the tree of life (Haddock et al. 2010). Bioluminescent light is the product of a chemical reaction involving the oxidation of a light-emitting molecule—luciferin—by a specific enzyme, luciferase (Shimomura 2012). In some cases, the luciferin is strongly bound to the luciferase and oxygen, forming a stable complex referred to as a photoprotein (Deheyn and Latz 2009; Shimomura 1985). Bioluminescent forms are found in many taxonomic groups, ranging from bacteria to vertebrates, but the great majority of luminous organisms are marine taxa (Shimomura 2012; Widder 2010). In fact, a recent study based on observations of more than 350,000 individuals in the water column reported that 76% of them were bioluminescent (Martini and Haddock 2017). A great number of these luminous marine organisms are invertebrates, including cnidarians, ctenophores, annelids, mollusks, and arthropods (Haddock et al. 2010) (Fig. 17.2d–f). The ecological

diversity of bioluminescent marine invertebrates is remarkable, with species occupying a great range of habitats, from coastal waters to the deep sea, in benthic and pelagic waters, from polar to tropical regions. This outstanding diversity is matched by the wide array of bioluminescent colors—including yellow light emitters (Fig. 17.2e), which are extremely rare in marine environments—as well as varying light patterns and chemistries (Verdes and Gruber 2017). Likewise, bioluminescence is associated with a variety of different functions, including defense, predation, and intraspecific communication (Bassot and Nicolas 1995; Gouveneaux and Mallefet 2013; Haddock et al. 2010; Oba et al. 2016).

Although all bioluminescent organisms convert the chemical energy of an oxidation reaction into light, their independent evolutionary origins have resulted in a great diversity of chemistries and biological systems (Conti et al. 1996). The luciferases characterized so far from different organisms have extremely diverse structures, substrate specificities, and mechanisms and do not generally share sequence similarities (Viviani 2002). Luciferins are also quite diverse; however, in many cases the same compound has been independently co-opted in unrelated organisms. For instance, coelenterazine is the light emitter in at least nine phyla including jellyfish, crustaceans, mollusks, and vertebrates, even though their luciferases are unrelated in sequence and structure (Gimenez et al. 2016; Haddock et al. 2010).

## 17.3 Biotechnological and Biomedical Applications

Recent scientific breakthroughs and technological advances such as next-generation sequencing, proteomics, and bioinformatics are accelerating our capacity to harness marine bioactive compounds for biotechnological and biomedical applications that have significant potential to improve human life and advance scientific knowledge. In the following sections, we describe some of the novel applications that have been developed from bioactive compounds isolated from marine invertebrate biochemical adaptations.

### 17.3.1 Pharmacological Applications of Venom Peptides

Many compounds derived from marine invertebrate biochemical innovations, in particular venom toxins, have become major sources of drug leads driving research efforts in the pharmaceutical industry and greatly advancing drug discovery and development efforts. The only drug derived from venomous marine snails, Prialt®, is based on a venom peptide from the species *Conus magus*, and it is used to treat chronic pain in HIV and cancer patients (Miljanich 2004). Prialt® (ziconotide; MVIIA) is a breakthrough drug as it not only realized the potential of venomous marine snails for drug discovery, but it also provided a new paradigm for treating

pain because it targets N-type calcium channels instead of opioid receptors (Fusetani et al. 2000; McGivern 2007). Until the discovery and development of Prialt®, most pharmaceutical companies looking for analgesic remedies investigated compounds that target opioid receptors, such as morphine. Prialt® revolutionized chronic pain treatment as it demonstrated that a peptide that modulates ion channels can be just as effective, or even more, than the gold standard morphine, with the additional benefit of not causing the undesirable side effect of drug addiction. However, Prialt® does not cross the blood-brain barrier, and it is currently administered via intrathecal injection, which represents a major drawback limiting its widespread application (Staats et al. 2004). Recent efforts to overcome the invasive delivery method of Prialt® involve a Trojan horse strategy in which the peptide is encapsulated in a viral nanocontainer and shuttled across the blood-brain barrier (Anand et al. 2015; Kelly et al. 2015). The Trojan horse strategy mimics how venom is stored and delivered by venomous snails in nature. Conoidean snails produce venom in a special gland referred to as the venom gland, where toxins are stored in capsule-like structures similar to a nanocontainer and later delivered to a hollow radular harpoon for injection into the prey (Holford et al. 2009; Terlau and Olivera 2004). The potential applications of conoidean venom peptides go well beyond pain management and include treatment of other disorders such as epilepsy and cancer, to name a few (Petras et al. 2015; Vetter and Lewis 2012). Most drug therapies being developed from marine snail venom peptides target ion channels and receptors (King 2011; Lewis and Garcia 2003; Olivera 1997; Ortiz et al. 2015; Vetter et al. 2011). Conoidean venom peptides are short disulfide-rich peptides with a characteristic structure consisting of a signal peptide, followed by a propeptide region and a terminal cysteine-rich mature peptide, and therefore they share some features of other peptide therapeutics, namely, poor pharmacokinetics and not being orally active (Uhlir et al. 2014). As a consequence, there are key areas pertaining to drug delivery that must be addressed to truly advance the potential of marine snail venom peptides for drug development.

### ***17.3.2 Applications of Light-Producing Molecules in Biophotonics***

Light-emitting compounds have also been widely used for biotechnological applications. The discovery of the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria* revolutionized the biological field leading to a variety of novel imaging tools and reporters (Chalfie et al. 1994; Tsien 1998). In a similar way, the constituents of bioluminescent systems, luciferin and luciferases, have been widely used in biotechnology for three primary applications: as probes for cellular biology, as tools to map and identify genes, and to track the progression of disease in laboratory animals (Widder and Falls 2014).



Bioluminescent molecules have been widely used in cell biology because many enzymes and metabolites can be measured by coupling them to bioluminescence cofactors such as ATP or H<sub>2</sub>O<sub>2</sub> (Widder and Falls 2014). For instance, the firefly luciferin-luciferase system can use the ATP produced by a reaction catalyzed by a kinase to produce light in proportion to the kinase (Lundin et al. 1976). This assay is the most sensitive detection method available, and because all living cells contain ATP, it has been used in a wide range of applications, from determining the impact of antibiotics on bacterial growth to the search for life on Mars (Widder and Falls 2014). In addition, many calcium-activated photoproteins isolated from marine invertebrates such as cnidarians and ctenophores have played a major role in understanding the function of calcium, a ubiquitous intracellular messenger in cell regulation (Bonora et al. 2013; Ottolini et al. 2013).

Bioluminescent proteins have also been used to replace fluorescent markers in genetic engineering experiments, to determine if a gene of interest inserted into a particular cell is actually being expressed. For this purpose, a luciferase gene is incorporated into the DNA region adjacent to the gene of interest, and both genes are expressed together when luciferin and any necessary cofactors are added (Contag and Bachmann 2002; Mezzanotte et al. 2017). The luciferases most commonly used for monitoring gene expression are those isolated from the North American firefly *Photinus pyralis* and from two marine invertebrates, the sea pansy *Renilla reniformis* and the copepod *Gaussia princeps* (Widder and Falls 2014).

Bioluminescent imaging reporters have also revolutionized the study of disease progression, for example, enabling the tracking of an infection in a single animal. Transforming pathogenic bacteria with luciferase and monitoring its bioluminescence made it possible to track the course of an infection in a single host; observe cell migration, proliferation, and apoptosis; and evaluate the effectiveness of antibiotics (Contag et al. 1995; Kim et al. 2015). Bioluminescence imaging has also greatly contributed to advances in cancer research, allowing to visualize tumor cells in living animals and making possible to evaluate the efficacy of chemotherapies and immune cell therapies in vivo (Madero-Visbal et al. 2012; Sweeney et al. 1999).

### ***17.3.3 Biomaterials Derived from Marine Invertebrates***

In recent decades, marine-derived biomaterials have gathered increased attention from the medical, pharmaceutical, and biotechnology industries for a variety of applications ranging from food additives to biodegradable plastics and bio-adhesives.

Cephalopod ink, for example, has been widely used by humans for many practical and commercial purposes, especially in medicine, cuisine, and art (Derby 2014). Several cosmetic products such as mascara or eyeshadow have been developed using squid ink, with the goal of developing products that are effective but also sustainable, safe, and respectful with the environment (Neifar et al. 2013). The food industry has also used cephalopod ink in various ways, including food flavoring,

food coloring, and even curing and preserving cuttlefish meat, due to its antimicrobial properties (Derby 2014; Xu et al. 2009).

Adhesive gels and their constituents, such as mussel adhesive proteins, are also being investigated as attractive biomaterials for various applications including electronic skin and wound dressings or to develop biomimetic analogues that function as antifouling coatings (Kord Forooshani and Lee 2017; Li and Zeng 2016). For example, the adhesion mechanism of mussels has inspired a free-standing, adhesive, tough, and biocompatible hydrogel that might be more convenient for surgical applications than currently used adhesives (Han et al. 2017).

## 17.4 Future Prospects

As outlined in this chapter, there are numerous biochemical adaptations that marine organisms have developed which could lead to important scientific advances, such as nonaddictive pain therapies as an alternative to opioids or novel surgical hydrogels. However, to realize the immense potential of marine invertebrate bioactive molecules for both basic research and biotechnological applications, much more must be done to support the study and discovery of the numerous secrets hidden in our oceans.

The Earth's oceans are a scientific phenomenon, full of amazing creatures that have evolved to master their domain and in doing so have provided a roadmap for discovery and innovation. While there are many ways to investigate the oceans, the path that puts the conservation of the environment and the organisms first should be the preferred one. As extinction rates increase, we are losing more than we can characterize with existing technologies. It would be a catastrophic human failure to treat our oceans as factories and not as a source of inspiration. To effectively develop applications based on successful marine products provided by nature, we need to invest in basic research to identify the most promising compounds and the best environmentally safe methods for extracting them. When discussing the biotechnology enterprise, the tendency is to highlight the human applications but not the relevance of the basic research that led to them. However consider, for example, the great scientific advances being achieved and conceived with CRISPR-Cas9 technology (Mei et al. 2016). Had it not been for the thoughtful and thorough investigations of the adaptive immune system of *Streptococcus thermophilus*, the powerful genome editing tool CRISPR-Cas9, as we know it, might not have happened (Lander 2016; Mojica and Rodriguez-Valera 2016). In the attempt to translate ocean products to goods and services to advance society, we must practice the concept of identifying the "usefulness of useless knowledge" (Flexner 1955). It is imperative that we bring to the table the inherent value of basic fundamental research in evolution and biodiversity, to allow the blue biotechnology enterprise to act as a true bridge between basic knowledge and applications to improve the quality of human life.

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# Chapter 18

## Coral Food, Feeding, Nutrition, and Secretion: A Review



Walter M. Goldberg

**Abstract** Tropical scleractinian corals are dependent to varying degrees on their photosymbiotic partners. Under normal levels of temperature and irradiance, they can provide most, but not all, of the host's nutritional requirements. Heterotrophy is required to adequately supply critical nutrients, especially nitrogen and phosphorus. Scleractinian corals are known as mesozooplankton predators, and most employ tentacle capture. The ability to trap nano- and picoplankton has been demonstrated by several coral species and appears to fulfill a substantial proportion of their daily metabolic requirements. The mechanism of capture likely involves mucociliary activity or extracoelenteric digestion, but the relative contribution of these avenues have not been evaluated. Many corals employ mesenterial filaments to procure food in various forms, but the functional morphology and chemical activities of these structures have been poorly documented. Corals are capable of acquiring nutrition from particulate and dissolved organic matter, although the degree of reliance on these sources generally has not been established. Corals, including tropical, deep- and cold-water species, are known as a major source of carbon and other nutrients for benthic communities through the secretion of mucus, despite wide variation in chemical composition. Mucus is cycled through the planktonic microbial loop, the benthos, and the microbial community within the sediments. The consensus indicates that the dissolved organic fraction of mucus usually exceeds the insoluble portion, and both serve as sources for the growth of nano- and picoplankton. As many corals employ mucus to trap food, a portion is taken back during feeding. The net gain or loss has not been evaluated, although production is generally thought to exceed consumption. The same is true for the net uptake and loss of dissolved organic matter by mucus secretion. Octocorals are thought not to employ mucus capture or mesenterial filaments during feeding and generally rely on tentacular filtration of weakly swimming mesozooplankton, particulates, dissolved organic matter, and picoplankton. Nonsymbiotic species in the tropics favor phytoplankton and weakly swimming zooplankton. Azooxanthellate soft corals are opportunistic

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feeders and shift their diet according to the season from phyto- and nanoplankton in summer to primarily particulate organic matter (POM) in winter. Cold-water species favor POM, phytodetritus, microplankton, and larger zooplankton when available. Antipatharians apparently feed on mesozooplankton but also use mucus nets, possibly for capture of POM. Feeding modes in this group are poorly known.

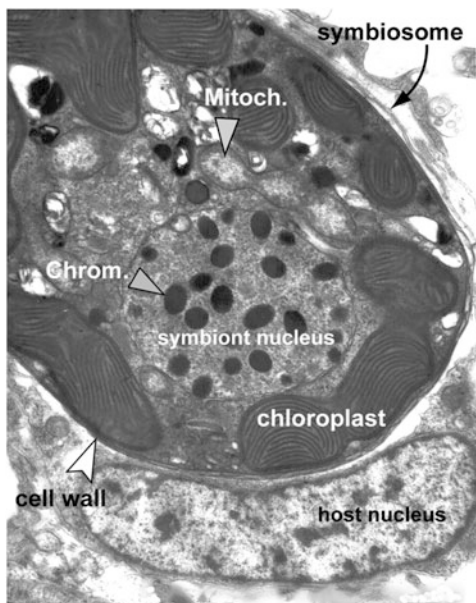
**Keywords** Mesoplankton · Microplankton · Nanoplankton · Picoplankton · Microbial loop · Dissolved and particulate organic carbon · Nitrogen · Phosphorus · Mucus secretion · Tentacle capture · Mesenterial filaments · Tropical · Temperate and polar scleractinians · Octocorals · Antipatharians

## 18.1 Introduction

Corals are ecological engineers capable of modifying their physicochemical environment by virtue of their growth, form, and metabolism. Corals and the reefs they support are among the richest, most complex, and astonishingly diverse ecosystems on Earth, although they compose only a fractional percent of its surface. To add incongruity, they also live in nutritionally poor waters, a paradox first noted by Charles Darwin (1842). A partnership with endosymbiotic algae enables tropical reef corals to flourish in such environments through the conservation of nutrients, including nitrogen and phosphorus, and by retention and recycling within the reef ecosystem. These complex processes are easier to describe generally than they are to fully explain. The diverse sources and forms of nutrition that are available to corals and other members of the reef benthos are additionally dependent on complex associations with heterotrophic bacteria, archaea, and viruses whose residence *in hospite* are not simply influential, but are vital to their hosts (e.g., Rosenberg et al. 2007; Silveira et al. 2017). Corals may also house an assortment of fungi, filamentous skeletal algae, and cyanobacteria (Ainsworth et al. 2010) whose roles in many cases have yet to be clarified. The host and its suite of typical residents are often referred to collectively as the holobiont, whose composition is environmentally influenced through a tangle of feedback loops that are only now beginning to be unraveled.

Corals are threatened by climate change, and research emphases have become more focused on that issue, particularly as warmer or eutrophic waters appear to promote the sequestration of nutritional resources by symbiotic algae at the expense of their hosts (Wooldridge 2017; Baker et al. 2018). Ultimately, this shift from symbiosis to parasitism may presage the well-known expulsion of the coral's symbiotic partners and a change in the nature of their microbial communities (e.g., Sokolow 2009; Bourne et al. 2009; Work and Meteyer 2014). This review focuses on the complexities presented by diverse sources of food, modes of feeding, secretory activities, and the nutritive sources available to corals in a variety of environmental situations, whether they act as hosts in symbiotic relationships or not. As used here, the term “corals” will refer to those that produce calcified

**Fig. 18.1** *Zooxanthella in hospite* showing cellular details and the symbiosome vacuole within the surrounding host cell. Courtesy of RK Trench, TC LaJeunesse, and Wikipedia



skeletons (stony corals, order Scleractinia); however, reference will also be made to octocorals including those that produce flexible collagenous or composite calcite/protein skeletons (gorgonian octocorals), a scleritic or hydraulic skeleton (soft corals, order Alcyonacea), and skeletons composed of chitin and protein (black or wire corals, order Antipatharia).

## 18.2 Autotrophic Nutrition: A Brief Overview

Most shallow-water tropical corals regardless of taxonomic alliance associate with photosynthetic dinoflagellate symbionts of the genus *Symbiodinium*, a phototroph commonly referred to as zooxanthellae (Figs. 18.1 and 18.2). The relationship is intimate with the symbiont housed inside a host-cell vacuole, the symbiosome (Fig. 18.1), through which the two partners typically exchange nutritive and other molecules. Carbon is fixed during photosynthesis by zooxanthellae, and 50 to >90% of it is translocated to the host within a few hours (Muscatine et al. 1981; Dubinsky and Jokiel 1994; Kopp et al. 2013), although different rates have been reported under suboptimal conditions (e.g., Tremblay et al. 2012a; Seeman 2013). Glycerol, lipids, and glucose are thought to be especially abundant among the translocated forms of carbon, and appear to provide more than enough for the respiratory needs of the zooxanthellae and the host (Battey and Patton 1984; Burriesci et al. 2012 and contained references). Indeed, when expressed on a daily basis, the photosynthetic/respiration (P:R) ratio can be greater than 1:1 suggesting that the symbiosis is autotrophic, i.e., that all daily metabolic

requirements including respiration and growth of the skeleton and tissue are met through photosynthesis and translocation of its products. However, photosynthetic or translocation capacity may differ within the same coral colony during the day due to self-shading, light fluctuation, depth, turbidity, and the genetic capacity of the *Symbiodinium* clade or density, among other factors (Warner et al. 2006; Ulstrup et al. 2007; Cooper et al. 2011).

Carbon translocation may also fail to account for periodic energy expenses including growth, gamete, or mucus production by the host (Muscatine et al. 1981; Davies 1984; Sorokin 1995) and may not be energetically balanced even when such requirements are taken into account (Falkowski et al. 1984; Edmunds and Davies 1989). While models suggest that 60% or more of the nitrogen available to the host can be recycled through autotrophy, it is clear that internal recycling cannot go on indefinitely without the influx of new sources to support growth and replace secretory losses (Tremblay et al. 2012a; Gustafsson et al. 2013). In addition, there may be differences in the use of products that are acquired through photosynthesis by the host and those obtained by heterotrophy. It has been suggested, for example, that simple autotrophic molecules are used for short-term energy requirements such as respiration, whereas more complex organic molecules are used for storage (e.g., Bachar et al. 2007; Tremblay et al. 2015). Moreover, translocated nutrients alone are deficient in phosphorous and nitrogen and thus provide “junk food,” calories without nutritional quality (Falkowski et al. 1984; Davies 1984). Thus, while shallow-water tropical species may be autotrophic to some degree, they must rely on prey items or on dissolved sources dissolved or particulate matter to replenish inorganic nitrogen, phosphorus, and other nutrients that cannot be adequately supplied by symbiotic algae (Muscatine and Porter 1977; Sorokin 1995).

### 18.2.1 *A Need to Feed*

Exogenous nutrient availability may be reflected by the density of zooxanthellae *in hospite* (Muscatine et al. 1989), by an increase in the size of symbiont cells, by the number of starch granules, and by the increased accumulation of intracellular lipids (Rosset et al. 2015). Lipogenesis is primarily a function of the chloroplasts (Crossland et al. 1980), and the assembly of these biomolecules may vary among zooxanthellae clades, the depth ranges of the host, or levels of environmental stress (e.g., Tchernov et al. 2004; Cooper et al. 2011). Synthesis begins with fatty acids and continues as storage lipids (i.e., triacylglycerol and wax esters), which are translocated to the host (Patton et al. 1977; Yamashiro et al. 1999; Grottoli et al. 2004). Lipids are an important indicator of coral vitality, and shallow-water coral tissue accumulates them as “fat bodies,” which may constitute 30–40% of the dry tissue weight (Stimson 1987). Such stores are important as food reserves that may compensate for a reduction in autotrophic output. Fatty acids can be synthesized endogenously by zooxanthellae but are most probably derived from heterotrophic feeding (Treignier et al. 2008; Imbs et al. 2010). However, there may be considerable differences among coral species in the amount of stored lipid reserves and in the

ability of the host to replenish them under stress (Stimson 1987; Ferrier-Pagès et al. 2011a; Imbs and Yakovleva 2012).

The relative ratio of heterotrophy to autotrophy varies greatly. In some cases, heterotrophy meets as little as 15% of the coral's daily metabolic requirement (Houlbrèque and Ferrier-Pagès 2009). In general, it appears that the importance of exogenous food for symbiotic corals becomes especially critical at low irradiance levels. Corals that have been assessed are able to maintain or increase levels of fatty acids, sterols, and protein concentrations as long as they are fed (Al-Moghrabi et al. 1995; Treignier et al. 2008). In *Stylophora pistillata*, heterotrophy combined with high irradiance levels results in high translocation rates of carbon to the host compared with fed colonies at lower irradiance levels. This "overproduction" in turn results in the release of excess organic compounds described below (Tremblay et al. 2014). Likewise, fed colonies of *S. pistillata* better resist irradiance stress and oxidative damage compared with unfed colonies (Levy et al. 2016). More specifically, other species have demonstrated the ability to resist temperature stress and its repercussions through the provision of a lipid-enriched diet (Tagliafico et al. 2017a).

Autotrophy may also differ seasonally among symbiotic coral species living in temperate and seasonally marginal environments. Colonies of the symbiotic *Cladocora caespitosa* form reefs in the Mediterranean Sea. This species reaches compensation irradiance in low-light conditions and is adapted to relatively high levels of turbidity. As a result, it can occur to a depth of 40 m. Summer conditions favor both skeletal and tissue growth, much of it from autotrophy. If colonies are experimentally fed under these conditions, they release excess carbon as mucus similar to tropical species (Hoogenboom et al. 2010; Tremblay et al. 2011, 2012b). However, zooplankton supplies are greater in cooler weather than in summer, and supplementation from heterotrophy is evident from the shifts in carbon and nitrogen isotope ratios during winter months. Thus, external sources of food compensate for photosynthetic limitations (Piniak 2002; Rodolfo-Metalpa et al. 2008; Ferrier-Pagès et al. 2011b). A similar compensation mechanism may be found among corals living on tropical mesophotic reefs (Schlichter et al. 1986; Eyal et al. 2015). Here at depths of up to 150 m, symbiotic species are likely to be light limited and more dependent on heterotrophy compared with their shallow-water relatives (e.g., Gattuso et al. 1993; Einbinder et al. 2009; Lesser et al. 2010). By contrast, symbiosis in the NW Atlantic species *Oculina arbuscula* is facultative. Colonies that retain their photosymbionts year-round obtain most of their nutrition from them, but naturally occurring aposymbiotic colonies rely on a diet of phyto- and zooplankton 12  $\mu\text{m}$  or smaller, in addition to organic matter in the sediment judging from the isotopic signatures (Leal et al. 2014a).

The symbiotic relationship is fraught with delicacy in the face of environmental change. Beyond their narrow range of temperature or irradiance, zooxanthellae become photosynthetically impaired and begin to undergo a process of expulsion and degrees of bleaching. Hosts in those conditions vary in their capacity to obtain heterotrophic nutrition (Palardy et al. 2008; Grottoli et al. 2006; Rodrigues and Grottoli 2007; Ferrier-Pagès et al. 2011a), and if bleaching is severe, heterotrophy must provide 100% of coral metabolic requirements. While some species are able to

make up for autotrophic impairment by heterotrophy, others cannot, even in the presence of food (e.g., Grottooli et al. 2006; Hughes and Grottooli 2013). Similarly, periodic episodes of turbid water may cause a shift from autotrophy to other forms of nutrition including the capture of particulate matter, even if it is a poor-quality food source. Under prolonged light reduction and fouling of the coral feeding surfaces, some species may be unable to obtain appropriate amounts or adequate forms of nutrition from these sources or may be overwhelmed by respiratory requirements imposed by nonnutritive particle removal (e.g., Anthony 1999; Anthony and Fabricius 2000; Browne et al. 2014).

## 18.3 Heterotrophic Nutrition in Scleractinians

### 18.3.1 *From the Water Column*

All scleractinian corals are heterotrophs that obtain at least part of their nutrition by suspension feeding. Corals are polytrophic feeders, obtaining food by several mechanisms. Tentacle capture and/or mucociliary entrapment of zooplankton and nanoplankton plays a prominent role. Most scleractinians exhibit characteristic responses to the presence of finely ground animal food (e.g., minced crab or clam tissue) including muscular activity of the tentacles, contraction of the oral disk, wide dilation of the oral region, secretion of mucus, and, in some, eversion of the mesenterial filaments (e.g., Carpenter 1910; Boschma 1925; Muscatine 1973) described below. Similar reactions occur when corals are exposed to millimolar (or lower) concentrations of amino acids (Mariscal and Lenhoff 1968; Goreau et al. 1971).

#### 18.3.1.1 Tentacle Capture and the Use of Cnidae

Suspension-feeding invertebrates consume plankton and other sources of organic material by straining, sieving, or otherwise trapping them from the water column. Active suspension feeders are those animals that employ a pumping mechanism to drive currents to trap food (e.g., bivalve molluscs, ascidians, and some annelid worms, among many other groups). Passive suspension feeders, by contrast, depend exclusively on ambient water movements to drive water past their food-gathering surfaces. With some exceptions (e.g., xeniid octocorals, described below), corals of all affinities fall into this category and employ tentacle capture, with assistance to varying degrees by mucus entrapment. Tentacles act as sieves that rely on their spacing, number, and shape to trap particles larger than their pass-through elements (LaBarbara 1984). Scleractinian coral tentacles are relatively simple conical structures, but they can be quite numerous (e.g., Fig. 18.2). Thus, the simple act of tentacle projection produces a canopy that slows the flow of water, thereby facilitating food capture (Johnson and Sebens 1993). However, relatively low flow rates

**Fig. 18.2** A field of conical tentacles from the scleractinian *Montastraea carvermosa*. Note white nematocyst batteries (inset) along the length. The white tips (acrospheres) are additional regions of cnidae concentration. Images courtesy of GP Schmahl, NOAA/Flower Gardens National Marine Sanctuary



are often crucial because higher flows can induce mechanical deformation of the exposed feeding surfaces and may cause detachment of prey (Wijgerde et al. 2012).

While some scleractinians exhibit a diminished number of cnidae and/or reduced tentacles (Schlichter and Brendelberger 1998; Goldberg 2002a, 2004), nematocysts and spirocysts are typically densely populated in the tentacles and are used to subdue and increase retention of prey items that might otherwise escape. Most scleractinian corals develop tentacles that are studded with wartlike “batteries” of nematocysts that may be helpful in taking relatively large planktonic prey (Fig. 18.2; Sebens et al. 1996). However, while venoms and neurotoxins are known to be associated with nematocysts (see below), those from corals have not been assessed, nor have adhesive spirocysts, the most abundant type of cnida in tentacles of several coral groups (Watson and Mariscal 1983; Goldberg and Taylor 1996; Strömberg and Östman 2017). Spirocysts characteristically develop internal tubules that appear to form a weblike mesh once they have been externalized by discharge, and this is thought to entangle prey (Mariscal et al. 1977). These are glutinant or adhesive cnidae that contrast with penetrant nematocysts (Mariscal 1984), but spirocysts have not been observed in the act of taking prey or particulate food. Interestingly, the cnidome of tropical octocorals may be diminished in number or in diversity compared with their stony relatives (Mariscal and Bigger 1977; Schmidt and Moraw 1982; Sammarco and Coll 1992). However, this generality may fail as more species are examined, and it may not apply to nonsymbiotic species (e.g., Piraino et al. 1993; Yoffe et al. 2012).

Satiation mechanisms have been examined in anemones, but not in corals, and appear to be controlled in part by the inhibition of discharge by certain types of nematocysts rather than by prey ingestion. However, there is also a marked decline in feeding efficiency due to the failure of discharged nematocysts to maintain adherence of food items to the tentacle in sated anemones, and this results in prey being dropped out of reach. Adhesive forces in turn, measured in micronewtons, are the sum of contributions arising from the stickiness of the tentacle mucus to the prey and the number of cnidae (nematocysts and spirocysts) discharged, multiplied by the mean force needed to pull individual cnidae out of the tentacle (Thorington and Hessinger 1996). Although numerous spirocysts discharge on stimulation, the adherence forces imparted by them are extremely low compared with nematocysts

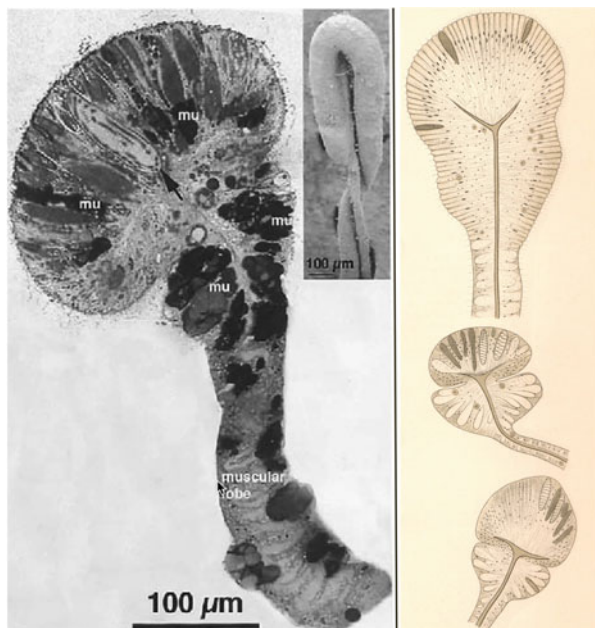
(Thorington and Hessinger 1998), leaving the question of spirocyst function in the dark. Furthermore, cnidae must be released for prey to be ingested, and this entails the wholesale disengagement of the nematocyst and its supporting cells from the tentacle as prey is consumed. This complex action of nematocyst release occurs in response to chemosensitization of prey-derived molecules (Thorington and Hessinger 1998). Both nematocyst discharge inhibition and adhesive failure are restored when no food has been available for 48 h or more (Thorington et al. 2010).

Although the venom of medically significant cnidarians has been examined (e.g., Hessinger 1988; Tezcan 2016), their composition is complex and is often not known in detail. They appear to contain about 250 types of peptides, proteins, enzymes, and proteinase inhibitors, as well as non-proteinaceous substances including purines, quaternary ammonium compounds, biogenic amines, and betaines (Frazão et al. 2012). Few of these have been characterized. Nematocyst venom from sea anemone cnidae contains phospholipases and cytolytic agents including membrane disruptors such as perforins and hemolysins (reviewed by Frazão et al. 2012; Jouiaei et al. 2015). This suggests a potential key role in extracellular digestion, but that aspect has yet to be examined. The contamination of nematocyst preparations with toxins of different origin is an additional confounding issue. For example, when cnidae are isolated en masse from tentacles for analysis, as they typically are, they may be contaminated with mucus discharge, and the mucus from certain anemones appears to contain toxins (Moran et al. 2012). Mucus secretion, whether toxic by itself or not, is an important aspect of coral feeding as described below.

### 18.3.1.2 Feeding and Digestion by Mesenterial Filaments

The mouths of each polyp are primed to open widely in the presence of food, and these openings lead to a muscular and ciliated pharynx before entering the coelenteric cavities. Each of these digestive regions is subdivided by mesenteries, muscular folds of the gastrodermis, and mesoglea whose edges become developed into longitudinal ribbonlike extensions. These “complete mesenteries” are arranged like spokes on a wheel in cross section, and among scleractinians, there are typically 8–12 of them arranged in pairs. In most species, the mesenteries extend half way or farther down the entire length of the coelenteron but rarely extend to its entire length as in anemones (Duerden 1902). There are also shorter partitions of the body wall (incomplete mesenteries) that do not reach the coelenteron. However, all mesenteries, whether complete or not, form thin, threadlike filaments that extend from the aboral ends of the mesenteries and coil toward the base of the coelenteron. A pair of mesenteries corresponds to the number of tentacles; thus, a polyp with 24 or 48 tentacles will have twice that number of mesenterial filaments, many of which have been illustrated in detail by Duerden (1902). Transverse sections typically reveal a bulbous tip, heavily invested with nematocysts and mucocytes, although the predominance of one cell type over the other changes along the filament length.

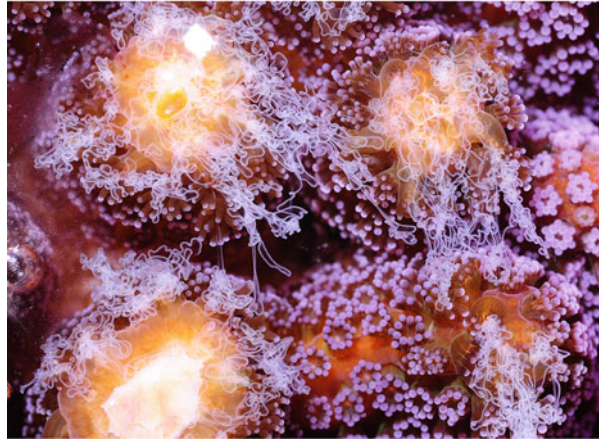




**Fig. 18.3** Left: Toluidine blue-stained transverse sections of a mesenterial filament from the scleractinian *Mycetophyllia reesi*. Numerous, darkly staining mucocytes (mu) occur at the tip; a single large nematocyst is shown (arrow), but many others are hidden below the surface. The muscular lobe and mucus cells are shown on the stalk. Scanning microscopy of the tip (inset, upper right) shows the grooved nature of the stalk, otherwise invisible in section (after Goldberg 2002b). Right: sections taken from varying depths along a filament from *Meandrina* sp. shows the changing nature of the cellular constituents, especially the proportion of nematocysts and mucocytes. The dark center extending through the filament represents mesoglea (after Duerden 1902)

Most coral species are capable of extracoelenteric digestion of food. Mesenterial filaments accomplish this by extrusion through the mouth, temporary openings on the colonial surface, or the column walls (Duerden 1902; Matthai 1918). Each filament exhibits a stalk 2–10 cm long and some exhibit a U-shaped groove containing ciliated cells (Muscatine 1973; Goldberg 2002b). The stalk described by the latter author is unequally bilobed with the larger side exhibiting a well-developed muscular band. The opposite lobe is densely packed with cnidae, mucocytes, collar cells, and zymogen cells. The filament tip is spatulate (Fig. 18.3) and is packed with characteristic zymogen, mucus cells and nematocysts similar to the bulbous filament tips reported from sea anemones (Van-Praët 1985; Shick 1991), although the predominance of one cell type over the other changes along the length of the filament. Zymogen granules are typical of digestive cells and include proenzymes, inactive forms of trypsin, pepsin, and other proteases. Boschma (1925) noted that after food is brought into the coelenteron, vacuoles in the mesenterial filaments become acidic and then alkaline after about 2 days, judging from pH-sensitive dyes, suggesting a pepsin-trypsin-like digestive sequence. Yonge (1930a) established that corals digest mucus-coated food

**Fig. 18.4** Mesenterial filament extrusion in *Stylophora pistillata* after stimulation with a bacterial biofilm. The filaments emerge through the mouths as well as the colonial surfaces. Image courtesy of Tim Wijgerde, Wageningen University, Netherlands



extracellularly by secretion of proteases from the mesenterial filaments. Particles were then phagocytosed or completely solubilized and transported within the filaments by either diffusion or by amoebocytes to complete the intracellular phase of digestion. Raz-Bahat et al. (2017) used histochemistry and RNA transcripts to determine that chymotrypsinogen occurs in the mesenterial filaments of *Stylophora pistillata*. They also report short and long filaments with different cellular arrangements emanating from incomplete and complete mesenteries, respectively, assigning a digestive role to the long ones. However, the fine structure of filaments implies that they have other functions. The occurrence of a ciliated groove along the filament length indicates a role in small particle transport, while the collar cells suggest a phagocytic capacity (Goldberg 2002b). Roles of absorption and egestion (e.g., of zooxanthellae) appear to be associated with the regions proximal to the filament tip as shown by Yonge (1930a). Similar work that extends what is known of coral digestion from the early twentieth century has returned little additional illumination.

The relatively shallow depth of information available on the structure-function relationships of mesenterial filaments has implications for properly assessing the nature and extent of heterotrophy. Filament extrusion can be an impressive event involving much of the colony (Fig. 18.4). Corals can use these structures for aggressive and competitive behavior by extending them onto nearby corals and digesting the tissue down to the skeleton within 12 h. A dominant hierarchy has been established showing that certain species are always winners compared with less aggressive species (Lang 1973; reviewed by Lang and Chornesky 1990). However, the cellular mechanics and event sequences of digestion, as well as the nutritive value to the aggressor, are unknown. Likewise, many scleractinians are unspecialized detritus feeders that use their mesenterial filaments to sweep the surrounding sediment for food (Goreau et al. 1971) or to clear the substratum for colony expansion (Roff et al. 2009). In addition, the role of the filaments in absorption of dissolved organic and particulate matter remains to be assessed, as does the entire potential scope of extracoelenteric feeding. The study by Wijgerde et al. (2011), for example, found that >96% of *Artemia* nauplii fed to *Galaxea*

*fascicularis* were not ingested, but instead were captured by mesenterial filaments and digested externally. Their study counterintuitively indicates that zooplankton digested extracoelenterically by *G. fascicularis* colonies provides them with far more carbon, nitrogen, and phosphorus than does coelenteric digestion. The role of zooplankton nutrition from the perspective of coelenteric digestion is reviewed below.

### 18.3.1.3 Capture of Meso zooplankton

A few corals are able to consume phytoplankton (e.g., Leal et al. 2014a), but most are thought to be active zooplankton predators (Porter 1974; Muscatine et al. 1981; Sebens et al. 1996). Such exogenous food supplies play a large role in the nutritive requirements of growth, skeleton formation, and respiration (Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004a; reviewed by Houlbrèque and Ferrier-Pagès 2009). Yet the degree to which corals are able to sustain themselves by zooplankton feeding is controversial, and some authors have found this food source inadequate for metabolic needs (Clayton and Lasker 1982; Yamamuro et al. 1995; Sammarco et al. 1999). Indeed a tentative ceiling for zooplanktonic heterotrophy as a carbon source for tropical scleractinians is thought to be about 60–66% (Grotoli et al. 2006; Palardy et al. 2008; Houlbrèque and Ferrier-Pagès 2009) although the contribution by nano- and picoplankton will be important as a means of reconciling the true scope of such calculations (see below). Corals may also face challenges of nutrition limitation for other reasons.

Meso zooplankton (Table 18.1) is the typical size range of food that is easily observed during coral feeding. Within this group, corals appear to select poor swimmers, and these plankters are taken disproportionately irrespective of colony morphology or polyp size (Palardy et al. 2005). The natural supply of plankton may vary considerably on a seasonal basis and during the lunar cycle. Thus, selective feeding on favored types of plankton may be cyclically limited (Palardy et al. 2006). While natural plankton is often experimentally supplied, *Artemia* nauplii or cysts often serve as surrogates. Freshly hatched nauplii range from 428 to 517  $\mu\text{m}$ , and hydrated cysts vary from 220 to 285  $\mu\text{m}$  in diameter depending on the strain (Vanhaecke and Sorgeloos 1980; Léger et al. 1987), and these fall within the size range of meso zooplanktonic prey.

Observations of coral feeding suggest that they are relatively inefficient mesoplanktonic predators, as significant numbers of them are often captured and then released, or escape (Hii et al. 2009; Wijgerde et al. 2011). Some types of zooplankton are more likely to contact tentacles at low flow speeds, either due to chance abetted by erratic swimming behavior or to settlement by gravity onto feeding surfaces (e.g., Johnson and Sebens 1993). Conversely, certain copepods that are strong swimmers may be able to avoid tentacles at low to moderate flow speeds, but may be unable to elude interception with stronger flow or more turbulent conditions (Sebens et al. 1996, 1998; Wijgerde et al. 2012). In addition, colony morphology modifies flow and feeding success. Branched corals appear to be more successful in relatively high flows compared with mound-shaped or flat coral colonies, due to drag and turbulence imposed by branching (Sebens and Johnson 1991).

**Table 18.1** Plankton size classes (after Sieburth et al. 1978; Omori and Ikeda 1992)

Macroplankton	Mesoplankton	Microplankton	Nanoplankton <sup>a</sup>	Picoplankton	Virioplankton
> 20 mm	200 µm– 20 cm	20–200 µm	2–20 µm	0.2–2 µm	<0.2 µm
Chaetognaths Salps Euphausiids Pteropods	Copepods Invert/fish Eggs Amphipods Mysids	Zooflagellates Ciliates Radiolarians Tintinnids Diatoms Dinoflagellates	Small autotrophic and heterotrophic flagellates incl. Crysomonads Choanoflagellates Euglenoids Kinetoplastids Coccolithophorids	Heterotrophic bacteria and Cyanobacteria Autotrophic and heterotrophic Picoeukaryotes	Free-living viruses and phages

<sup>a</sup>For example, illustrations in Fenchel (1982)

Laboratory experiments have found that capture is proportional to prey density, increasing with availability until satiation (e.g., Palardy et al. 2006; Tagliafico et al. 2017b). Corals that recently consumed prey fed less frequently than those that had not eaten for a few days (Ferrier-Pagès et al. 2003). There are species-specific differences in prey capture, perhaps due to feeding mechanisms, colonial morphology, number and arrangement of tentacles, polyp density, or surface to volume ratio (Lewis and Price 1975; Johnson and Sebens 1993; Sebens et al. 1996; Hii et al. 2009). In addition, the capture of zooplankton is also dependent on polyp expansion and feeding times. Depending on the species and the environment, coral polyps may be expanded both day and night, only during the day, or only at night when zooplankton concentrations are highest (Lewis and Price 1975). This may be due to the emergence of demersal plankton at dusk, migration of plankton from the open ocean, or release of gametes or larvae (e.g., Heidelberg et al. 2004; Yahel et al. 2005). However, expansion itself is inducible and can be modulated by the interaction of light, current flow, and the presence of food (Levy et al. 2000, 2003).

#### 18.3.1.4 Capture of Micro-, Nano-, and Picoplankton

Several studies have examined the contribution of small plankton groups to the scleractinian coral diet. Nanoplankton includes a diverse group of photosynthetic and heterotrophic flagellates, including dinoflagellates, as well as coccolithophorids, ciliates, and diatoms that range in size from 2 to 20  $\mu\text{m}$ . Picoplankton is the fraction of the plankton community that is an order of magnitude smaller and includes bacterial as well as eukaryotic heterotrophs and phototrophs. One particularly important group of algal picoplankton is a group of coccoid cyanobacteria less than a micron in diameter, which is responsible for 80–90% of the total carbon production annually or daily in oligotrophic tropical marine waters (Longhurst and Pauly 1987; Stocker 1988; Partensky et al. 1999).

The small planktonic groups (micro-, nano-, picoplankton) contribute >50% of the chlorophyll biomass in reef waters whether from Japan (Tada et al. 2003), the Great Barrier Reef (Furnas and Mitchell 1986), French Polynesia (Legendre et al. 1988), or Fiji's Great Astrolabe Reef (Charpy and Blanchot 1999). Picoplankton in these environments may contain up to  $10^6$  bacterial cells  $\text{ml}^{-1}$ ,  $10^4$ – $10^5$  cyanobacteria  $\text{ml}^{-1}$ , and as many as  $10^4$  flagellates  $\text{ml}^{-1}$  (Sorokin 1991; Ferrier-Pagès and Gattuso 1998). Micro-, nano-, and picoplankton may thus constitute an important food source for corals, but verifying their consumption has been indirect by necessity. Field studies have examined the depletion of these organisms as incoming flows pass over reef communities (Patten et al. 2011), but more specific experimental evidence is hampered not only by their small size, but also by their delicate and readily digested architecture. Thus, assessing the fate of these organisms requires radiotracers, chlorophyll autofluorescence, and DAPI stains to demonstrate their incorporation into coral tissue (Sorokin 1973; Ferrier-Pagès et al. 1998a; Houlbrèque et al. 2004b; Tremblay et al. 2012a). In most cases, feeding on picoplankton is deduced from the clearance rate of flow tanks after comparing those with and without corals. Ferrier-Pagès et al. (1998b)

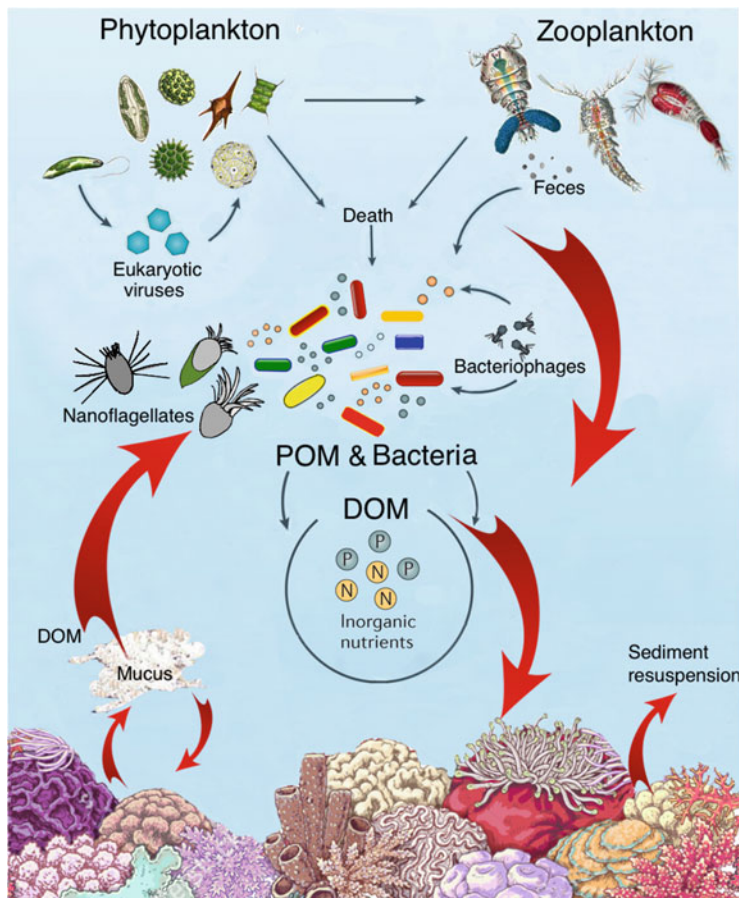
incubated *Stylophora pistillata* with  $^3\text{H}$ -labeled bacteria and ciliates ( $<30\ \mu\text{m}$ ) and found that the amount of radioactivity in coral tissue is proportional to the amount disappearing from the medium. Bak et al. (1998) observed that fluorescently labeled bacteria are taken up and captured by mesenterial cells of the Caribbean coral *Madracis mirabilis*. Further studies of three different coral species revealed that autotrophic and heterotrophic nanoflagellates represent 84–94% of the total ingested carbon and 52–85% of the total ingested nitrogen (Houlbrèque et al. 2004b, 2006; reviewed by Houlbrèque and Ferrier-Pagès 2009). Although a source of dietary nitrogen, cyanobacterial picoplankton is not clearly selected as a component of the scleractinian diet. However, this does not appear to be the case for sponges (e.g., Hanson et al. 2009; McMurray et al. 2016), ascidians (Ribes et al. 2005; Jacobi et al. 2017), or certain octocorals described below.

### 18.3.1.5 The Microbial Loop, POM, and DOM in the Water Column: A Brief Overview

Water column activities in the plankton include multiple feedback loops beginning with carbon fixation by phytoplankton, a group including photosynthetic diatoms, dinoflagellates, and others shown in Table 18.1. Particulate organic matter (POM) is derived from primary producers and zooplanktonic grazing food webs among other sources, despite early studies concluding that such contributions were minimal. However, the importance of this material has been demonstrated by examining reef nutrient budgets (Genin et al. 2009; Sorokin and Sorokin 2010; Wyatt et al. 2013). In part, the reason for the underestimate was the focus on larger particles, often sampled using plankton nets, when in fact most particulates in oligotrophic waters are  $<5\ \mu\text{m}$  (Ferrier-Pagès and Gattuso 1998; Wyatt et al. 2013). Conversely, the relative contribution of allochthonous particulates and other nutrients on reefs compared with those originating in situ may be dependent on local factors including riverine input, upwelling, runoff, offshore plankton blooms, and other factors. Thus, in some environments, the import of allochthonous particulates may be substantial (Wyatt et al. 2010, 2013; Briand et al. 2015), but others suggest that phytoplankton-derived organic carbon and import from oceanic currents, for example, contribute to only a minor fraction, perhaps up to 13% of the total reef primary production (e.g., Alldredge et al. 2013). Regardless of the amount, phytoplankton and grazing zooplankton are important sources of POM as well as dissolved organic matter (DOM).

Up to half of the carbon fixed by phototrophs in the water column is released as DOM (Roshan and DeVries 2017), which includes simple sugars, amino acids, lipids, vitamins, chlorophyll, humic acids, and larger organic molecules up to 100,000 Da. This material is the largest exchangeable reservoir of organic material on Earth and is defined operationally as the fraction of organic matter that is not retained by filtration. This is often a glass fiber filter with a pore size of  $0.7\ \mu\text{m}$ , but there is no universally agreed upon filter or pore diameter (Carlson and Hansell 2015).

The autotrophs supply plumes of nutrients in the form of POM and DOM through leakage of photosynthates, cell lysis and death, or the production of cellular exudate



**Fig. 18.5** The microbial loop of the water column and its relations to the reef benthos including mucus production and consumption in the water column by heterotrophic picoplankton, or incorporation into the sediments and resuspension of organic matter as described in the text

(e.g., Passow 2002; Stocker 2012). The latter material is a sticky, nearly invisible mucus produced by some species, especially diatoms, that causes an increase in size through flocculation, and the sinking of the resulting particulates. These materials and processes initiate the microbial loop (Azam et al. 1983) as illustrated in Fig. 18.5. Larger zooplankton (mesozooplankton) and microplankton feed by grazing on phytoplankton cells as well as on microplanktonic ciliates and detritus (Calbet and Saiz 2005; Nakajima et al. 2017a). They contribute to POM and DOM by death, as well as by ammonium and phosphate leakage, ‘sloppy grazing’, and fecal matter (e.g., Møller et al. 2003). Small fecal pellets from 3 to 150  $\mu\text{m}$  in diameter are ubiquitous in the upper reaches of the ocean and exceed larger fecal particulates by three orders of magnitude in the upper 2 km in some areas. These “minipellets” contain intact bacteria and picoplankton and originate from microzooplanktonic

grazers including ciliates, protozoans, heterotrophic dinoflagellates, radiolarians, and foraminiferans (Turner 2002).

Dissolved organic matter is taken up by phytoplankton but more so by heterotrophic bacteria and archaea due to their absorptive capacity and high surface to volume ratios. In addition, the secretion of hydrolases solubilizes and reduces the size of POM, thereby releasing more DOM (reviewed by Azam and Malfatti 2007). It is estimated that heterotrophic flagellates and ciliates consume roughly half of picoplankton production, which in turn is grazed by higher trophic levels, especially nanoplanktonic microflagellates (Ferrier-Pagès and Gattuso 1998; Ferrier-Pagès and Furla 2001). In addition, some autotrophic nanoflagellates are capable of supplementing their photosynthetic output by bacterial phagocytosis (Laybourn-Parry and Parry 2000).

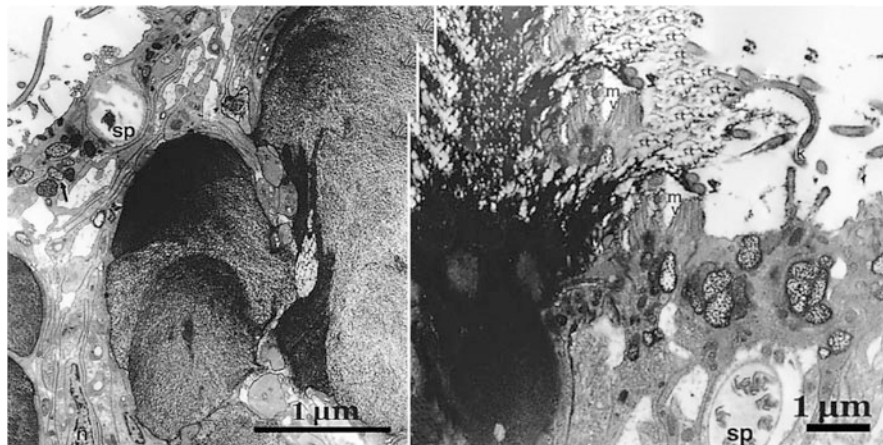
Lastly, the importance of viruses in the microbial loop (Fig. 18.5) cannot be understated. At concentrations of approximately ten million per milliliter of surface seawater, viruses are the most abundant biological entities in the oceans (Breitbart 2012). The majority are phages that lyse their bacterial hosts and may be responsible for the turnover of 20–120% of the daily standing stock of bacteria and archaea depending on location. Likewise, there are also large populations of cyanophages that infect and lyse the smallest and most abundant members of the autotrophic picoplankton (Aylward et al. 2017). In addition, while eukaryotic viruses are of ecological importance everywhere, some of them infect important taxa of phytoplanktonic primary producers. Indeed, phytoplankton blooms appear to be curtailed in part due to large-scale viral infections that are transported by winds over long distances in marine aerosols (e.g., Sharoni et al. 2015). Conversely, these infective agents release cellular DOM and inorganic material into the seawater, which can serve as fuel for further microbial growth and nanoflagellate feeding (reviews by Suttle 2005; Thurber et al. 2017).

The importance of allochthonous particulate organic matter (POM) is increasingly apparent. Recent studies have demonstrated that POM may contribute significantly to reef nutrient budgets (Genin et al. 2009; Sorokin and Sorokin 2010; Wyatt et al. 2010), influence coral calcification (e.g., Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004a), and increase resilience to stress (e.g., Grottole et al. 2006; Ferrier-Pagès et al. 2010).

## 18.4 Benthic and Epibenthic Organic Matter Production and Consumption

The origins of marine POM (and DOM) on shallow coral reefs are varied and include primary producers such as macroalgae and turf algae (Purcell and Bellwood 2001; Wilson et al. 2003; Haas and Wild 2010; Haas et al. 2010; Haas et al. 2011). Seagrass and mangrove communities in reef-associated environments also export organic matter and augment these sources (e.g., Heck et al. 2008; Granek et al.





**Fig. 18.6** Left: Discharge of mucus from coral epidermis. Left: Darkly stained, intact mucocytes. Right: Discharge of mucus cell in response to food. Spirocyst (sp), microvilli (mv), kinocilium (k), after Goldberg (2002a)

2009). POM may also originate from zooplanktonic and piscine fecal matter, its bacterial decay, and coprophagy by planktonic fishes (Robertson 1982; reviewed by Turner 2002; Smriga et al. 2010). Coprophagy continues in the sediments as benthic scrapers and shredders feed on settled fecal matter (Wotton and Malmqvist 2001). While organic particulates are typically carbon-rich, they may also contain organic and inorganic forms of phosphorus and nitrogen that may become sources of nutrition for corals in the water column and the sediments that surround them (e.g., Houlbrèque and Ferrier-Pagès 2009). These nutrients are further considered below.

### 18.4.1 Release of Mucus

Coral tissue is often heavily invested with cells that produce copious amounts of mucus (e.g., Marshall and Wright 1993; Fig. 18.6). Secretions from mucocytes have distinct functions including sediment cleansing, wound healing, and protection from invasive microbes, as well as a shielding from desiccation and UV damage (reviewed by Brown and Blythell 2005). Mucus on the surface of corals is also associated with a diverse and incompletely known core microbiota including viruses, bacteria, and archaea, some of which are thought to be species-specific (e.g., McKew et al. 2012; Nguyen-Kim et al. 2014; Rubio-Portillo et al. 2018). A highly diverse mucus microbiome is thought to perform vital services including nitrogen metabolism, sulfur cycling, and antimicrobial defense, among other functions (e.g., Thurber et al. 2017; Rohwer et al. 2002; Kellogg 2004). Stresses such as elevated temperature, pH, or UV radiation may cause a change in the composition of mucus (Lee et al.

2016), which may induce changes in the microbiome leading to the prevalence of potentially pathogenic species (e.g., Sato et al. 2009; Lee et al. 2015; Sweet and Bythell 2016; Glasl et al. 2016). This area of research is very important for an understanding of anthropogenic effects on corals and is the focus of much current research, but is not further considered here.

Stable isotope signatures suggest that photosynthates translocated by endosymbiotic zooxanthellae represent the foundation of mucus production (Naumann et al. 2010). However, even though the energetic price is quite high at 20–45% of the daily net photosynthate produced (Davies 1984; Edmunds and Davies 1989; Brown and Blythell 2005), coral mucus is continually released into the water column and may contribute as much as half of the total mucus released by all of the benthic organisms (animal and vegetable) on coral reefs (Crossland 1987; Naumann et al. 2010). *Acropora* spp., the dominant scleractinian genus on the Great Barrier Reef, can exude up to 4.8 L of mucus per square meter of reef area per day (Wild et al. 2004a). High light levels can induce such elevated releases, which include both particulate mucus and dissolved organic matter, primarily organic carbon, DOC (Crossland 1987; Naumann et al. 2010). However, mucus release differs among tropical species and conditions. Tanaka et al. (2009), for example, found that only about 5% of the net daily photosynthetic production was released from *Acropora pulchra*. By contrast, Muscatine et al. (1984) found that 6–50% of newly fixed carbon was lost as DOC from *Stylophora pistillata* depending on irradiance levels. Tremblay et al. (2012a) found that the free availability of heterotrophic food as well as high light levels were required for the accumulation of autotrophic carbon, which was then released as DOC in *S. pistillata*. Conversely, release rates of  $^{13}\text{C}$ -labeled fixed carbon dropped under low light levels even when corals were fed (Tremblay et al. 2014). Mucus release appears to constitute a dominant form of organic matter generated in the reef ecosystem (e.g., Hatcher 1988; Ferrier-Pagès et al. 1998a; Bythell and Wild 2011) and represents the main pathway for coral primary production to enter the food web (Hatcher 1988). It is also an important energetic and ecological link between corals, reef waters, and carbonate sediments (reviewed by Garren and Azam 2011). Even cold-water corals release DOC in approximately the same quantities as their shallow-water counterparts (Wild et al. 2008).

Mucus is a complex of very large glycoproteins (mucins), which tend to be sulfated in coral exudates and form gel-like strands that have varying properties of viscosity, polymerization, and flow (Jatkar et al. 2010; Coddeville et al. 2011). Freshly released coral mucus differs among species due to variations in lipid, sugar, and amino acid composition (Ducklow and Mitchell 1979; Crossland 1987; Meikle et al. 1988; Wild et al. 2010b). Particulate mucus may also contain high levels of phosphate if mucus release is coincident with egestion of food from the coelenteron (Nakajima et al. 2015). The nitrogen content of mucus is also variable and may be influenced by planktonic food availability, by the colonization of picoplanktonic organisms, and by nitrogen-fixing bacteria as described below. In addition, mucus may contain zooxanthellae, nematocysts, and coral tissue (Crossland 1987; Wild et al. 2004a, b).

At one time, coral mucus was thought to be a negligible source of nutrients (e.g., Krupp 1984; Coffroth 1990). However, it is now widely accepted that strand or weblike mucus acts as a particle trap for sediment and microorganisms in which pico- and nanoplankton have been shown to increase five- to sixfold (Ferrier-Pagès et al. 2000; Wild et al. 2004a; Allers et al. 2008; Naumann et al. 2009). This growth is up to 50 times faster than in the open ocean (Sharon and Rosenberg 2008; Silveira et al. 2017). Cycles of mucus production, aging, and shedding are accompanied by an increase in total microbial abundance. It is now clear that this colonization enhances the food value of released mucus in terms of carbon (Ferrier-Pagès et al. 1998a; Nakajima et al. 2009) and nitrogen (Grover et al. 2014; Bednarz et al. 2017 and contained references). As this enriched exudate is concentrated or fragmented by currents and cast adrift across the reef, it becomes a downstream pelagic food source for mucus-feeding fishes (Benson and Muscatine 1974) and zooplankton (Gottfried and Roman 1983), and after sinking into the sediment, it is cycled to members of the benthic community (Wild et al. 2005; Huettel et al. 2006; Mayer and Wild 2010; Tanaka et al. 2011; Naumann et al. 2012). It is thus a mechanism by which the pelagic food supply is coupled to the benthos (Fig. 18.5; Naumann et al. 2009; Bythell and Wild 2011).

### 18.4.2 *Mucus and Coral Feeding*

Mucus sheets several hundred microns thick are thought to be a means by which certain scleractinian and gorgonian corals avoid fouling by trapping and then releasing particulate sediments (e.g., Duerden 1906; Coffroth 1990; Tseng et al. 2011; Bessell-Brown et al. 2017). However, mucus is also a major mechanism for the entrapment and ingestion of small nutritive particles (e.g., Carpenter 1910; Yonge 1930b). Its production can serve as an adjunct to more selective zooplankton feeding by the tentacles, or it can be an important or perhaps the primary mode of food acquisition. Lewis and Price (1975) showed that mucus secretion by the epidermis and from the mouth was a common response to the presence of food by corals within several groups and included the production of dense nets, thick strands, or thin filaments depending on the species and the vigor of the water flow. These secretions entangle visible zooplankton as well as fine particulate material (visible as aggregates in Fig. 18.7). Experiments with  $^3\text{H}$ -labeled ciliates and bacteria have shown that the label appears in the mucus before becoming incorporated into the tissues of *Stylophora pistillata* (Ferrier-Pagès et al. 1998b). Corals of various affinities produce and re-ingest their own secretions by guiding mucus toward the mouth with ciliary currents, in some cases whether or not the polyps are expanded (Muscatine 1973; Lewis and Price 1975, 1976; Schlichter and Brendelberger 1998). This mechanism of capture can thus be quite subtle.

The action of ciliary currents by themselves may also become a means of obtaining food. Shapiro et al. (2014) described tracts of  $\sim 12\ \mu\text{m}$ -long cilia on the epidermal surface of corals that they examined using high-speed video microscopy.

**Fig. 18.7** The scleractinian *Galaxea fascicularis* has trapped particulate matter within mucus strands, which are then transported to the mouth and ingested. Image courtesy of Tim Wijgerde, Wageningen University, Netherlands



The cilia created strong counter-rotating vortices that extended up to 2 mm from the coral surface. These in turn drove food particles 5  $\mu\text{m}$  or less parallel to the coral epidermis, suggesting a possible role of self-generated currents in nutrition, and less of a reliance on transport by ambient flows. The function of mucus in conjunction with such ciliary activity has yet to be evaluated.

### 18.4.3 Feeding by Cold-Water Corals

Cold- and deepwater corals rely on carbon input from the ocean surface. Food sources may include zooplankton, bacteria, POM, and DOM (Dodds et al. 2009; Mueller et al. 2014a; Orejas et al. 2016) from shallow-water phytoplankton, zooplankton feeding, and fecal production as well as viral-mediated lysis of bacteria and phytoplankton (Fig. 18.5). Particulate organic matter is also derived from shallow water, especially “marine snow,” primarily composed of sinking polymeric exudates colonized by bacteria, as well as by heterotrophic flagellates and zooplanktonic fecal pellets (Waite et al. 2000; Turner 2015). Coral mucus from shallow water may also be a nutrient source for cold-water corals (Huettel et al. 2006; Rix et al. 2016). This organic rain produced in the epipelagic undergoes dissolution and disaggregation as bacteria consume it, but sinking particulates may also aggregate (reviewed by Lee et al. 2004) and contribute to the food supply for deep-sea communities.

Corals in these environments are frequently reported from sites with relatively high concentrations of organic particulate material. These are transported vertically by internal waves, tidal currents, and the gradients presented by the continental slope or topographic high spots. Such locations may produce an optimal environment for

suspension feeders despite the lack of zooxanthellae (Roberts et al. 2006; Wheeler et al. 2007). One of the common reef-forming corals in the deep North Atlantic and elsewhere is *Lophelia pertusa*, a branched scleractinian that exhibits an opportunistic feeding strategy. While this species feeds readily on zooplankton in low-flow conditions (Purser et al. 2010; Naumann et al. 2011), it is thought to thrive mainly on POM judging from the lipid profiles, stable isotope signatures, and observations of localized mucus secretion in response to the presence of food particles (Mortensen 2001; Duineveldt et al. 2012; Zetsche et al. 2016). The particulates also include phytoplankton resuspended from the bottom by turbulence and that appears to be a preferred food (Mueller et al. 2014a; van Oevelen et al. 2016). In addition, *L. pertusa* is able to take up free amino acids (Gori et al. 2014; van Oevelen et al. 2016) and may also fix inorganic carbon through chemoautotrophic activity of its associated microbiome (Middleburg et al. 2015). Less is known of other species, but fatty acid profiles in two different cold-water corals from >460 m in the Mediterranean suggest that they consume living zooplankton rather than POM (Naumann et al. 2015).

## 18.5 Dissolved Organic and Inorganic Matter

### 18.5.1 *Mucus and the Production of Dissolved Organic Carbon*

Several authors have found that dissolved organic carbon (DOC) constitutes more than half to 80% of the organic content of freshly released mucus (Wild et al. 2004a; Nakajima et al. 2009; Fonvielle et al. 2015). Net release of DOC by corals on a daily basis has been reported for some species (e.g., Crossland 1987; Wild et al. 2010a; Naumann et al. 2010), in particular if the colonies had been fed (Ferrier-Pagès et al. 1998a). Thus, corals are often thought to be sources of DOC rather than sinks (e.g., Hata et al. 2002; Nakajima et al. 2009, 2010). However, in other cases, corals took up more DOC than they released or produced more particulate organic carbon (POC) than DOC, that rapidly settled to the bottom (e.g., Tanaka et al. 2008; Naumann et al. 2010). Thus, the generalization that corals are net producers of DOC remains unclear. In addition, the amount of DOC production by corals under normal circumstances may vary with conditions of light, temperature, and the nutritional state of the colony (Wild et al. 2010b; Mueller et al. 2014b; Courtial et al. 2017). There may also be a delay in the time it takes to convert DOC to an assimilable form (e.g., simple sugars described below). Dissolved organics that are decomposed within hours or days are referred to as labile, compared with semi-labile material that may require a month. Refractory DOC may persist for many months or longer while resisting bacterial growth, perhaps due to antibiotic properties, organic composition, or other factors that influence its bioavailability (Tanaka et al. 2008, 2011; Taniguchi et al. 2014).

Tropical coral reefs survive in oligotrophic waters by means of efficient recycling mechanisms (Wild et al. 2004b; Bourne et al. 2016) including the disposition of mucus. Mucus DOC that is hydrolyzed by microbial activity releases simple sugars, typically including arabinose, mannose, galactose, glucose, and N-acetyl-glucosamine, along with rhamnose, fucose, and xylose in some (Wild et al. 2005). The dissolved fraction may also contain amino acids (Wild et al. 2010b; Nelson et al. 2013). Coral-derived DOC is an excellent medium that induces a rapid increase in bacterial growth (Allers et al. 2008; Nakajima et al. 2015). It also influences the growth and abundance of heterotrophic nanoflagellates that are able to transfer carbon to higher trophic levels through the microbial loop (Nakajima et al. 2017b).

Although mucus DOC is thought to be predominantly recycled and remineralized by microbes in the sediment (e.g., Wild et al. 2004a), it is not clear how much of that process takes place in the water column above the corals (e.g., Walsh et al. 2017), in the coral mucus or tissues, in calcareous reef sands, or within the porous structure of the reef itself (Scheffers et al. 2004; Rusch et al. 2009; Schöttner et al. 2011). Indeed, while coral-associated bacteria may play a key role in the uptake of dissolved organic matter by the holobiont (e.g., Ferrier-Pagès et al. 1998a), coral tissues also appear well suited to that task. The epidermis of their column walls, tentacles, as well as their oral disk and pharynx are similar to the absorptive epithelia of the mammalian kidney and duodenum as Goreau et al. (1971) have pointed out.

Although the ability of corals to take up glucose has been documented (references in Houlbrèque and Ferrier-Pagès 2009), it can be metabolized by zooxanthellae and incorporated into lipids, an activity that is light enhanced (e.g., Oku et al. 2003). This is interesting because lipid production, while vital for growth and storage, is typically envisioned as a function of either photoautotrophy or heterotrophy by the capture of zooplankton (e.g., Baumann et al. 2014). Sorokin (1973) found that radiolabeled DOC constituted up to 20% of the total daily carbon assimilated by several important coral genera. Thus, while the production of DOC by corals is well known, DOC uptake appears to be relatively small compared with sponges, which take up >80% of their carbon as dissolved organic matter (e.g., de Goeij et al. 2008; Mueller et al. 2014c), an ability that may reflect the function of their associated microbiota (e.g., Hoer et al. 2018).

### ***18.5.2 Sources of Dissolved Organic Nitrogen***

Corals can obtain organic nitrogen from a variety of sources including protein from digested plankton (Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004b) or ingestion of nitrogen-rich sediment particles (Mills and Sebens 2004; Mills et al. 2004a). Mucus production and release may represent a loss of dissolved organic nitrogen (DON) for corals that produce it, although the composition is quite variable, ranging from 5 to 59% by dry weight (Ducklow and Mitchell 1979; Meikle et al. 1988), and release rates are greatly influenced by colonial light and feeding history (Ferrier-Pagès et al. 1998a). Mucus nitrogen is enhanced by the activity of N<sub>2</sub>-fixing microbes, which can increase

the nitrogen content 79-fold after only 3 h (e.g., Huettel et al. 2006; Grover et al. 2014). Other sources of DON (and DOC) include high molecular weight compounds released by benthic reef algae including proteins, and other forms of organic nitrogen (Haas and Wild 2010), as well as estuarine input of humic and fulvic acids (Jaffé et al. 2004), all of which are high molecular weight compounds that are unavailable as nutrients for corals without microbial intervention. Urea,  $C=O(NH_2)_2$ , is an important, low-molecular-weight source of nitrogen on coral reefs and is available from the excreta of various organisms including fishes (Meyer and Schultz 1985; Schantz et al. 2017) and seabirds (Lorrain et al. 2017), as well as from heterotrophic feeding by corals. Fishes and seabirds may create ephemeral hotspots of urea that can be up to 20 times the ambient concentration (Crandall and Teece 2012) even though the typical urea levels in seawater are 0–13  $\mu\text{mol}^{-1}$  (e.g., Suzuki and Casareto 2011). Zooxanthellae are capable of using nitrogen in this form, even in nanomolar concentrations (Houlbrèque and Ferrier-Pagès 2009), but some corals can assimilate urea at least fourfold over uptake by the algal fraction (Grover et al. 2006).

Free amino acids represent another form of nitrogen that is available to zooxanthellae (e.g., Sorokin 1973; Ferrier 1991). Grover et al. (2008) found that  $^{15}\text{N}$ -labeled amino acids are taken up within 2 h at 200–500 nmol, concentrations similar to those found in situ. Together with inorganic sources of nitrogen (considered below), dissolved free amino acids are capable of supplying nearly 100% of nitrogen required for tissue growth in *Stylophora pistillata*. Cold-water corals are also capable of taking up amino acids, albeit at lower rates than their tropical counterparts (Gori et al. 2014).

### 18.5.3 Dissolved Inorganic Nitrogen (DIN)

Nitrogen is ordinarily a limiting nutrient for coral growth and metabolism in oligotrophic tropical seas (Hatcher 1990). However, both the coral host and their zooxanthellae are able to assimilate DIN primarily in the form of ammonium ( $\text{NH}_4^+$ ), and nitrate ( $\text{NO}_3^-$ ) to a lesser extent, even at very low concentrations (Muscatine and D'Elia 1978; D'Elia et al. 1983; Grover et al. 2003). Ammonium is the favored form of nitrogen assimilation by corals, and it is most readily available from the digestion of prey in the coelenteron (Grover et al. 2002, 2008). Although the hosts have the ability to assimilate DIN, uptake is more rapid in algal cells, and they accumulate this form of nitrogen 14–23 times more quickly compared with host tissue (Grover et al. 2002; Pernice et al. 2012; Tanaka et al. 2015). Nitrogenous waste products from heterotrophic feeding by the host are either lost to the environment or recycled to zooxanthellae, which may store assimilated nitrogen in the form of uric acid crystals if conditions allow, or the symbionts may actively translocate simple N-compounds to their hosts (Reynard et al. 2009; Kopp et al. 2013).

Coral reefs are sources of fixed nitrogen, that is, conversion of elemental  $\text{N}_2$  to ammonium, and the process has been reported from corals (Lesser et al. 2004, 2007) as well as from other members of the benthic reef community (e.g., Charpy-Roubaud

et al. 2001). While initially thought to be primarily the work of cyanobacteria, recent studies have shown that corals harbor diverse communities of heterotrophic bacteria and archaea that fix nitrogen and produce a distinctive diazotroph-derived nitrogen, DDN (e.g., Olson et al. 2009; Olson and Lesser 2013; Benavides et al. 2016, 2017). A large number of these microbes are found within the surface mucus layer where they produce 400 times the amount of ammonium in seawater, perhaps due to the enhanced organic carbon and inorganic phosphate content of the mucins. Phosphate is required for nitrogen fixation as described below. Corals may capture diazotrophs during feeding, although this activity appears to be dependent on the environment and the nutritional condition of the host, among other factors (Bednarz et al. 2017). Tropical symbiotic corals in shallow water are capable of DDN production, especially during summer months when inorganic nutrient concentrations are lowest (Cardini et al. 2015). However, symbiotic corals in shallow water take up little DDN, perhaps only 1% of their total nitrogen requirements. Those that depend more on heterotrophy (e.g., corals in low-light environments, corals that are bleached, and asymbiotic deepwater corals) assimilate 15-fold more DDN than their shallow-water counterparts (Cardini et al. 2014; Bednarz et al. 2017).

A majority of the  $N_2$ -fixation gene sequences from several corals are related to *Rhizobium*, the diazotroph responsible for nitrogen nutrition in the roots of leguminous plants. Legume-associated rhizobia only fix nitrogen under oxygen-limited conditions; thus, it is possible that the process may be limited to nighttime. However, not all diazotrophs are rhizobia-like and oxygen levels do not limit all nitrogenases (reviewed by Benavides et al. 2017).

Other elements of the nitrogen cycle including nitrification, the oxidation of ammonium to nitrite ( $NO_2^-$ ) or nitrate ( $NO_3^-$ ), have been found associated with coral mucus, tissues, and the skeleton, and are abundant in the microbiomes of several species. The substrate appears to be ammonium derived from coral metabolism and may represent a mechanism of preventing nitrogen loss (reviewed by Rådecker et al. 2015). Likewise, nitrite reduction to ammonia, denitrification, and nitrification all appear to be active within carbonate reef sediments, but further work will be required to determine the ecological significance of these nitrogen cycle elements for corals and the reef ecosystem (Erler et al. 2014; Cook et al. 2017).

#### ***18.5.4 Dissolved Forms of Phosphorus***

Dissolved organic phosphorus in open tropical and subtropical waters exhibit a distinct seasonality due to plankton blooms where dissolved organic phosphate (DOP) often accounts for >80% of the total dissolved phosphorus pool (Lomas et al. 2010). It is a key nutrient for phytoplankton growth, particularly in the production of nucleic acids and phospholipids, as well as for intracellular energy through the production of ATP. By contrast, oligotrophic surface reef waters are typically depleted of phosphorus with concentrations often well below  $0.5 \mu\text{mol l}^{-1}$  (Ferrier-Pagès et al. 2016). Corals typically take up phosphorus as dissolved



inorganic phosphate (DIP), in the form of orthophosphate,  $\text{PO}_4^{3-}$ . Sources include plankton lysis within the microbial loop, runoff from rain, upwelling, sediment resuspension, and microbial degradation of coral mucus (e.g., Wild et al. 2005; Nakajima et al. 2015; den Haan et al. 2016). Zooxanthellae take up significant quantities of this nutrient and have a high affinity for it. They can store phosphorus (see below) when abundant, but it is most often rapidly metabolized and rendered undetectable (D'Elia 1977; Jackson and Yellowlees 1990; Yellowlees et al. 2008). While phosphorus cannot be fixed as nitrogen can, phosphorus is required for  $\text{N}_2$  fixation due to the considerable ATP demands of nitrogenase production by diazotrophs (Mills et al. 2004b; reviewed by Sohm et al. 2011). Thus, the two nutrients are linked, and the addition of small amounts of phosphorus enhances the uptake of nitrogen (Davy et al. 2012; Tanaka et al. 2015). Phosphate is critical for photosynthesis, likely from its multiple products in the Calvin cycle (e.g., Martin et al. 2000), although this has not been specifically examined in zooxanthellae. Likewise, phospholipids are incorporated into the organic matrix of corals and may be vital for the control of nucleation and crystal growth of the skeleton (Ferrier-Pagès et al. 2016 and contained references). However, an undersupply of phosphate coupled to an increase in inorganic nitrogen disrupts photosynthesis and causes a loss of coral biomass, ultimately leading to coral disease and bleaching (Rosenberg et al. 2007; Wiedenmann et al. 2012; Rosset et al. 2017).

Even at low concentrations of DIP, uptake by the symbiotic association is both rapid and is typically light enhanced. Host symbiosome membranes that surround the zooxanthellae *in hospite* are associated with ATPase activity and perhaps represent an active transport mechanism for phosphate. This suggests a way to control the flux of nutrients across the symbiotic interface by the host (Godinot et al. 2009, 2011; Ferrier-Pagès et al. 2016). However, uptake is influenced by a variety of factors including the availability of external and internal reserves and feeding history (D'Elia 1977; Godinot et al. 2009; Ferrier-Pagès et al. 2016). Internal reserves may be different when comparing host to symbiont. Godinot et al. (2016) found that feeding increased intracellular phosphate in host tissue, but primarily in the form of phosphonates, compounds in which P is linked to carbon, along with smaller amounts of phosphate esters. The formation of these potential storage forms of phosphorus appears to be independent of zooxanthellae, which become enriched with intracellular phosphate when their culture media is augmented with inorganic phosphate. However, intracellular phosphate becomes depleted in the presence of *organic* phosphate sources, indicating that they are unable to use that form (Godinot et al. 2016).

Corals are able to obtain phosphate from feeding on plankton including picoplankton, presumably from digestion and reduction to DIP (Sorokin 1973). Indeed, heterotrophic and autotrophic picoplankton are capable of appreciable DOP uptake (Karl and Björkman 2015), suggesting that these organisms may be a rich source of source phosphate. Interestingly, some coral species, or perhaps their symbionts, apparently are able to assimilate DOP directly (Wijgerde et al. 2011). However, a balance sheet for phosphate uptake will have to be reconciled with the release of organic phosphates into the environment, which apparently occurs during

both day and night (D'Elia 1977; Sorokin 1992). The formation of phosphorus compounds, their apportionment between host and symbiont, and environmental feedbacks that may control them are complex and currently are poorly understood.

## 18.6 Heterotrophic Feeding in the Octocorallia

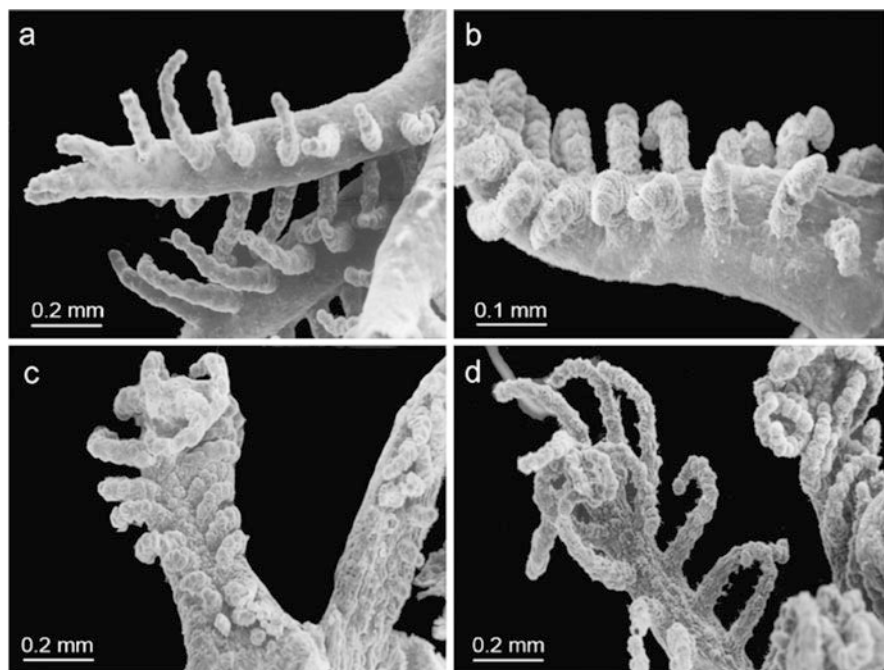
Octocorals in general appear to feed on zooplankton, especially epibenthic copepods and particulate material. The survey undertaken by Lewis (1982) found that food was captured by tentacles in a raptorial manner and was then taken to the coelenteron by ciliary currents in the mouth and pharynx. No mucus or other capture devices (i.e., mesenterial filaments) were employed, as octocorals are not known to protrude them in pursuit of prey (e.g., Fabricius and Alderslade 2001). Octocorals include those with flexible skeletons (gorgonians) tend to orient their branches perpendicular to the prevailing current direction, thereby maximizing the volume of water passing the polyps (Wainwright and Dillon 1969). Whether on flexible skeletons or not (e.g., alcyonacean soft corals), the polyps are bent by moderate flows and likewise are able to feed downstream either at the tentacle tips or on the pinnules (Lasker 1981; Patterson 1991). As in all passive suspension feeders, feeding efficiency is flow-dependent, and differences may be related to polyp morphologies. Those octocorals with tall polyps encounter higher drag and are more easily deformed in currents with flow velocities  $> 10 \text{ cm sec}^{-1}$ , thus interfering with food capture. Conversely, octocorals with short polyps are less readily deformed in currents and feed in a wider range of flow forces, up to  $40 \text{ cm sec}^{-1}$  (Dai and Lin 1993).

Unlike the simple conical tentacles of scleractinians (Fig. 18.2), the arrangement of multiple polyps and their pinnate tentacles form a comb-like filter that can function efficiently as a passive plankton trap depending on pinnule length, shape, and density (Fig. 18.8).

Denser pinnules have a greater chance of capturing small particles. Elongated pinnules have a larger surface area and thus have greater capture efficiencies (Lasker et al. 1983; Grossowicz and Benayahu 2012). Correspondingly, the octocoral diet differs from scleractinians, even though there may be differences among asymbiotic polar, temperate, and tropical species, and those within temperate and tropical regions that are symbiotic.

### 18.6.1 *Alcyonacean Soft Corals*

Alcyonaceans can dominate the shallow waters of the Indo-Pacific. They are typically zooxanthellate, and while some may exhibit low photosynthesis and respiration rates compared with scleractinians (Fabricius and Klumpp 1995), that is not always the case. The P:R ratio in most symbiotic alcyonaceans studied by Sorokin (1991) was more than



**Fig. 18.8** Tentacles from two species of *Dendronephthya*, azooxanthellate soft corals from the Red Sea are shown, *D. hemprichi* (a, b) and *D. sinaiensis* (c, d). Pinnule density and tentacle length likely represent differences in capture efficiency. After Grossowicz and Benayahu (2012) courtesy of the authors

1 (1.4–2.7), perhaps suggesting that they are autotrophs. Likewise, xeniid octocorals are zooxanthellate, but unlike other corals, they actively pulsate, thrusting polyps upward into the boundary layer (Kremien et al. 2013). This movement increases the oxygen content of the surrounding water and enhances both net photosynthesis and feeding efficiency, the expenditure of energy in motion notwithstanding. Zooxanthellate alcyonaceans that have been studied are mixotrophic and deplete the water of particulate matter passing over them (Fabricius and Dommissse 2000). Examination of the gut contents from several species has shown that they feed on zooplankton, especially epibenthic copepods (Lewis 1982). However, using radiocarbon labeling, Sorokin (1991) concluded that most of the octocorals he examined appeared to be poor consumers of microzooplankton and fed instead on bacterioplankton and dissolved organic matter. Likewise, xeniids supplement their photoautotrophic nutrition with particulate organic carbon and nitrogen and DOM (Schlichter 1982; Bednarz et al. 2012). Thus, the diet and the degree to which these corals depend on zooplankton are not yet clear. Tropical soft corals *without* zooxanthellae have an eclectic diet. Those with a pinnule mesh of 60–80  $\mu\text{m}$  capture weakly swimming invertebrate larvae, but >90% of the carbon demand was met by phytoplankton (Fabricius et al. 1995a, b). Following  $^{14}\text{C}$ -labeled diatoms and dinoflagellates, Widdig and Schlichter (2001) found that phytoplankton

were digested in the coelenteron and were apparently transported there by ciliary activity of the mouth and pharynx without the assistance of mucus. The same authors noted that detritus was also part of the diet of this group. By contrast, tropical azooxanthellate soft corals studied by Sorokin (1991), as well as those in the genus *Alcyonium* (Migne and Davout 2002), consumed nearly five times as much zooplankton as phytoplankton in temperate waters. An *Alcyonium* congener similarly appeared to show strong affinity for the capture of small zooplankton (~300 µm) and detritus particles, but not phytoplankton. This approximately corresponded with its 200–280 µm pinnule spacing, although the tentacle tips intercepted most particulate material (Sebens and Koehl 1984; Sebens 1984). Digestion has been little studied in this group although Pratt (1905) noted that *Alcyonium digitatum* incorporated carmine-stained fish macerate into digestive vacuoles of the mesenterial filaments. From there, food particles were then carried through the colony by amoeboid cells in the solenial canals.

### 18.6.2 *Gorgonian Octocorals*

Studies of feeding in zooxanthellate gorgonian octocorals have reported the capture and ingestion of particulate matter (*Artemia* cysts) as measured in flow tank experiments (e.g., Lasker 1981; Lasker et al. 1983). However, examination of the coelenteron has shown that zooplankton in the range of 100–700 µm also are captured, with larger prey items corresponding to species with larger polyps. In addition, microplankton and nanoplankton (diatoms, dinoflagellates, and ciliates) were consumed, but the contribution of these items to heterotrophically derived carbon was about half that of the larger zooplankton. Picoplankton was not a significant part of the diet (Ribes et al. 1998; Coma et al. 1998). Coffroth (1984) found that one tropical species readily ingested mucus. It appears that the carbon balance sheet for tropical symbiotic gorgonians has yet to be directly reconciled, although the consumption of particulate matter by nonsymbiotic species (see below) suggests an additional potential food source.

Temperate gorgonian species show distinct seasonal changes in their diet. The coelenteron contents of a totally heterotrophic species from the Mediterranean (*Paramuricea clavata*) indicate that zooplankton accounts for an important share of the diet. Nauplii, eggs from copepods and other invertebrates, and other small prey items of low motility (100–200 µm) account for 78% of prey items, though adult calanoid copepods (600–700 µm) were also captured. Phytoplankton (diatoms, dinoflagellates) and nanoeukaryotic flagellates and ciliates were also an important part of the diet, as was particulate organic matter with and without living cells, especially during the winter season when it accounted for 96% of the total ingested carbon (Ribes et al. 1999; Coma et al. 1994, 2001). *Leptogorgia sarmentosa*, another asymbiotic Mediterranean species, consumed a similar diet composed of microplanktonic prey including invertebrate eggs and low-motility larvae, as well as nanoeukaryotes, dinoflagellates, diatoms, ciliates, and detrital POC (Ribes et al. 2003; Rossi et al. 2004). Isotopic signatures suggest a seasonal shift from

zooplankton in summer to organic matter in winter (Cocito et al. 2013). As with other temperate gorgonians, picoplankton and DOM consumption was not significant (Coma et al. 1994; Ribes et al. 1999). Similarly, a congener from the temperate Atlantic region, *L. virgulata*, displayed a seasonal isotopic shift in its diet that signaled a change from heterotrophic nanoplankton to microplankton (Leal et al. 2014b).

Particulate organic matter was found throughout the year in the coelenteron of *Corallium rubrum*, another Mediterranean octocoral, and was the main source of dietary carbon along with secondary amounts of copepods, invertebrate eggs, and phytoplankton (Tsounis et al. 2006). There were distinct shifts in prey size and ingestion rate (smaller and lower respectively in winter) in this symbiotic species. However, in addition Picciano and Ferrier-Pagès (2007) demonstrated that detrital organic particulates account for ~93% of their carbon requirements, along with phyto- and zooplanktonic nanoflagellates, and an apparently atypical consumption of picoplankton. A similar seasonal change in diet was noted in another temperate species (*Eunicella singularis*), a relationship with zooxanthellae notwithstanding (Ferrier-Pagès et al. 2015). Zooxanthellae in this species supply considerably less carbon to their hosts than scleractinians in the same habitat, but reduced respiration rates appear to compensate.

Antarctic octocorals seem to show the same trophic behavior as those studied in other latitudes by feeding primarily on a mixed diet of microplankton. Autotrophic nanoflagellates and small diatoms dominate the Southern Ocean in summer (e.g., Bathmann et al. 1997), and a corresponding menu should not be surprising. However, the evidence suggests that when summer sun and the plankton supply fade, the diet changes to heterotrophic nanoflagellates, dinoflagellates, and ciliates, which are present practically all year. In addition, several species supplement this diet either by feeding on sediment in a bent-over position or by feeding on resuspended bottom material. Stable isotopes suggest that winter meals include phytodetritus that accumulates in the winter as a “green carpet” and microzooplankton (Slattery et al. 1997; Orejas et al. 2003; Elias-Piera et al. 2013). Similar seasonal changes in diet have been noted in an Antarctic sea pen (Servetto et al. 2017). Likewise, fatty acid profiles and stable isotopes of carbon and nitrogen suggest a varied and perhaps opportunistic diet among deepwater octocorals. Specimens from the family Paragorgiidae on the continental slopes of Labrador (Newfoundland) and the Sea of Okhotsk likely feed on fresh phytodetritus, whereas primnoids among other gorgonian families appear to supplement their diet of POM by feeding on herbivorous zooplankton (Sherwood et al. 2008; Imbs et al. 2016).

## 18.7 Heterotrophic Feeding in the Antipatharian Corals

Limited observations suggest that antipatharian corals capture zooplankton as a major component of their diet (Lewis 1978; Wagner et al. 2012) with the use of tentacular nematocysts and spirocysts, which appear to be present in abundance (e.g., Goldberg and Taylor 1989a, b; Ocaña et al. 2006; Bo et al. 2008). The

coelenteron contents indicate that large copepods, polychaetes, amphipods, ostracods, and chaetognaths are the most common prey (Tazoli et al. 2007). Likewise, the coelenteric cavities of four species were found with the remains of copepods and small zooplankton (Warner 1981; Carlier et al. 2009). Mucus nets are secreted all over the colony surface and likely entrap food particles, as do the mesenterial filaments (Lewis 1978). Stable isotope analyses likewise suggest that particulate organic matter may be an important source of nutrition for these animals (Williams and Grottoli 2010).

## 18.8 Questions for Future Research

Scleractinian corals have long been used as models of intracellular symbiosis and the mutual exchange of nutrients. However, further work will be required to determine which symbiotic species can upregulate heterotrophic feeding as a means of compensating for a reduction in photosynthesis. Likewise, the sources and use of nutrients by the holobiont, especially phosphorus, will require further work. The roles of nitrogen fixation in coral nutrition and the participation of the nitrogen cycle on reefs remain to be clarified. The manner in which nano- and picoplankton are captured and brought to the coelenteron is also unclear, however likely it may be that mucociliary mechanics are involved. Likewise, the production of mucus by scleractinian corals is prolific, but how much of either the dissolved or particulate forms of this secretory material they re-consume is still unresolved. Many scleractinians employ mesenterial filaments to procure food, either zooplanktonic or particulate. However, little is known of their functional morphology or chemistry. The tentacles possess spirocysts as the principal type of cnidae, but other than characterization as a glutinant, no studies have shown how they are used to obtain prey. The diet of octocorals is distinct from scleractinians, and less is known about zooxanthellate forms in the tropics than those that have been examined from cold-water and temperate environments. Likewise, the degree of reliance on particulate feeding by octocorals and mucus net feeding by anthipatharians would benefit from further study.

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# Chapter 19

## The Suitability of Fishes as Models for Studying Appetitive Behavior in Vertebrates



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**Abstract** Fish have proven to be valuable models in the study of the endocrine control of appetite in response to peripheral signals of energetic and nutritional status. In parallel, a growing body of literature points to the importance of sensory experiences as factors affecting food choice in fish, with a special focus on visual and chemical signals allowing discrimination of potential foods within a 3D environment. Accordingly, waterborne compounds, such as monosaccharides or amino acids, are regarded as the main “olfactory” cues driving fish alimentary behavior. However, we recently suggested that hydrophobic molecules also allow food identification in aquatic environments and that fish actually explore a larger variety of chemosensory cues, including the olfactory/volatile compounds, when determining food palatability. In this study, we show that both homeostatic and chemosensory mechanisms involved in food intake are highly conserved in vertebrates and that the chemosensory world of fish is less different from that of terrestrial mammals than commonly thought. As a result, we support a more integrated and synthetic view of the mechanisms of chemical communication in both terrestrial and aquatic systems, which could help to ensure greater translatability of the fish models, such as the zebrafish (*Danio rerio*), the turquoise killifish (*Nothobranchius furzeri*), the goldfish

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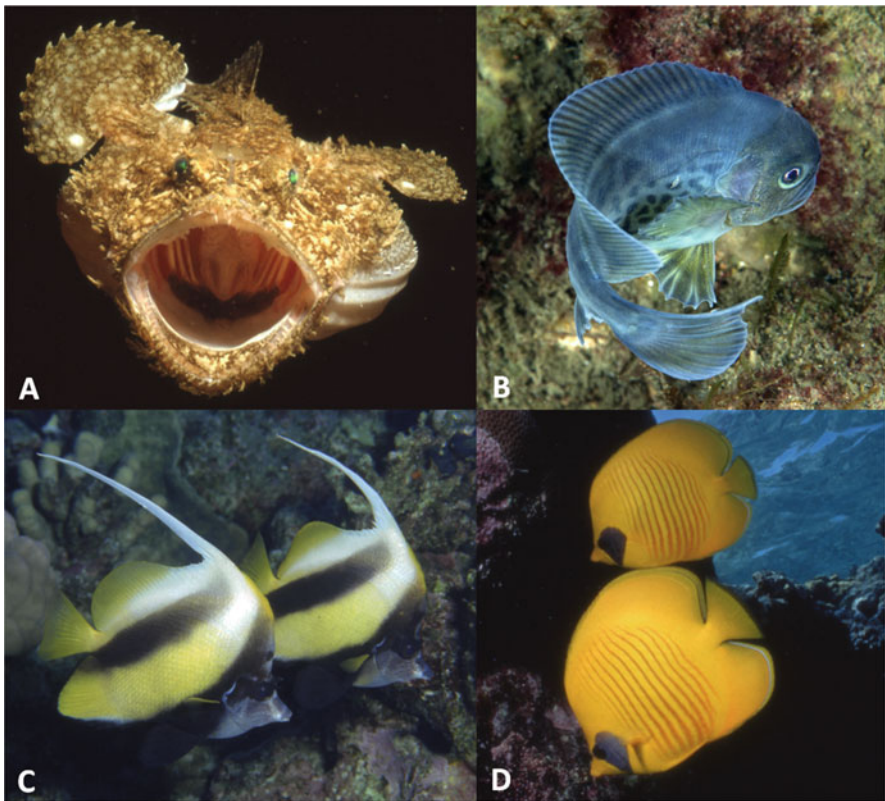
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(*Carassius auratus*), or the Japanese medaka fish (*Oryzias latipes*) to terrestrial vertebrates when approaching complex dynamic patterns in alimentary behavior.

**Keywords** Teleosts · Animal models · Chemical signaling · Foraging behavior

## 19.1 Introduction

Fish feeding behavior includes a variety of feeding habits (da Silva et al. 2016). Some species show generalist habits. Among these are fish with opportunistic and nonselective feeding habits, such as the sit-and-wait angler *Lophius piscatorius* (Fig. 19.1A), which does not search for food in the environment but rather waits for the prey to approach and then quickly swallows it. On the other hand, other species are highly specialized in terms of food items, even feeding on selected benthic invertebrates among sponges and cnidarians, well known to contain species-specific metabolites that deter the generalist fish. As an example,



**Fig. 19.1** The opportunistic feeder *Lophius piscatorius* (A), the jellyfish feeder *Schedophilus medusophagus* (B), the planktivorous *Heniochus diphreutes* (C), and the masked butterflyfish *Chaetodon semilarvatus* (D), which feeds on benthic invertebrates

*Schedophilus medusophagus* (Fig. 19.1B) shows a specialist predation on jellyfish. Consequently, along with shared homeostatic aspects, fish alimentary behavior also depends on the ability of both generalist and specialist fish to discriminate among a vast variety of environmental signals when searching for food.

Current trends indicate that the traditional dichotomy between homeostatic and sensory/hedonic aspects involved in appetite control in vertebrates “should be replaced by a much larger, highly interactive system that unifies homeostasis with reward, cognition, and emotion” (Berthoud et al. 2017), involving a cross talk between the neurochemical substrates of the different involved systems (Higgs et al. 2017). In particular, fish feeding behavior is already known to be regulated by a huge variety of nutrients, visual shapes, colors, and chemosensory molecules in the surrounding environment. This allows fish to make their choices in response both to contingent energy demand and to their innate or adaptive sensitivity to the different signals.

“Optimal foraging theory” assumes that at least some components of foraging behavior must be heritable (Wallin 1988; Gibbons et al. 2005) and that economically advantageous foraging patterns are selected through natural selection (Werner and Hall 1974). However, it has been also proposed that learned behaviors are advantageous over genetically based behaviors, since they have greater adaptability (Immelmann 1975). Apparently, effective learning helps animals in the discrimination among a huge variety of potential food items, given that a well-distinguishable signal favors learning and memory phenomena. For example, a vast number of volatile terpenoids account for species-specific olfactory profiles of many plants. This enables terrestrial herbivores to decode and memorize their “olfactory messages” and eventually eat or avoid the emitters (olfactory aposematism) according to their preferences. On the other hand, we should also expect that heritable feeding preferences of specialist feeders are mediated by species-specific signals. In all cases, the environmental signals must be integrated with endogenous signals that come to the brain from the periphery of the body, mostly coming from the gastrointestinal tract, indicating the metabolic and energetic state, to determine the resulting final alimentary behavior. Consequently, the translatability of studies on the alimentary behavior across a broad range of vertebrate species, from fish to terrestrial mammals, should necessarily proceed through comparison of conserved homeostatic and chemosensory mechanisms.

## 19.2 The Endocrine Signals

The basic vertebrate mechanisms involving central and peripheral signaling in the control of appetite has been recognized in several teleost species, making them suitable models for investigating the neurophysiology of appetite control when mediated by internal stimuli. Furthermore, fish are attractive models because they have different feeding habits and have evolved different strategies for food intake and different adaptive morphological organizations of the digestive apparatus (D’Angelo et al. 2016a, b). As an example, zebrafish, goldfish, and medaka fish are omnivorous



and agastric, whereas turquoise killifish are omnivorous, albeit preferring lipid-enriched diet, but have a well-developed stomach (D'Angelo et al. 2016b).

Terrestrial vertebrates and fish differ in many aspects of their physiology, especially in terms of metabolic rates, energy storage, and growth patterns (Volkoff 2016). However, in spite of these differences, they share similar endocrine signals (mainly hormones and neuropeptides) regulating physiological mechanisms involved in appetite control (Rønnestad et al. 2017). Such signals regulate food ingestion by stimulating (orexigenic) or depressing (anorexigenic) appetite, thus acting as modulators of hunger or satiety under peripheral/gastrointestinal control. Central orexigenic neuropeptides co-occurring in mammals and fish include neuropeptide Y (NPY), orexin A and B (OX-A and OX-B), galanin (GAL), and melanin-concentrating hormone (MCH). The anorexigenic signals include instead cocaine- and amphetamine-regulated transcript (CART), the corticotropin-releasing hormone (CRH), and melanocortin peptides processed from the polypeptide precursor pro-opiomelanocortin (POMC). The above neuropeptides are produced by hypothalamic neurons, which are located, both in mammals and fish, in proximity of the ventricle. Here, peripheral chemicals (e.g., glucose or fatty acids) or endocrine factors (e.g., gastrointestinal hormones) circulating in the blood can more easily cross the blood-brain barrier, exerting a direct action on those neurons. These latter are, in fact, also responsive to hormones such as leptin, insulin, ghrelin, cholecystokinin, and other gastrointestinal hormones, which bind to specific receptors (Delgado et al. 2017). Central neuropeptides and related receptors (i.e., OX-A and OX-2R) are also localized at intestinal levels (Russo et al. 2011; D'Angelo et al. 2016a) and open up to a potential bidirectional signaling of the gut-brain axis in fish.

### 19.3 The Aminergic and Endocannabinoid Systems

Monoamine neurotransmitters act as major regulators of food intake in vertebrates (Lamb 2007). In particular, serotonin (5-hydroxytryptamine, 5-HT) administration is reported to suppress food intake both in mammals (Blundell 1977; Wierucka-Rybak et al. 2016), and several fish (Ortega et al. 2013). Similarly, the endocannabinoid system also plays a major role in the regulation of appetite in terrestrial vertebrates, by modulating serotonin signaling in rats (Wierucka-Rybak et al. 2016). Orthologs of the mammalian cannabinoid CB1 and CB2 receptors have been also identified in fish (Valenti et al. 2005) where they contribute to the control of food intake too. In particular, administration of anandamide (AEA), the endogenous agonist of cannabinoid receptors, causes a dose-dependent effect on food intake in the goldfish (Valenti et al. 2005) and in the sea bream *Sparus aurata* (Piccinetti et al. 2010). In the latter case, AEA administration stimulates the expression of the orexigenic peptide NPY (Piccinetti et al. 2010). It has also been suggested that the endocannabinoid system modulates associative learning and memory in zebrafish (Ruhl et al. 2015). On the other hand, CB1 receptor promotes food intake in mice by increasing odor detection (Soria-Gómez et al. 2014). Overall, the endocannabinoid

system has thus emerged as a major mediator of hedonic aspects of reward processing (Friemel et al. 2014), including those associated with odor or taste perceptions. However, an unbiased approach to reward processing in fish will require overcoming the current prejudice that terrestrial and aquatic vertebrates perceive extremely different classes of chemosensory signals (Tierney 2015).

## 19.4 The Chemosensory Receptors and Their Affinity for Ligands

Most molecular chemosensory modalities are well conserved in vertebrates. In particular, olfactory receptor (OR) genes belonging to the vast family of G-protein-coupled receptors (GPCR) have been identified both in terrestrial vertebrates and in several fish species (Hino et al. 2009), including zebrafish (Niimura and Nei 2005) and the coelacanth fish *Latimeria chalumnae* (Picone et al. 2013). Taste GPCRs are also well conserved from fish to terrestrial vertebrates (Oike et al. 2007; Bachmanov et al. 2014; Morais 2017). Given that smell and taste receptors allow discrimination among different chemical signals, their specific affinities for specific ligands must be also conserved. Instead, this patently obvious consideration is commonly contradicted in the literature, where the idea that the stimulating chemicals for fish are necessarily in solution and only water-soluble molecules are regarded as olfactory signals, widely prevails. Currently, in fact, “waterborne” substances are considered the main ORs-binding substances detected by the olfactory system of fish (Shiple et al. 2003; Niimura and Nei 2005; Derby and Sorensen 2008; Hino et al. 2009; Niimura 2012). As in the case of vision, the olfactory sensory experience is widely associated with searching for distant objects within a 3D space. This implies that ORs of aquatic and terrestrial vertebrates detect opposite bouquets of odorants and that “terrestrial vertebrates were given the ‘evolutionary option’ to sense odorants that were volatile and not soluble in water” (Tierney 2015). On the other hand, this also implies that a dramatic change in the patterns of affinity of chemosensory molecules suddenly occurred during transition from aquatic to terrestrial olfaction. However, in the light of the highly combinatorial character of the molecular mechanisms of olfaction, such a dramatic “saltation” seems to be highly unlikely. The same sort of reasoning would also apply to taste, which is instead strictly considered as a proximal sense. We believe that such a classification of sensory perceptions based on spatial range, which limits the chemosensory world of fish to water-soluble rather than to volatile chemical stimuli, is conceptually misleading (Mollo et al. 2014, 2017). It also presents several negative practical implications, including the creation of an insurmountable obstacle to the translatability of data on eating behavior from fish to terrestrial vertebrates. As underlined in our recent article, a first step toward overcoming this obstacle is to recognize the ability of aquatic organisms to perceive typical taste molecules at a distance and olfactory substances by contact (Giordano et al. 2017).

## 19.5 The Natural Cues

Gustation and olfaction in teleost fish are both believed to respond to aqueous chemical stimuli (Lamb 2007). Accordingly, waterborne amino acids and sugars, which are commonly detected by taste receptors in terrestrial vertebrates, are also considered potent stimulants of fish olfactory systems. This generates a confused picture that can only be sustained by denying any specificity in the interactions between chemosensory signals and their macromolecular counterparts, namely, olfactory and gustatory receptors. We have recently proposed, instead, that perhaps it is time to repudiate the traditional and widespread practice of differentiating between gustation and olfaction on the basis of their spatial range, eventually to adopt a generalized view of chemoreception (Mollo et al. 2017). However, the problem is not limited to this aspect, of relatively easy resolution by better defining the affinities of taste receptors for specific ligands. Amino acids, nucleotides, steroids, prostaglandins, and bile acids are commonly accepted as main odor classes detected by the olfactory system of fish (Hara 1994; Shipley et al. 2003; Derby and Sorensen 2008; Hino et al. 2009; Buchinger et al. 2014; Zufall and Munger 2016), while the possible ability of fish to detect the vast variety of natural compounds occurring in aquatic environments not included in the above categories is almost ignored in the literature. Moreover, it is worth mentioning that none of the above classes of compounds guarantee a univocal identification of possible food sources. Most importantly, their putative high solubility in water does not correspond to reality in the case of steroids and prostaglandins (Mollo et al. 2017).

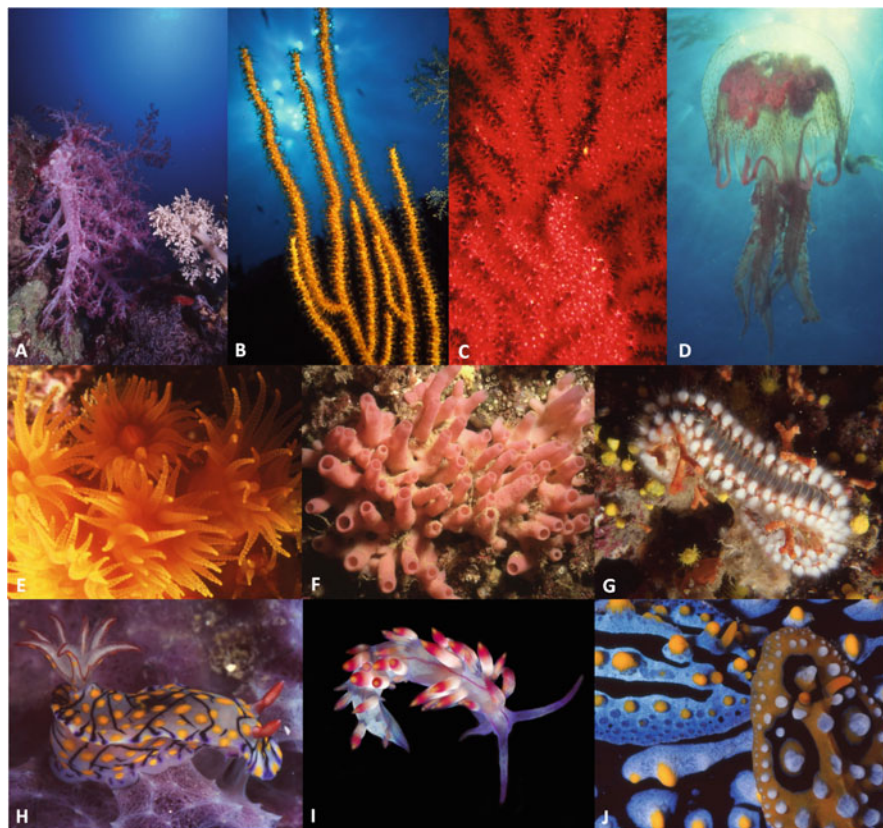
Amino acids can certainly indicate the presence of food sources, but, given their ubiquity, they cannot allow easy discrimination among them. Consequently, they influence the alimentary behavior of both terrestrial vertebrates and fish, and they do so through interactions with well-conserved taste receptors (Oike et al. 2007; Bachmanov et al. 2014; Morais 2017), by providing important but non-specific information. Similarly, water-soluble nucleotides are ubiquitous in many foods both on land and in water, and they can intensify the umami sensation caused by glutamate, potentiating umami taste (Chaudhari et al. 2009). Steroids, instead, are a major class of compounds characterized as being fat-soluble organic compounds that can easily pass through cell membranes. Despite their very low solubility in water, they are believed to be sensed by the “olfactory organs” of fish (Mollo et al. 2017). Furthermore, their best-studied receptors are indeed nuclear transcription factors rather than membrane chemosensory receptors. Also prostaglandins are generally insoluble in water (Inaba et al. 1984), so that solvents such as methanol and DMSO have been used for their solubilization when recording both electro-olfactograms and behavioral responses in fish (Sorensen et al. 1988; Yabuki et al. 2016). Although it has been proposed that prostaglandin  $F_{2\alpha}$  can also activate olfactory receptors in zebrafish, prostaglandins generally activate specific GPCRs, called prostanoid receptors, exerting a variety of actions in various tissues and cells (Narumiya et al. 1999). Finally, bile salts are also considered potent olfactory stimuli in fish (Buchinger et al. 2014). However, it has also been shown that they act as effective taste stimuli in

channel catfish (Rolen and Caprio 2008). Adding to the list, the naturally-occurring sulfur-containing compounds dimethyl- $\beta$ -propiothetin, dimethylthetin, dipropyl (di) sulfide, dimethylsulfide (DMS), and dimethyl sulfone, act as feeding stimulants for freshwater fish (Nakajima et al. 1989). Overall, the above classes of compounds are believed to mostly influence the feeding behavior of fish searching for food in a 3D space, first activating the so-called olfactory sense, then inducing feeding motion and prey capture.

However, we should ask ourselves if an immense variety of volatile and insoluble olfactory compounds not included in the above categories and contributing to the flavors of most foods on land could also be perceived by fish, driving their alimentary choices in water. On the one hand, a vast variety of biogenic volatile organic compounds (BVOCs) have an enormous potential for mediating significant ecological interactions in land plants and animals (Penuelas et al. 1995). On the other hand, a wide array of terpenoids, including the volatile ones, also occur in aquatic organisms, especially in cnidarians (Fig. 19.2A–E) and sponges (Fig. 19.2F), playing important roles in chemical defense, competition for space and reproduction, and in the maintenance of an unfouled surface (Gross and König 2006). Some of those compounds are exactly the same occurring on land (Mollo et al. 2017). Some of them are toxic, and their toxicity assumes a relevant ecological significance and impact on feeding behavior especially when it is associated with the presence of visual aposematic or chemosensory aposematic signals (Eisner and Grant 1981), which favor avoidance learning in fish that experienced postingestive negative effects (Giordano et al. 2017). Among benthic animals, many brightly colored nudibranchs (Fig. 19.2H–J) are known to contain compounds acting as toxins, avoidance-learning inducers, and chemosensory cues (Cimino and Ghiselin 2009; Haber et al. 2010; Carbone et al. 2013; Mudianta et al. 2014; Cheney et al. 2016).

Nudibranchs, however, are trophic specialists that obtain their defensive weapons from chemically defended sponges and cnidarians, or from other animals such as bryozoans, and reuse the dietary compounds for their own defense against predators, including fish. It is worth mentioning here, that many fish species also exhibit dietary preferences for sponges and cnidarians that are rich in terpenoids (Sano 1989; Alino et al. 1992; Ruzicka and Gleason 2008; Powell et al. 2014; Loh and Pawlik 2014). Little is known, however, about the ability of those fish of discriminating among different hydrophobic terpenoids when searching for food. This is a crucial issue not only for generalist fish to avoid defensive molecules contained by many benthic organisms (Cimino et al. 1983; Nagle et al. 1996; Chanas et al. 1997; Marin et al. 1997; Wilson et al. 1999; Manzo et al. 2004; Garson 2010; Mollo et al. 2014; Giordano et al. 2017; Fisch et al. 2017) but also for the trophic specialists, the survival of which depends on the availability and proper recognition of the preferred prey.

Similar considerations can be extended to other classes of compounds, such as flavonoids, widespread in aquatic (Al-Saif et al. 2014; Zidorn 2016) and terrestrial plants (Miean and Mohamed 2001; Szewczyk et al. 2016) and representing a large and rather heterogeneous class of molecules endowed with broadly ranging water



**Fig. 19.2** Images show chemically defended marine invertebrates. Various species of soft corals (A). The brightly colored gorgonians *Eunicella cavolini* (B) and *Paramuricea clavata* (C). The mauve stinger jellyfish *Pelagia noctiluca* (D). The hexacorals *Astroides calycularis* (E). The sponge *Haliclona mediterranea* (F). The bearded fireworm *Hermodice carunculata* (G). A spongivorous nudibranch belonging to the genus *Hypselodoris* (H). The aeolid nudibranch *Coryphellina rubrolineata* that feeds on hydroids (I). The highly toxic phyllidiid nudibranchs (J)

solubilities. Furthermore, they can occur either in free form or as more soluble glycoside derivatives.

To further complicate matters, most studies on the influence of flavonoids and terpenoids on fish feeding behavior have been performed on plant parts or extracts (often containing both classes of compounds), and they have been mostly directed to practical applications in aquaculture or fishing (Venkateshwarlu et al. 2009; Zhu et al. 2010).

Nonetheless, some results clearly indicate that some compounds or fractions characterized by high hydrophobicity and from poor solubility to almost complete insolubility in water act as feeding attractants or appetizing ingredients. Among them, two particularly relevant examples of convergence between terrestrial vertebrate and

fish appetitive behavior are represented by anise essential oil and kaempferol. Anise oil is widely used in fish baits,<sup>1</sup> zootechnics (El-Deek et al. 2002; Charal et al. 2016), and as attractant for invertebrates such as bees (Waller 1970). Kaempferol is a fish feeding stimulant (Zhu et al. 2010), as also are terrestrial plants and their parts in which this compound is particularly abundant, such as the rhizomes of *Kaempferia galangal* (Venkateshwarlu et al. 2009), and many algae, including red algae such as *Gracilaria dendroides* (Al-Saif et al. 2014) and *Acanthophora spicifera* (Zeng et al. 2010), consumed both by herbivorous fish (Weijerman et al. 2008) and by humans (South 1993; Trono 1999; Payri et al. 2000; Mahadevan 2015). Kaempferol is also present in other terrestrial plants or their parts endowed with well-known appetizing properties for humans, such as caraway (Kunzemann and Herrmann 1977), clove (Shan et al. 2005), cumin (Pandey et al. 2015), fennel (Kunzemann and Herrmann 1977), sage (Shan et al. 2005), and cape (Inocencio et al. 2000).

Certainly, compounds almost insoluble in water cannot efficiently convey information in water within a 3D space, which is a prerogative of waterborne compounds allowing fish to identify distant prey items as much as the visual appearance does (Cole and Endler 2015). We recently proposed that the behavior of fish to repeatedly take food into their oral cavity and then reject it, before swallowing or refusing it, is due to the crucial need for sensing by contact substances that cannot be perceived at a distance by the fish “nostrils” (Mollo et al. 2014; Giordano et al. 2017). Therefore, we believe that in-depth exploration of this countercurrent hypothesis will be needed to open up new avenues to better understand the fish feeding behavior, finally clarifying that their chemosensory world is larger and more diversified than currently thought.

## 19.6 Anthropogenic Xenobiotics

Examples of anthropogenic xenobiotics that influence the feeding behavior of both fish and terrestrial vertebrates through endocrine disruption, increase of hunger, accumulation in chemosensory organs, and their consequent functional alteration are also reported in the literature. These contaminants can have large-scale effects in aquatic ecosystems even at sublethal doses, disturbing fish alimentary behavior and altering the food web.

Insecticides belonging to the class of acetylcholinesterase inhibitors (AChEIs) have a critical impact on both fish (Baldwin et al. 2009) and human health (Mandour 2013). However, AChEIs are also used to treat the cognitive symptoms of dementia and can induce appetite loss due to gastrointestinal side effects in patients with Alzheimer’s disease (Droogsma et al. 2013; Soysal and Isik 2016). Among AChEI insecticides, chlorpyrifos reduces feeding activity in salmonids (Sandahl et al. 2005),

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<sup>1</sup>Examples of related Patents: CA2000186A1, DE102013014777A1, US1922841, US7296378, US5827551, US5333405A, US4828829, US3421899, US5089277.

while malathion has been proposed to disrupt both endocrine functions and the olfactory sensations responsible for food intake in a freshwater catfish, *Clarias batrachus* (Lal et al. 2013).

Cadmium from industrial and agricultural sources has been recognized as an important health hazard (Järup and Åkesson 2009). This toxic heavy metal has also been tested on the rainbow trout, *Oncorhynchus mykiss*, for its effects on the fish feeding behavior. It has been noticed that the accumulation of cadmium mainly takes place in the olfactory tissues of the fish and that the disruption of the feeding behavior would be caused by the alteration of the olfactory system (Scott 2003).

The effects of butyl benzyl phthalate (BBP) and p,p-DDE, which is a DDT metabolite, on feeding behavior have been evaluated in three-spined stickleback *Gasterosteus aculeatus*, showing that fish feeding behavior may be efficiently used in the detection of the effects of pollutants (Wibe et al. 2004).

Pharmaceuticals and personal care products such as triclosan, diclofenac, and carbamazepine, which represent potentially toxic chemicals in aquatic environments, have also been shown to affect the feeding performances of Japanese medaka fish (Nassef et al. 2010).

## 19.7 Conclusions

Chemical signaling has critical impact on the alimentary behavior of all terrestrial and aquatic forms of life, from unicellular organisms to humans. We have shown that both fish and land vertebrates are equipped with similar chemosensory and homeostatic systems regulating their feeding behavior. However, we have also shown that fish, together with all terrestrial mammals, not only sense waterborne compounds determining food palatability but also perceive a wide variety of chemical signals including species-specific lipophilic terpenes. The different kinds of perceived chemical signals are thus also conserved in the vertebrates. Consequently, model fish could be efficiently used to help scientists understand the complex patterns of alimentary behavior, including those involving the perception of insoluble compounds. This opens new avenues leading to a better understanding of feeding behavior in vertebrates and to employ fish models in virtually all fields of biological and biomedical research focusing on modulation of eating behavior and the optimization of food intake.

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# Chapter 20

## Glycans with Antiviral Activity from Marine Organisms



I. D. Grice and G. L. Mariottini

**Abstract** There remains today a critical need for new antiviral agents, particularly in view of the alarming increase in drug resistance and associated issues. The marine environment has been a prolific contributor towards the identification of novel therapeutic agents in the recent few decades. Added to this, glycans (or carbohydrate- or sugar-based compounds) have in very recent decades made outstanding contributions to the development of novel therapeutics. This review brings together these significant facets of modern drug discovery by presenting the reported literature on glycans derived from marine organisms that possess antiviral activity.

The glycans have been grouped together based on the marine organism they were isolated from, namely, (1) bacteria, (2) chromists, (3) plants and (4) animals. For chromists, glycans are further subsectioned into Ochrophyta (brown algae), Miozoa (according to [www.algaebase.org](http://www.algaebase.org); also called Myzozoa according to WoRMS, [www.marinespecies.org](http://www.marinespecies.org)) (dinoflagellates) and Bacillariophyta (diatoms). For plants, glycans are further subsectioned into Chlorophyta, Rhodophyta and Tracheophyta. Glycans isolated to date are reported as alginates, chitosan, extracellular polysaccharides, fucans (e.g. fucoidans), galactans (e.g. carrageenans), glycolipids, glycosaminoglycans, glycosides, glycosylated haemocyanin, laminarans, mannans, polysaccharides (not defined), rhamnans and xylomannans. Interestingly, many of the glycans displaying antiviral properties are sulfated.

Reports indicate that marine-sourced glycans have exhibited antiviral activity against African swine fever virus, cytomegalovirus, dengue virus, Epstein-Barr virus, encephalomyocarditis virus, human immunodeficiency virus, hepatitis C virus, herpes simplex virus, human cytomegalovirus, human papilloma virus, human rhino virus, influenza virus, Japanese encephalitis virus, murine leukaemia

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virus, murine sarcoma virus, Newcastle disease virus, parainfluenza virus, respiratory syncytial virus, Semliki Forest virus, tobacco mosaic virus, vaccinia virus, varicella zoster virus, viral haemorrhagic septicaemia virus and vesicular stomatitis virus. Selected representative glycan structures are presented in Fig. 20.1.

## Abbreviations

ASFV	African swine fever virus
CMV	Cytomegalovirus
CS	Chondroitin sulfate
DENV	Dengue virus
EBV	Epstein-Barr virus
ECMV	Encephalomyocarditis virus
EPS	Exopolysaccharide
Fuc	Fucose
GAGS	Glycosaminoglycans
Gal	Galactose
Glc	Glucose
GlcA	Glucuronic acid
GS	Galactan sulfate
GulA	Guluronic acid
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSV	Herpes simplex virus
HRV	Human rhino virus
IduA	Iduronic acid
IFV	Influenza virus
JEV	Japanese encephalitis virus
Man	Mannose
ManA	Mannuronic acid
MuLV	Murine leukaemia virus
MuSV	Murine sarcoma virus
NDV	Newcastle disease virus
PIFV	Parainfluenza virus
PS	Polysaccharide
Qui	Quinovose
Rha	Rhamnose
RSV	Respiratory syncytial virus
SF	Sulfated fucan
SFG	Sulfated galactan
SFV	Semliki Forest virus

SG	Sulfate groups
SGF	Sulfated galactofucan
SGP	Sulfated galactan polysaccharide
SP	Sulfated polysaccharide
SPMG	Sulfated polymannuroguluronate
SQDG	Sulfoquinovosyldiacylglycerol
SXM	Sulfated xylomannan
TMV	Tobacco mosaic virus
VC	Vaccinia virus
VHSV	Viral haemorrhagic septicaemia virus
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
Xyl	Xylose

## 20.1 Introduction

In our so-called modern era, viral infections remain a major global threat to human health. This situation exists despite the significant success that has been achieved in the development of antiviral drugs for treatment of some viruses. For some viral diseases, vaccines and antiviral drugs have allowed us to keep infections from spreading widely and have helped virally afflicted to recover. However, some patients suffering from viral infections experience treatment failures, due largely to virulent recombinant viruses, drug resistance and cell toxicity (Irwin et al. 2016). Such failures fuel the threat of a global spread of emerging, re-emerging and drug-resistant viral strains. Examples include human immunodeficiency virus (HIV) (Leong et al. 2017), hepatitis C virus (HCV) (Lazarus et al. 2014; Saleem et al. 2016), dengue virus (DENV) (Mayer et al. 2017) and influenza virus (IFV) (Yen 2016), which affect large proportions of the world's population. In response to the limitations of current antiviral agents, there is a constant search for new potent and effective antiviral drugs capable of negating or circumventing these issues.

Nature and in particular terrestrial resources (e.g. plants and microorganisms) have provided significant therapeutics to the drug market. However, it is reported that in the 30-year period prior to 2010, only 18 out of the 110 antiviral drugs on the market were derivatives of natural products. Although the percentage of drugs that were synthetic mimics and analogues of natural products was much higher, the figures clearly indicate the important role of natural resources in providing templates for new therapeutics (Newman and Cragg 2012).

In more recent times, attention has been directed to the marine environment, which contains estimated 50% of all species on the planet (Vo and Kim 2010). Interestingly though it was reported in 2015 that less than 0.01–0.1% of all marine microbial species were known to scientists, clearly indicating the under-investigated resource the oceans present in regard to identifying novel chemicals as potential



therapeutic drugs (Simon and Daniel 2011). In recognition of this, there have been significant efforts in recent decades directed towards exploration of the earth's oceans for novel chemicals, and consequently there have been significant increases in the number of novel products isolated from marine organisms. The decade prior to 1985 produced on average less than 100 novel compounds isolated annually from the marine environment, while the period between 1985 and 2005 averaged out to approximately 500 novel compounds annually. The period post 2005 saw a sharp rise in the number, with more than 1000 novel compounds a year discovered post 2008, although interestingly those with antiviral activity made up only around 2% of the number possessing known biological activity (Hu et al. 2015).

The marine environment is regarded as extreme. Depending upon their own living environment and consequently the latitude, ocean depth, etc., organisms are exposed to conditions such as high salinity, poor nutrition, low temperatures (although thermal vent temperatures would be of extreme elevation) and high pressures. Collectively these conditions result in unique metabolisms and therefore unique metabolites and materials, differing markedly from those in the terrestrial environment. Added to this, the oceans are hyper-abundant with viruses. It is reported that virus numbers reach up to  $10^7$  particles/mL, outnumbering bacteria by a factor of 10. In this extreme hyper-virulent environment, it is estimated that bacteria suffer  $10^{23}$  viral infections/second throughout the planet's oceans (Suttle 2007; Sanchez-Paz et al. 2014). Yet many marine organisms achieve outstanding life spans, despite living in such virus-rich conditions. For example, the ocean quahog (*Arctica islandica*), an edible bivalve clam, routinely lives for over 400 years (Philipp and Abele 2010).

It is well known that animals, plants, fungi and bacteria produce chemicals to protect themselves against such pathogens as viruses. To date, marine organisms from five of the six marine kingdoms, namely, bacteria, chromists, fungi, plants and animals (none from protozoans)—as described in recent taxonomy (Cavalier-Smith 1998; Ruggiero et al. 2015)—have reports on antiviral agents from diverse chemical classes, including alkaloids, anthraquinones, glycans, lactones, peptides (including lectins), steroids, terpenoids and xanthenes, to name a few (Uzair et al. 2011; Cheung et al. 2014; Ahmadi et al. 2015; Dang et al. 2015; Moghadamtousi et al. 2015). It is in this context that the present review focuses on structural and biological activity aspects of glycans from marine sources possessing antiviral activity. The selection of this class of chemicals is reflective of the ever-increasing significance that glycans play in vital human biology and human-pathogen interactions, along with the enormous diversity of glycans in nature in general (Varki and Lowe 2009).

For taxonomical purposes in this review, we refer to the above cited papers (Cavalier-Smith 1998; Ruggiero et al. 2015), as well as to Guiry and Guiry, [www.algaebase.org](http://www.algaebase.org) (Guiry and Guiry 2017), and WoRMS, [www.marinespecies.org](http://www.marinespecies.org) (WoRMS 2008).

### **20.1.1 Antivirals: Definition and Therapeutic Activity**

Viruses are intracellular obligate parasites, whose replication depends on cell metabolism and biosynthetic processes performed in the host cell. Effective antiviral drugs are active against infections caused by viruses, either blocking cellular virus entry, blocking cellular exit, or interfering with the replication process (Wang et al. 2012). Often a close analogy exists between cell metabolic pathways and viral-specific macromolecular synthesis. This aspect is a significant complicating feature for the development of drugs that are able to selectively inhibit viral replication without damaging host cell metabolism. For this reason, the possible damage suffered by host cells following antiviral regimen must be considered (Kang et al. 2015).

Almost all viruses are sensitive to the inhibition activity exerted by structural analogues of amino acids or nucleotides, which induce the synthesis of non-functional proteins or nucleic acids. However, because this activity can also be directed against host cells, research in recent decades has focused on discovering possible mechanisms to selectively target and penetrate virus-infected cells, which have alterations in their cell membrane.

It's known that some antivirals are virustatic, as they are effective only against replicating viruses, but are inactive against latent viruses (Jiao et al. 2011). However, antivirals can act at different stages of viral infection/replication; some act by preventing adhesion of viruses to cells, others act on the translation of viral mRNA, some prevent the replication of viral nucleic acids, and others show inhibitory activity of virus progeny assembly and escape (Kang et al. 2015). Additionally, antivirals can inhibit viral DNA synthesis, DNA and RNA polymerase activity, reverse transcriptase activity, protease activity, integrase activity (Jiao et al. 2011) and neuraminidase activity (expressed mainly in influenza viruses) (Wang et al. 2014b; Li et al. 2008) or can act as antiretrovirals (anti-HIV). Interestingly, interferons have also been studied in a number of clinical situations. Currently antiviral drugs are available to actively combat and control the majority of viruses, although of great significance—eradication of viral infections is not complete (Jiao et al. 2011).

### **20.1.2 Glycan Definition**

Glycan is a generic term for any sugar or assembly of sugars, where they may be linked (via glycosidic covalent linkages) to another molecule. The term glycan is often used interchangeably with saccharide and carbohydrate, although in many references it refers to a sugar or carbohydrate polymer. A sugar polymer is made up of monosaccharides, joined together to make oligosaccharides (2–20 units) or polysaccharides (PS) (>20 units). The term glycoconjugate is often used to describe a macromolecule containing monosaccharides, oligosaccharides or even PSs linked glycosidically to a protein or lipid moiety. The prefix *glyco-* and the suffixes *-saccharide* and *-glycan* indicate the presence of sugar or carbohydrate component/s.

The term *simple* carbohydrate or sugar indicates that the glycan contains a singular type of monosaccharide, usually in a linear arrangement, whereas a *complex* carbohydrate indicates that the glycan contains more than one type of monosaccharide, may have non-linear branching, hydroxyl group modifications or be covalently linked to a protein, or lipid (glycoconjugate). In this review, all these various carbohydrate species will be referred to as *glycans*. The focus of this review on marine antiviral glycans is a summary of their (1) source, (2) reported glycan and known structural details (or reference to these) and (3) outline of reported antiviral activity.

## 20.2 Marine Glycans: Structural Features and Reported Antiviral Activity

The majority of glycans, including those with antiviral activity from the marine environment, are PSs, and there exist numerous review papers on the structural features identified to date, along with their known biological activities (Li et al. 2008; Vo and Kim 2010; Jiao et al. 2011; Uzair et al. 2011; Vo et al. 2011; Wang et al. 2012; Ahmed et al. 2014; Cheung et al. 2014; Novio et al. 2014; Wang et al. 2014a, b; Ahmadi et al. 2015; Dang et al. 2015; Kang et al. 2015). General details on these antiviral glycans from marine organisms have been collected together in Tables 20.1, 20.2, 20.3 and 20.4 (vide infra). They have been grouped based on the kingdom of their source organism. The specific references to each organism, the structure (what is known) and the displayed antiviral activity are indicated, but further details are presented in the relevant references. It is noteworthy that no glycans from fungi are reported with antiviral activity.

What is clearly evident is that there is unique specificity and a high level of complexity inherent in these glycans and for larger glycans (PSs) the composition (of sugar monosaccharides) may be known, but the specific and unique structure giving rise to the antiviral activity (amongst other activities in many instances) is not presently known. Therefore, an overview of the structural features and known mechanism of antiviral glycan activity is here presented. More detailed structural information can be obtained, along with reported specifics of the mechanisms and antiviral activities from the cited references below and in Tables 20.1, 20.2, 20.3 and 20.4. It is not the focus of this review to recount these exact details for every organism listed in Tables 20.1, 20.2, 20.3 and 20.4, but rather to collect together the glycans from marine organisms where antiviral activity has been reported and present some structural details, plus a brief indication of the specific antiviral activity. The glycans as named in Tables 20.1, 20.2, 20.3 and 20.4 are the names/terms used by the authors in the respective papers. It is acknowledged that there are some instances where these names could justifiably be renamed. Additionally, there are many reports of water extracts that exhibit antiviral activity [e.g. Vo et al. (2011)]; however, the specific identification of glycans or even glycan classes present within the extract have not been determined. Also of note is that many

**Table 20.1** Glycans from marine *Bacteria* and *Cyanobacteria* with reported antiviral activity

	Marine organism	Glycans	Reported antiviral activity	Comments	References
Bacteria	<i>Geobacillus thermodenitrificans</i>	EPS	HSV-2		Arena et al. (2009)
	<i>Pseudoalteromonas</i> sp. AM	EPS	HSV-1	EPS mainly of Glep repeats	Al-Nahas et al. (2011)
Blue-green algae (Cyanobacteria)	<i>Lyngbya lagerheimii</i>	SQDG (glycolipids)	HIV-1		Gustafson et al. (1989)
	<i>Nostoc flagelliforme</i>	Nostaflan (acidic PS)	HSV-1, HSV-2, IAV, HCMV	Inhibits binding and entry (contains Glep, Xylp, GleAp, Glep, Galp)	Kanekiyo et al. (2005) and Kanekiyo et al. (2007)
	<i>Phormidium tenue</i>	SQDG	HIV-1		Gustafson et al. (1989)
	<i>Scytonema</i> sp.	Sulfoglycolipids	HIV-1	Reverse transcriptase and DNA polymerase inhibition	Loya et al. (1998)
	<i>Spirulina platensis</i> (renamed as <i>Arthrospira platensis</i> )	Calcium spirulan (SP-Ca), SQDG	HSV-1, measles, mumps, IV, polio, Coxsackie, HIV-1, HCMV	Inhibits entry (contains Man, Rib, Fuc, Glc, Xyl, Gal, Rha, GalA, GlcA, Ca, sulfate).	Hayashi et al. (1996a, b), Chirasuwan et al. (2009) and Lee et al. (2000)

**Table 2.2** Glycans from marine *Chromists* with reported antiviral activity

	Marine organism	Glycans	Reported antiviral activity	Comments	References
Brown algae (Ochrophyta: Phaeophyceae)	<i>Adenocystis urticularis</i>	Sulfated galactofucan PS	HIV-1, HSV-1, HSV-2		Ponce et al. (2003) and Trinchero et al. (2009)
	<i>Ascophyllum nodosum</i> , <i>Fucus vesiculosus</i> , <i>Saccharina longicuris</i>	Laminarans	HIV	Glucan, common PS in wide variety brown algae	Vera et al. (2011) and O'Doherty et al. (2010)
	<i>Cladostiphon okamuranus</i>	Fucoidans (SP)	DENV-2, NDV	GluAp, sulfated-Fuc	Nagaoka et al. (1999), Hidari et al. (2008) and Elizondo-Gonzalez et al. (2012)
	<i>Dilophus fasciola</i>	Glycolipids	HSV-1		El Baroty et al. (2011)
	<i>Eisenia arborea</i>	SP	Measles virus		Moran-Santibanez et al. (2016)
	Extensively found in brown algae (e.g. <i>Dictyota mertensii</i> , <i>Lobophora variegata</i> , <i>Fucus vesiculosus</i> , <i>Spatoglossum schroederi</i> , <i>Sargassum piluliferum</i> , <i>Cystoseira indica</i> )	Fucans (fucoidans)	HIV, DENV-2, HCMV, HSV-1, HSV-2, IFV, Sindbis virus	Antiviral activity attributed to viral adsorption inhibition	Chevolot et al. (2001), Mandal et al. (2007), Hidari et al. (2008), Queiroz et al. (2008) and Chollet et al. (2016)
	Extensively found in brown algae	SPMG (sulfated alginates)	HIV	Inhibition adsorption/entry. Unique ratio of ManA:GulA-PS	Meiyu et al. (2003)
	<i>Laminaria hyperborea</i> , <i>L. digitata</i>	Alginates (PS)	HIV, IAV, HBV (911-alginate-PS drug)	(Linear anionic cell wall PS)	Wang et al. (2012)
	<i>Laminaria japonica</i>	SPMG, alginates	HIV-1		Hui et al. (2008)
	<i>Leathesia difformis</i>	Sulfated fucans	HSV-1, HSV-2		Feldman et al. (1999) and Hui et al. (2008)
	<i>Macrocystis pyrifera</i>	Alginates (PS), SP	HIV, IAV, HBV, Measles virus		Wang et al. (2012) and Moran-Santibanez et al. (2016)
	<i>Padina pavonica</i>	SP	HSV, Hepatitis A		Mohamed and Agli (2013)

(Ochrophyta: Eustigmatophyceae)	<i>Pelvetia compressa</i>	SP	Measles virus			Moran-Santibanez et al. (2016)
	<i>Sargassum horneri</i>	Sulfated fucan	HSV-1			Hoshino et al. (1998)
	<i>Sargassum mcclurei</i>	SP (fucoidans)	HIV-1	Pre-incubation with virus		Thuy et al. (2015)
	<i>Sargassum patens</i>	SGF	HSV-1, HSV-2			Zhu et al. (2006)
	<i>Sargassum polycystum</i>	SP (fucoidans)	HIV-1	Pre-incubation with virus		Thuy et al. (2015)
	<i>Sargassum vulgare</i>	SQDG (glycolipid)	HSV-1, HSV-2			Plouguerné et al. (2013)
	<i>Sphacelaria indica</i>	Xylogalactofucan	HSV-1	Inhibition of viral adhesion		Bandyopadhyay et al. (2011)
	<i>Spilachnidium rugosum</i>	Sulfated fucan	HSV-1, HSV-2	Potent virucidal activity		Harden et al. (2009)
	<i>Stoechospermum marginatum</i>	Sulfated fucan	HSV-1, HSV-2			Adhikari et al. (2006)
	<i>Taonia atomaria</i>	Glycolipids	HSV-1			El Baroty et al. (2011)
	<i>Turbinaria ornata</i>	SP (fucoidans)	HIV-1	Pre-incubation with virus		Thuy et al. (2015)
	<i>Undaria pinnatifida</i>	SGF	HSV-1, HSV-2			Hayashi et al. (2008) and Harden et al. (2009)
	<i>Ellipsoidon</i> sp.	SP (unconfirmed)	VHSV, ASFV	Replication inhibition		Fabregas et al. (1999)
	<i>Cochlodinium polykrikoides</i>	A1 and A2 (SPs)	HIV-1, HSV-1, IFV-A and IFV-B, RSV-A, RSV-B, PIFV-2	Extracellular SP (contains Glc, Gal, Man, UroA, SGs)		Hasui et al. (1995)
	<i>Gyrodinium impudicum</i>	p-KG03 (exo-SP)	EMCV, IFV-A, IFV-B	Inhibits adsorption IFV-A and IFV-B		Yim et al. (2004) and Kim et al. (2012)
<i>Navicula directa</i>	Naviculan (SP)	HIV-1, HSV-1, HSV-2, IFV-A	Blocking of adsorption. Contains Gal, Xyl, Rha, Fuc, Man, sulfate		Lee et al. (2006)	
(Bacillariophyta: Bacillariophyceae)						

**Table 20.3** Glycans from marine *plantae* with reported antiviral activity

	Marine Organism	Glycans	Reported antiviral activity	Comments	References
Green algae (Chlorophyta: Ulvothyceae)	<i>Acrosiphonia orientalis</i>	PS-undefined	White spot syndrome virus (shrimp)		Manilal et al. (2009)
	<i>Caulerpa brachypus</i>	SP	HSV-1	Post-viral addition. SP1:Rha, (Xyl, Glu), SP2: Gal	Wang et al. (2014a, b)
	<i>Caulerpa okamurae</i>	SP	HSV-1	Post-viral addition. Gal (Xyl, Man)	Lee et al. (2004)
	<i>Caulerpa racemosa</i>	SP, SQDG	HSV-1, HSV-2, DENV-2 (DENV-1,3,4)	Branched hetero-GalAra	Ghosh et al. (2004), Wang et al. (2007a, b) and Pujol et al. (2012)
	<i>Caulerpa scalpelliformis</i>	SP	HSV-1	Post-viral addition. Gal (Xyl, Man)	Lee et al. (2004) and Wang et al. (2007a, b)
	<i>Caulerpa spiralis</i>	SP	HSV-1	Post-viral addition. Ara, Xyl, Glu	Lee et al. (2004)
	<i>Chaetomorpha crassa</i>	SP	HSV-1	Ara, Xyl, Glu	Lee et al. (2004)
	<i>Codium adhaerens</i>	SP	HSV-1	Ara (Glu, Xyl)	Lee et al. (2004)
	<i>Codium fragile</i>	SFG	HSV-1	Ara (Xyl)	Ohta et al. (2009) and Lee et al. (2004)
	<i>Codium latum</i>	SP	HSV-1	Ara	Lee et al. (2004)
	<i>Enteromorpha compressa</i>	SP	HSV-1	Rha (Xyl, GlcA, Rha)	Lee et al. (2004)
	<i>Gayralia oxysperma</i>	SP	HSV	Branched sulfated hetero Rha	Cassolato et al. (2008)
	<i>Monostroma latissimum</i>	Rhamnan sulfate	HCMV, HIV-1, HSV-1	Rha. Inhibition virus adsorption	Lee et al. (1998, 1999)

	<i>Monostroma nitidum</i>	SP	HSV-1, HSV-2	Rha (Glu)	Lee et al. (2004, 2010)
	<i>Ulva fasciata</i>	Glycolipids	HSV-1		El Baroty et al. (2011)
	<i>Ulva intestinalis</i>	SP	Measles virus		Moran-Santibanez et al. (2016)
	<i>Ulva lactuca</i>	Ulvan PS	IFV-A, JEV	Adsorption and entry inhibition	Ivanova et al. (1994) and Chiu et al. (2012)
Chlorophyta: Trebouxiophyceae	<i>Chlorella autotrophica</i>	SP (unconfirmed)	VHSV, ASFV	Replication inhibition	Cassolato et al. (2008)
	<i>Chlorella vulgaris</i>	PS-rich fraction	HSV-1	Inhibits attachment, replication	Santoyo et al. (2010)
Chlorophyta: Chlorophyceae	<i>Haematococcus pluvialis</i>	PS-rich fraction	HSV-1	Inhibits attachment, penetration, replication	Santoyo et al. (2012)
Red algae (Rhodophyta: Florideophyceae)	<i>Acanthophora spicifera</i>	Agaran sulfate	HSV-1, HSV-2		Duarte et al. (2004)
	<i>Agardhiella tenera</i> , <i>Callophyllis variegata</i> , <i>Schizymenia binderi</i>	Galactans, SFGs	CMV, DENV, DENV-2, HAV, HIV-1, HIV-2, HSV-1, HSV-2, RSV, Sindbis virus, VV	Extracellular SGP. Agaran-carrageen hybrid	Witrouw and De Clercq (1997), Estevez et al. (2001), Matsuhira et al. (2005), Rodriguez et al. (2005), Pomin (2010) and Ahmadi et al. (2015)
	<i>Asparagopsis armata</i>	PS	HSV-1	CHCl <sub>3</sub> /methanol extract	Bouhhal et al. (2010)
	<i>Boergeseniella thuyoides</i>	SP	HIV, HSV-1		Bouhhal et al. (2011)
	<i>Bostrychia montagnei</i>	SFG	HSV-1, HSV-2		Duarte et al. (2001)
	<i>Callophyllis variegata</i>	Galactans	DENV-2, HSV-1, HSV-2	Carrageenan-type backbone	Rodriguez et al. (2005)
	<i>Ceramium rubrum</i>	PS	HSV-1, HSV-2	CHCl <sub>3</sub> /methanol extract	Bouhhal et al. (2010)
	<i>Constantinea simplex</i>	PS	HSV-1, HSV-2, VV		Richards et al. (1978)

(continued)



Table 20.3 (continued)

Marine Organism	Glycans	Reported antiviral activity	Comments	References
<i>Cryptonemia crenulata</i>	D,L-SFG hybrid	DENV-1,2,3,4, HSV-1, HSV-2	Virus adsorption/internalisation	Talarico et al. (2004, 2007a)
<i>Cryptopleura ramosa</i>	Agarans	HSV-1, HSV-2		Carlucci et al. (1997b)
<i>Eucheuma cottonii</i>	$\kappa$ -Carrageenans	CMV, HSV-1, HSV-2, HIV-1, Sindbis virus, VV, hepatitis A virus		Baba et al. (1988) and Girond et al. (1991)
<i>Eucheuma spinosum</i>	$\iota$ -Carrageenan	ECMV, HSV-1, HSV-2, Semliki Forest virus, swine fever virus, VV, hepatitis A virus		Gonzalez et al. (1987) and Girond et al. (1991)
<i>Farlowia mollis</i>	PS	HSV-1, HSV-2, VV		Richards et al. (1978)
<i>Galaxaura cylindrica</i>	Glycolipids	HSV-1		El Baroty et al. (2011)
<i>Gelidium pulchellum</i>	PS	HSV-1	CHCl <sub>3</sub> /methanol extract	Bouhhal et al. (2010)
<i>Gelidium spinulosum</i>	PS	HSV-1	CHCl <sub>3</sub> extract	Bouhhal et al. (2010)
<i>Gigartina aciculata</i>	$\lambda$ -Carrageenans	HSV-1, HSV-2, HIV, Sindbis virus, VV		Baba et al. (1988)
<i>Gigartina pistillata</i>	$\lambda$ -Carrageenans	CMV, HSV-1, HSV-2, HIV-1, Sindbis, VV		Baba et al. (1988)
<i>Gigartina skottsbergii</i>	Carrageenans ( $\iota$ , $\kappa$ , $\lambda$ , $\mu$ , $\nu$ ) (anionic-SPs)	IFV, DENV, HIV, HPV, HRV, HSV-1, HSV-2	Structural classification mainly due to sulfation pattern	Carlucci et al. (1997a, 2004), Pujol et al. (2006) and Talarico et al. (2007b)
<i>Gigartina atropurpurea</i>	SGF, ( $\lambda$ and $\kappa$ -Carrageenans)	HSV-1, HSV-2		Harden et al. (2009)
<i>Gigartina tenella</i>	Glycolipids	HIV-1		Ohta et al. (1998)
<i>Grateloupia indica</i>	SFGs	HSV-1, HSV-2		Chattopadhyay et al. (2007)

<i>Grateloupia filicina</i>	SFGs, amylose	DENV-2, HIV-1, HSV-2	HSV-2 (amylose), HIV-1 (SFGs)	Wang et al. (2007a, b)
<i>Grateloupia longifolia</i>	SFGs	HIV-1, HSV-1, HSV-2	Inhibition	Wang et al. (2007a, b)
<i>Gracilaria corticata</i>	SP	HSV-1, HSV-2		Mazumder et al. (2002) and Chattopadhyay et al. (2008)
<i>Gymnogongrus griffithsiae</i>	Carrageenans (ι, ν, κ), SFGs	HSV-1, HSV-2, DENV-2, 3, 4		Talarico et al. (2004, 2005)
<i>Gymnogongrus torulosus</i>	D,L-SFG, Galactans	HSV-2, DENV-2		Pujol et al. (2002)
<i>Halophytis incurva</i>	PS	HSV-1	CHCl <sub>3</sub> /methanol extract	Bouhhal et al. (2010)
<i>Laurencia papillosa</i>	Glycolipids	HSV-1		El Baroty et al. (2011)
<i>Meristella gelidium</i>	Carrageenans (ι, ν, κ)	HSV-2, DENV-2	Inhibition	de S.F.-Tischer et al. (2006)
<i>Nothogenia fastigiata</i>	SXM	CMV, HIV-1, HIV-2, IFV-A, IFV-B, Junin virus, RSV, simian immunodeficiency virus, Tacaribe virus, HSV-1, HSV-2		Damonte et al. (1994) and Kolender et al. (1997)
<i>Osmundaria obtusiloba</i>	SQDG (glycolipids)	HSV-1, HSV-2		Gustafson et al. (1989), Marchetti et al. (1995) and de Souza et al. (2012)
<i>Plocamium cartilagineum</i>	SFGs, SGF	HSV-1, HSV-2	CHCl <sub>3</sub> /methanol extract	Harden et al. (2009) and Bouhhal et al. (2010)
<i>Pterosiphonia complanata</i>	PS	HSV-1	CHCl <sub>3</sub> /methanol extract	Bouhhal et al. (2010)
<i>Pterocladia capillacea</i>	GS	CMV, HSV-1, HSV-2, Pseudorabies virus		Pujol et al. (1996)
<i>Schizymenia dubyoi</i>	Glucuronogalactans	HIV-1, HSV-1, HSV-2, VSV		Bourgougnon et al. (1993, 1996)

(continued)

Table 20.3 (continued)

	Marine Organism	Glycans	Reported antiviral activity	Comments	References
	<i>Schizymenia pacifica</i>	SP	HIV		Nakashima et al. (1987a, b)
	<i>Scinaia hatei</i>	SP	HIV-1, HSV	Man (Xyl)-sulfated	Lu et al. (2007) and Mandal et al. (2008)
	<i>Sebenia polydactyla</i>	SXM	HSV-1		Ghosh et al. (2009)
	<i>Soliteria chordalis</i>	SP (Carraguard)	HIV-1, HSV-1, HSV-2	Linear PS Gal and 3,6-anhydroGal	Skoler-Karpoft et al. (2008)
	<i>Soliteria filiformis</i>	SP	Measles virus		Moran-Santibanez et al. (2016)
	<i>Sphaerococcus coronopifolius</i>	SP	HSV-1, HSV-2, HIV-1		Bouhhal et al. (2010) and Bouhhal et al. (2011)
	<i>Stenogramma interruptum</i>	PS	HSV-1, HSV-2	Primarily <i>t</i> -Carrageenan, $\lambda$ & $\kappa$ -carrageenan	Caceres et al. (2000)
	<i>Tichocarpus crinitus</i>	$\kappa$ & $\beta$ -carrageenans	TMV		Nagorskaia et al. (2008)
Rhodophyta: Porphyridiophyceae	<i>Porphyridium cruentum</i>	Exo-SP	HSV-1, HSV-2	Inhibits penetration, replication	Raposo et al. (2014)
	<i>Porphyridium purpureum</i>	SQDG (glycolipids)	Antiviral—not specified		Naumann et al. (2007)
	<i>Porphyridium</i> sp.	SP	HSV-1, HSV-2, VZV	Inhibits adsorption, replication (HSV)	Huheihel et al. (2002)
	<i>Porphyridium</i> sp.	PS (purified)	MuLV, MuSV	Inhibits proliferation of retrovirus	Talyshinsky et al. (2002)
Sea grass (Tracheophyta)	<i>Thalassia testudinum</i>	Thalassiolins A–C	HIV	HIV integrase inhibitory activity	Rowley et al. (2002)

**Table 20.4** Glycans from marine animals with reported antiviral activity

	Marine organism	Glycans	Reported antiviral activity	Comments	References
Sponge (Porifera)	<i>Myxilla rosacea</i>	PS	HIV	PS named Rosacelose	Cimino et al. (2001)
Sponge (Porifera)	<i>Siliquariaspongia mirabilis</i>	Mirabamides A and D	HIV-1	Inhibition—neutralisation and fusion	Plaza et al. (2007)
Peanut worm (Sipuncula)	<i>Sipunculus nudus</i>	PS (named SNP)	HBV	Down regulation of HBV DNA	Su et al. (2016)
Clam (Mollusca)	<i>Meretrix petechialis</i>	PS, SFG	HIV-1	Cell infusion inhibitory activity, inhibition of syncytia formation	Amornrut et al. (1999) and Woo et al. (2001)
Mussel (Mollusca)	<i>Perna viridis</i>	PS	IFV	MDCK cell cultures	Zhang et al. (2008)
Scallop (Mollusca)	Not specified	Glycosaminoglycan	HSV-1		Yu et al. (2008)
Squid (Mollusca)	Squid cartilage	CS-E (glycosaminoglycan)	HSV, DENV	Inhibition of viral attachment. (GlcAβ1-3GalNAc(4S,6S)-predominant repeat disaccharide	Bergefall et al. (2005) and Kato et al. (2010)
Veined rapa whelk (Mollusca)	<i>Rapana venosa</i>	Glycosylated haemocyanin FU-RtH2-e	RSV, HSV-1, HSV-2, EBV	Inhibition of viral attachment	Nesterova et al. (2011) and Dolashka et al. (2010)
Crustacea (Arthropoda)	Crustacean—not defined	Chitosan	FCV-F9, bacteriophage MS2		Davis et al. (2012) and Adhikari et al. (2006)
Tunicate (Chordata)	<i>Didemnum molle</i>	PS (kakekolose)	HIV-1	Sulfated Man homo-PS	Riccio et al. (1996)
Sea cucumber (Echinodermata)	<i>Stauracium liouvillei</i>	Glycosides (liouvillosides A and B)	HSV-1	Virucidal	Maier et al. (2001) and Antonov et al. (2008)

glycans that have been isolated from marine organisms have evidently not been tested for antiviral activity to date, although they may well present such activity with further investigations.

The below classes of glycans are those entered in Tables 20.1, 20.2, 20.3 and 20.4, where antiviral activity has been reported.

### 20.2.1 *Alginates (Sulfated Alginates)*

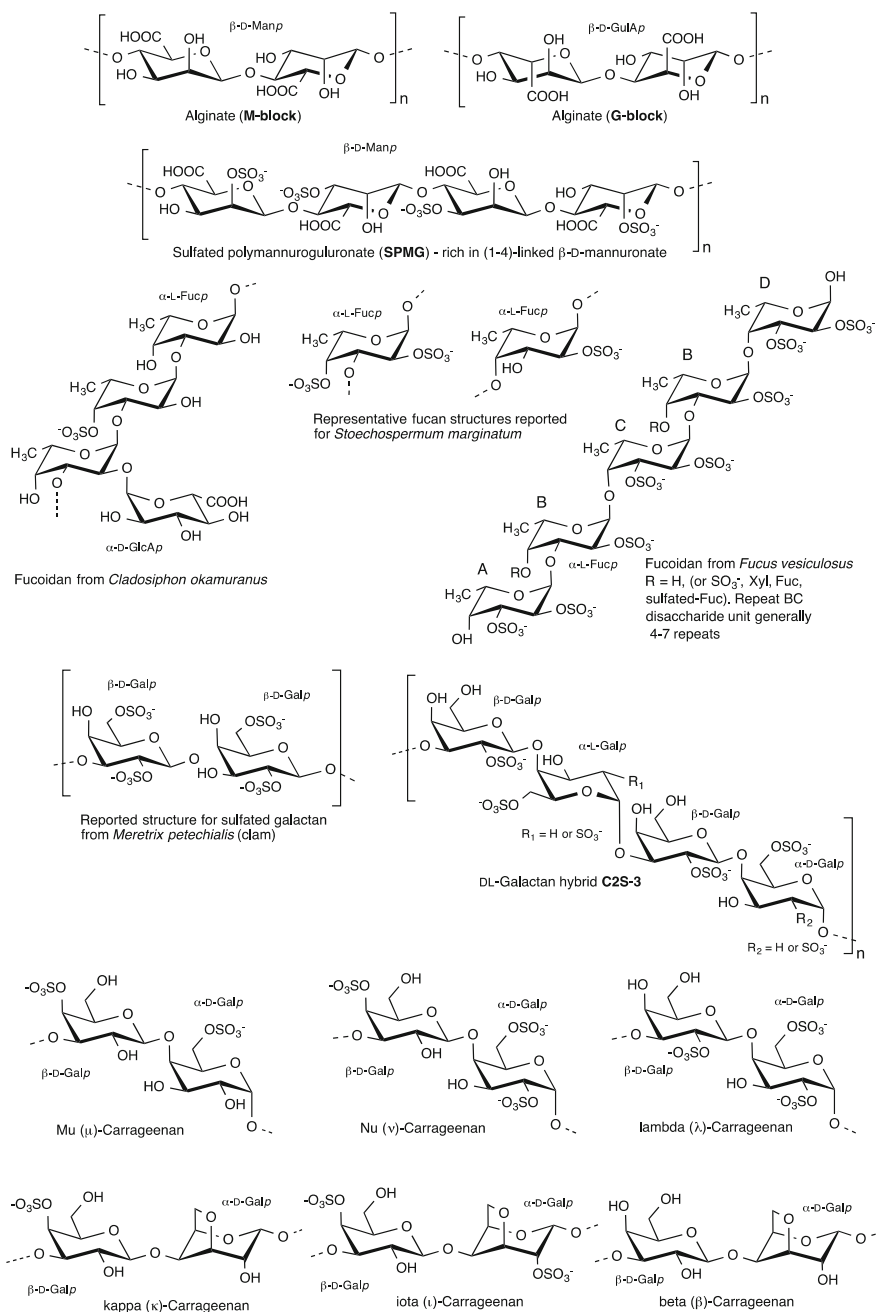
Alginates (sulfated alginates) are the principal cell wall and intercellular matrix linear acidic PSs widely distributed in brown algae (Phaeophyceae). They are composed of a central backbone of G-blocks (poly- $\alpha$ -L-GulAp) or M-blocks (poly- $\beta$ -D-ManAp) or alternating G-blocks and M-blocks (GM-blocks) (Wang et al. 2012). The ratios of each block are species specific, differing amongst the various alginates. The block ratios can also vary across the seasons within a given alga and even across different regions of the individual alga. Differing block ratios provide varying functional aspects for protection against waves and dehydration at low tide. Alginates can be found as mixed salts with  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$  or  $\text{Na}^+$  cations and may contribute up to 47% w/w of the alga dry weight (Usov 1999). This extensive variation in the alginates reportedly impacts the antiviral activity (Florez et al. 2017).

*Sulfated alginates*: Of particular note is the sulfated polymannuroguluronate (SPMG) isolated from the brown alga *Laminaria japonica*, which has shown HIV inhibitory activity, with an octasaccharide unit showing further specific activity. The *antiviral activity* of alginates in general is exerted via their interfering with virus adsorption, suppression of DNA polymerase activity and improving the host cells defence mechanisms. The antiviral activity of SPMG is reported as being principally due to its ability to block the interaction between the virus gp120 protein and the CD4 molecule on the T-cell surface (Meiyu et al. 2003; Hui et al. 2008).

See Fig. 20.1 for representative structures of alginate M-block and G-block disaccharide repeat units.

### 20.2.2 *A1 and A2 SP*

A1 and A2 SP were isolated from the marine microalga *Cochlodinium polykrikoides*, being identified as extracellular sulfated PSs. They were shown to contain Galp, Glcp, Manp and uronic acids, plus a specific distribution of sulfate groups (SGs). Antiviral activity was demonstrated against numerous virus targets (Hasui et al. 1995).



**Fig. 20.1** Selected reported structures of marine glycans displaying antiviral activity (see Tables 20.1, 20.2, 20.3 and 20.4 vide supra)

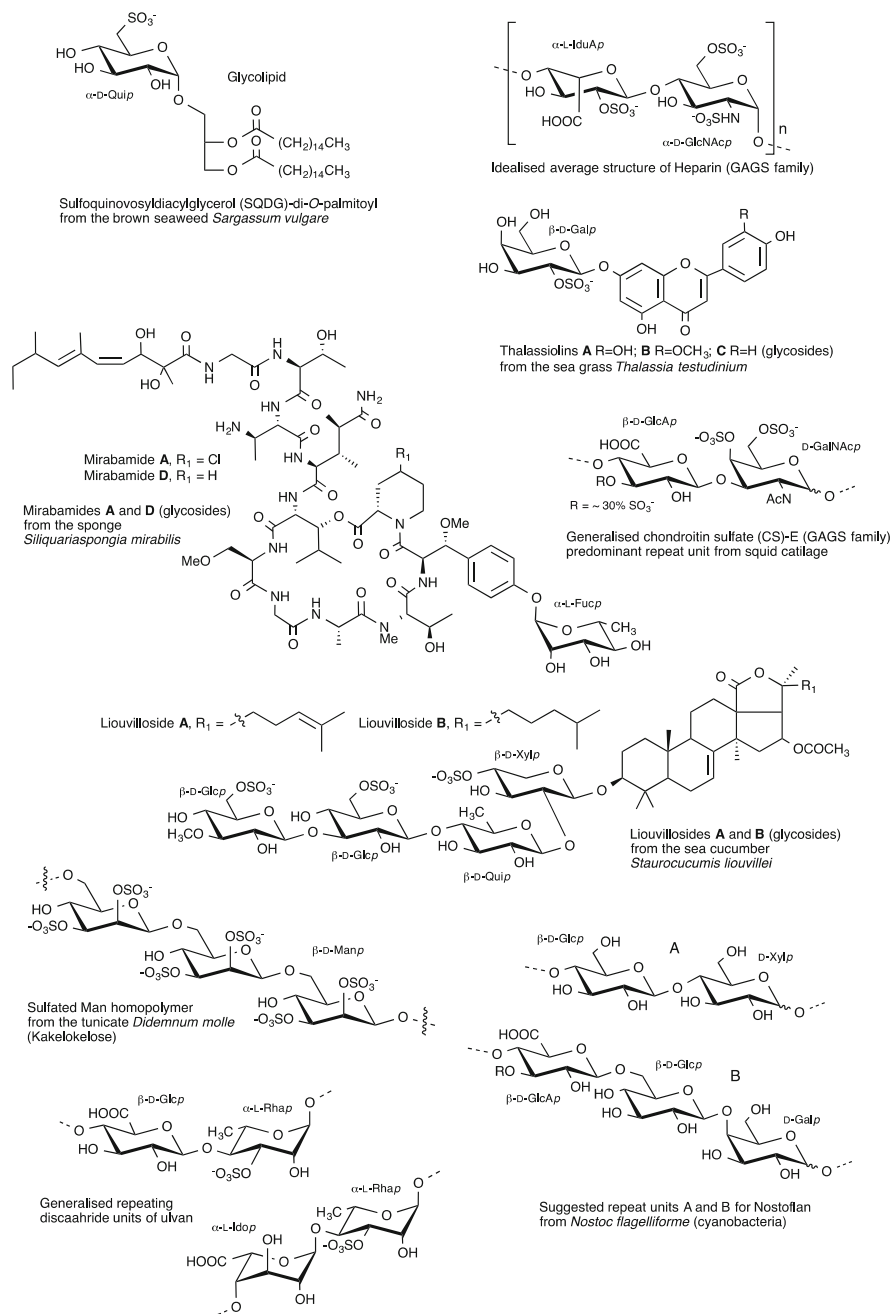


Fig. 20.1 (continued)

### 20.2.3 Chitosan

Chitosan is a linear PS composed of randomly distributed  $\beta$ -(1 $\rightarrow$ 4)-linked D-GlcN<sub>p</sub> (deacetylated unit) and D-GlcNAc<sub>p</sub> (acetylated unit). Its source is generally shrimp and other crustaceans, where the hard shell is hydrolysed. The molecular weight of chitosan is usually in the range 50–1000 kDa.

Antiviral activity of this hydrolysed marine PS was evidenced by its ability to reduce infectivity of two human enteric viral surrogates, feline calicivirus F-9 and the (ssRNA) bacteriophage MS2 (Su et al. 2009; Davis et al. 2012). While the PS is not in its native form (the PS being truncated), it seems reasonable in this instance to class the material as being from a marine organism.

### 20.2.4 Extracellular PS

Extracellular PS (EPS) was isolated from the bacteria *Geobacillus thermodenitrificans* from a shallow marine vent of a volcanic island and was reportedly composed of a trisaccharide repeat of Man<sub>p</sub> and displayed anti-HSV-2 activity (Nicolau et al. 2000; Arena et al. 2009).

*Pseudoalteromonas* sp. AM was isolated from a Red Sea sponge (Egypt) and is reported as being primarily composed of Glc<sub>p</sub> and possessing anti-HSV-1 activity (Al-Nahas et al. 2011).

### 20.2.5 Fucans/Sulfated Fucans

Fucans/sulfated fucans (SF) can be classified into three major groups: fucoidans, xylofucoglycuronans and glycuronogalactofucans. They are widely distributed in species of brown algae and in some red algae, being composed primarily of repeat fucose chains (Wang et al. 2012). Many of the examples given in Tables 20.1, 20.2, 20.3 and 20.4 indicate that fucans or sulfated fucans are of a non-defined structure, but are responsible for the observed antiviral activity (see Table 20.2).

*Fucoidans*: They are complex and heterogeneous sulfated fucose-rich PSs (heterofucans) that have been primarily found in the fibrillar cell walls and intracellular spaces of brown algae/seaweeds (Florez et al. 2017). Interestingly, they have also been found in some marine invertebrates, such as sea urchins (egg jelly coat) and sea cucumbers (Chizhov et al. 1999; Bilan et al. 2002). When first isolated in 1913 by Kylin, they were named as ‘fucoidins’; however, the IUPAC name ‘fucoidan’ is now recognised. The terms fucan, fucosan or sulfated fucan are at times used interchangeably to indicate a fucoidan (Ahmadi et al. 2015). Fucoidans generally contain (1 $\rightarrow$ 3) and some alternating (1 $\rightarrow$ 4)-linked sulfated  $\alpha$ -L-Fuc<sub>p</sub> along with lower percentages (usually less than 10%) of other monosaccharides such as



Manp, Galp, Xylp, uronic acids, additional acetyl groups and even protein. Of note though, some fucoidans have been identified as having comparable amounts of Fuc and Gal and were therefore regarded as fucogalactans (Wang et al. 2010). Fucoidans can account for between 5 and 32% w/w dry alga biomass (Florez et al. 2017). Fucoidans have to date been identified in more than 70 species of brown algae (Phaeophyceae) (Chollet et al. 2016). The structures isolated from algae sources are generally highly variable and complex and excellent reviews exist on a more detailed presentation of the diversity of structure and biological activity (Li et al. 2008; Chollet et al. 2016).

Fucoidans belong to the large family of marine sulfated PSs named fucans. The fucan family also includes xylofucoglycuronans and xylofucomanuronans (from ascophyllans, as associated with *Ascophyllum* genus) and glycuronofucogalactan (from sargassans, associated with *Sargassum* genus) (Chollet et al. 2016).

**Antiviral activity:** Fucoidans exhibit (as do most SPs) low cytotoxicity compared to most other antiviral drugs currently in clinical use. They have exhibited antiviral activity both in vitro and in vivo generically regarded as being due to the fucan's ability to inhibit HIV reverse transcriptase activity (Queiroz et al. 2008) and inhibit virus adsorption, thereby inhibiting viral-induced syncytium formation (Mandal et al. 2007). While sulfation patterns are variable, they are mainly found at *O*-2 and *O*-4 and are regarded as necessary for activity. Reduction of carboxylic acid groups (GlcAp  $\rightarrow$  Glcp) results in loss of antiviral activity (Wang et al. 2012; Queiroz et al. 2008).

The first fucoidan structure to be elucidated was that of *Fucus vesiculosus* in 1950 (Conchie and Percival 1950), although the structure was later refined (Chevolot et al. 2001). See exemplar fucoidan structures from *Fucus vesiculosus* and *Cladosiphon okamuranus* in Fig. 20.1.

## 20.2.6 Galactans/Sulfated Galactans

Galactans/sulfated galactans (SFG) are from red seaweeds (Rhodophyta algae) being high-molecular-weight sulfated linear PSs, consisting of linear backbones of repeating units of 3-linked  $\beta$ -D-Galp (A-unit) and 4-linked  $\alpha$ -D (or L)-Galp (B-unit) with potential replacement 3,6-anhydroGalp residues (AnGal-units). The sugars in the A-unit always belong to the D-series, while the sugars in B-units are in both the D- and L-configurations for carrageenans and agarans, respectively.

The carrageenans are classified depending upon the location and number of sulfate groups and the presence or absence of AnGal on B-units. For example,  $\kappa$  (kappa),  $\iota$  (iota),  $\lambda$  (lambda),  $\mu$  (mu) and  $\nu$  (nu) carrageenans vary according to the presence of 1, 2 or 3 sulfate ester groups, respectively, per repeat disaccharide unit (Jiao et al. 2011). There are at least 15 different carrageenan structures that have been reported to date (Lahaye 2001). Natural carrageenans typically occur as mixtures of different hybrids, such as  $\kappa/\beta$ -hybrids,  $\kappa/\iota$ -hybrids,  $\kappa/\mu$ -hybrids or  $\omega$ (omega)/ $\iota$ -hybrids (van de Velde et al. 2005; Jouanneau et al. 2010; Yang et al. 2011), along with additional

methyl, pyruvic acid acetal and monosaccharide sugars adding to the unique complexity already inherent in the glycans (Yu et al. 2010). Carrageenans  $\iota$ ,  $\kappa$ ,  $\lambda$ ,  $\mu$ ,  $\nu$  and  $\beta$  have been identified in marine algae possessing antiviral activity. Carrageenans  $\iota$ ,  $\kappa$ ,  $\lambda$ ,  $\mu$ ,  $\nu$  and  $\beta$  structures are given in Fig. 20.1. For specific structural insights, further details can be found in the cited references (see Table 20.3).

Much less information is reported on the structural features of agarans with antiviral activity. Only two reports exist on agarans from red algae displaying antiviral activity, namely, *Acanthophora spicifera* and *Cryptopleura ramosa*, both exhibiting anti-HSV activity (see Table 20.3).

Recently, an additional class to the carrageenans and agarans has been identified, namely, the DL-hybrids. This group has B-units in both the D- and L-configurations (mixture). The galactan backbones are in general also variously modified by substituents such as sulfate, methoxy, 4,6-O-pyruvate and monosaccharide groups. They can also be found as  $K^+$ ,  $Na^+$ ,  $Mg^{2+}$  and  $Ca^{2+}$  sulfate esters (Laurienzo 2010). A representative DL-galactan hybrid structure is given for C2S-3 isolated from the red alga *Cryptonemia crenulata* (see Fig. 20.1).

Of additional interest is the SFG reported from the clam *Meretrix petechialis*. This homogeneous SFG is composed of (1 $\rightarrow$ 3) linked  $\beta$ -D-Galp sulfate, with 2-O and 6-O-sulfation and displayed anti-HIV activity (Amornrut et al. 1999).

The antiviral SFG from *Codium fragile* (green algae) was reported to mainly consist of  $\beta$ -D-Galp (1–3, and 1–3,6-linked) with pyruvic acid (~12%) and ~11% sulfation (primarily at O-4 of Gal). The antiviral potency of *Codium fragile* SFG was indicated to be exerted via inhibition of HSV-2 replication, due to interference by the SFG in virus adsorption and penetration into host cells (Ohta et al. 2009).

While no singular mechanism of antiviral activity for the SFGs is apparent, it seems that much of this activity is exerted at the early stages of virus adhesion and that observed antiviral activity has a high correlation to molecular weight and the specific sulfation pattern (Neyts et al. 1992).

### 20.2.7 Glycolipids/Sulfated Glycolipids

Glycolipids/sulfated glycolipids non-specific glycolipids and sulfoquinovosyldiacylglycerols (SQDG) possessing antiviral activity have been reported in brown, blue-green, green and red algae (see Tables 20.1, 20.2, 20.3 and 20.4 and cited references). Glycolipids are a less studied class of antiviral secondary metabolites (de Souza et al. 2012). Seaweeds are known to synthesise three major types of glycolipids: monogalactosyldiacylglycerides (MGDG), digalactosyldiacylglycerides (DGDG) and sulfoquinovosyldiacylglycerides (SQDG), with  $\beta$ -D-Galp,  $\alpha$ -D-Galp-(1 $\rightarrow$ 6)- $\beta$ -D-Galp-(1 $\rightarrow$ )glycerol and  $\alpha$ -D-Quip-6-O-sulfo sugars in MGDG, DGDG and SQDG, respectively (de Souza et al. 2012). The glycerol moiety may then be mono- or diacylated with fatty acids. Palmitoyl (C16) fatty acid is reported as being the most common acylation moiety, although substitutions of margaroyl (C17), oleoyl (C18:1), nonadecanoyl (C19) and

tricosanoyl (C23) are reported for DGDGs and SQDGs displaying antiviral activity (El Baroty et al. 2011; Plouguerné et al. 2013).

See Fig. 20.1 for the structure of sulfoquinovosyl di-*O*-palmitoyl glycerol from *Sargassum vulgare* (brown algae).

With regard to the antiviral activity of DGDG and SQDG, recent reports demonstrate that palmitic acid is capable of binding to the CD4 cell receptor and therefore blocking HIV-1 entry and infection (Lee et al. 2009). Additionally, the sulfate group of the SQDGs is suggested as being involved and inhibiting the interaction between heparin sulfate and viral glycoproteins, thereby blocking binding and adsorption (Neys et al. 1992).

### 20.2.8 Glycosaminoglycans

Glycosaminoglycans (GAGS) are linear PSs made up of disaccharide repeat units. The repeat units being an amino sugar and a uronic acid, with the uronic acid linked by alternating  $\beta$ -(1 $\rightarrow$ 4) and  $\beta$ -(1 $\rightarrow$ 3) glycosidic linkages.

Chondroitin sulfate (CS) chains are composed of glucuronic acid (GlcAp) and *N*-acetylgalactosamine ( $\alpha$ -D-GalNAcp) at alternating positions. The GlcAp can be *O*-sulfated at C2 or rarely at C3, whereas the  $\alpha$ -D-GalNAcp can be sulfated at *O*4 and/or *O*6. Several types of CS chains have been classified based on the sulfation pattern on the disaccharide units. In particular, CS-E is predominantly composed of  $\beta$ -D-GlcAp-(1 $\rightarrow$ 3)-GalNAcp (4S,6S), where 4S and 6S indicate for 4-*O*- and 6-*O*-sulfation. CS-E has been purified from squid cartilage, where reportedly its chains were comprised of ~61% of the E-disaccharide unit, with a notable ~30% additional sulfation on *O*3 of GlcAp. It is of interest that the GlcA(3S)-containing CS-E disaccharides have never been reported previously in mammalian cells or tissues (Bergefall et al. 2005; Kato et al. 2010).

Antiviral activity of GAGS isolated from scallop (not specified) and squid cartilage (CS-E) were both reported as having antiviral activity against HSV-1, and additionally squid cartilage CS-E also had activity against DENV (Bergefall et al. 2005; Yu et al. 2008; Kato et al. 2010). CS-E reportedly blocked HSV invasion of cells, implying that certain structural features present in CS-E may fit better than heparin to the attachment/entry domains of viral components (Bergefall et al. 2005).

### 20.2.9 Glycosides

*Thalassiolins* A–C from the sea grass *Thalassia testudinum* are flavone glycosides and more specifically luteolin-glycosides, with a unique C7 substitution of the aglycone with  $\beta$ -D-Glcp(2S). Thalassiolin A exhibiting most potency (between A and C) in anti-HIV assays (Rowley et al. 2002). Structures for these thalassiolins are given in Fig. 20.1.

*Mirabamides A and D* from the sponge *Siliquariaspongia mirabilis* are a small group of known compounds named cyclic depsipeptides-glycosides, where the glycone is an  $\alpha$ -L-Fucp-(1→linked) to the phenolic moiety of the tyrosine functionality on the larger aglycone, as seen in Fig. 20.1. Mirabamide A was reportedly the most potent in HIV-1 fusion and neutralisation assays, indicating their activity is connected with the early stages of infection (Plaza et al. 2007).

*Liouvillosides A and B* from the sea cucumber *Staurocucumis liouvillei* are trisulfated triterpene tetrasaccharides, where the glycone is  $\beta$ -D-Glcp(3-O-methyl,6S)-(1→3)- $\beta$ -D-Glcp(6S)-(1→4)- $\beta$ -D-Quip-(1→2)- $\beta$ -D-Xylp(4S)-1-triterpene. Liouvilloside A has a double bond at C24/25 of the distal alkyl group, while liouvilloside B is without the unsaturation (Maier et al. 2001). Both liouvillosides were reported to possess virucidal activity against HSV-1. Structures are given for liouvillosides A and B in Fig. 20.1.

### 20.2.10 Glycosylated Haemocyanin

Glycosylated haemocyanin from the veined rapa whelk *Rapana venosa* is a very large glycoprotein, involved with oxygen transport. The structure of the oligosaccharides of the structural subunit RvH2 was found to be a highly heterogeneous mixture of glycans attached to the polypeptides. The functional unit RvH2-e was reported as possessing anti-HSV activity via inhibition of viral attachment (Dolashka et al. 2010; Nesterova et al. 2011).

### 20.2.11 pKG03 PS

pKG03 PS from the microalgae-dinoflagellate *Gyrodinium impudicum* is a highly sulfated exopolysaccharide (EPS) of unique feature. It has a molecular weight of  $1.87 \times 10^7$  Da and is categorised as a homopolymer of Galp linked to uronic acids, including sulfation of the disaccharide repeat unit—although the specific details are not reported (Yim et al. 2004).

Antiviral activity is indicated as being primarily targeted to the inhibition of viral adsorption and internalisation (Kim et al. 2012).

### 20.2.12 Laminarans

Laminarans are glucans (comprised of Glcp repeat units) considered a food reserve commonly found in brown algae. The generalised structure is regarded as a 20–25 Glc repeat unit, comprised of (1→3)- $\beta$ -D-glucan with (1→6)- $\beta$ -D-Glcp branches (Wang et al. 2012). To date, two types of laminarans have been identified.

One type, the G-series, is comprised of Glcp residues, while the second type, the M-series, is terminated by D-mannitol (Ahmadi et al. 2015). The ratios then of the G- or M-series in any isolated laminaran and the consequent structural features are indicated as being associated with the reported biological activity of each unique PS (Chizhov et al. 1998).

Antiviral HIV activity is reported via inhibition of virus adsorption to lymphocytes and HIV reverse transcriptase activity (Ahmadi et al. 2015; Wang et al. 2012).

### **20.2.13 Mannan/Sulfated Mannan**

Mannan/sulfated mannan was isolated from the tunicate *Didemnum molle* (referred to as kakelokelose). This sulfated mannan reportedly possessing antiviral activity consists of 2,3-disulfated Manp units (1→6) linked (Riccio et al. 1996). See reported structure in Fig. 20.1.

### **20.2.14 Naviculan**

Naviculan was isolated from the green microalgae (diatom) *Navicula directa*. It is a high-molecular-weight SP containing Galp, Fucp, Manp, Rhap, Xylp and sulfate groups. It has demonstrated potent antiviral activity against HIV, HSV-1, HSV-2 and IFV. This activity it is suggested is exerted via its ability to inhibit initial stages of viral adsorption and internalisation (Lee et al. 2006).

### **20.2.15 Nostoflan**

Nostoflan was isolated from the blue-green algae (cyanobacterium) *Nostoc flagelliforme*. Nostoflan is an acidic PS mainly composed of (→4)-β-D-Glcp-(1→4)-D-Xylp-(1→ and →4)-[β-D-GlcAp-(1→6)-β-D-Glcp-(1→4)-D-Galp-(1→). Its multi-virus (HSV-1, HSV-2, IAV, HCMV) inhibitory activity is attributed to its perturbation of virus binding and internalisation (Kanekiyo et al. 2007).

### **20.2.16 Polysaccharide/Sulfated Polysaccharide**

Polysaccharide (PS)/sulfated polysaccharide (SP) of non-structurally specified nature, has been reported in extracts with antiviral activity from blue-green algae, brown algae, clam, dinoflagellates, diatoms, green algae, mussels, red algae, sipunculid, sponge and tunicates (see Tables 20.1, 20.2, 20.3 and 20.4 for references).

### 20.2.17 *Spirulan SP*

Spirulan SP from the cyanobacteria *Spirulina platensis* (renamed as *Arthrospira platensis*) is a novel SP reportedly composed of fructose, Galp, Glcp, GalAp, GlcAp, Manp, ribose, Xylp, Ca<sup>2+</sup> and sulfate groups. Antiviral activity is associated with blockade of virus entry into host cells. Of note, the presence of calcium was indicated as being essential for features of antiviral behaviour (Dolashka et al. 2010).

### 20.2.18 *Rhamnan Sulfate*

Rhamnan sulfate isolated from the green alga *Monostroma latissimum* was determined to be made up of large amounts of Rhap residues. Therefore a Rhap homopolysaccharide consisting of 1,3- and 1,2-linked Rhap in a ratio of 3:2, with sulfation mainly at O-3 or O-4 in the 1,2-linked rhamnose residues (Lee et al. 1998). The rhamnan sulfate displayed potent antiviral replication activity against HCMV, HIV-1 and HSV-1 and also appeared to be involved in inhibiting the later steps of viral replication in host cells (Lee et al. 1999).

### 20.2.19 *Ulvan SP*

Ulvan SP from the green algae *Ulva lactuca* is a highly variable and complex branched SP. It is composed mainly of D-GlcAp, L-Rhap and D-Xylp with sulfates predominantly located at O-3 or O-2 on Rhap units. GlcAp in the disaccharide repeat unit is present predominantly as aldobiouronic acid (L-IdoAp) and sulfated aldobiouronic acid (with the disaccharide repeat unit of D-GlcAp-L-Rhap being the major disaccharide repeat unit) (Jiao et al. 2011; Wang et al. 2014a).

### 20.2.20 *Xylomannan/Sulfated Xylomannan*

Xylomannan/sulfated xylomannan (SXM) from the red alga *Nothogenia fastigiata* (Rhodophyta) follows the structural pattern of other xylomannans of this seaweed, i.e. -(1→3)- $\alpha$ -D-mannans-2- and 6-sulfation and having single stubs of -(1-2)-linked  $\beta$ -D-Xylp. Both antiviral SXMs isolated from *N. fastigiata* are obtained as complexes with a galactan and xylan (Kolender et al. 1997).

The major SXM from *Sebdenia polydactyla* (also from a red alga) had 0.6 sulfate groups per monomer unit (molecular weight of 150 kDa) and contains a backbone of -(1→3)-linked- $\alpha$ -D-Manp residues, substituted at position 6 with a single stub of  $\beta$ -D-Xylp residues (Ghosh et al. 2009). The conformation of the mannan backbone, the

molecular size and the sulfation patterns were indicated as being responsible for the multi-virus antiviral activity of these SXMs (Kolender et al. 1997; Ghosh et al. 2009).

### 20.3 General Comments on Antiviral Activity of Glycans from Marine Organisms

The inhibition mechanisms of polysaccharides from marine organisms involve interference with either the virus replication cycle or stimulating the immune response of the host. There exists great diversity in the life cycles of specific viruses; however, there are a number of essential features to each virus life cycle, namely, (1) viral adsorption, (2) penetration, (3) capsid uncoating, (4) biosynthesis, (5) assembly and (6) release, and additionally, a glycan can act in (7) a virucidal manner. The specific unique complexity of each glycan from a particular organism correlates to the specific mechanism of action. It is noteworthy that glycan structures even from the same organism can be site specific and even season specific. Interestingly, there are no glycans from marine organisms that are reported to exert their antiviral activity at the viral assembly and release stages of the life cycle.

*Virucidal activity and inhibition of viral adsorption:* This is due to either direct non-reversible interaction of the SP with the virus surface via anionic interactions, indicating that the sites on the viral envelope required for viral attachment to host cells are occupied or inactivated by the SP (Damonte et al. 2004; Harden et al. 2009; Wang et al. 2012), or by interacting with the virus receptors on the host cell surface. SPs can also mask the cationic architecture of host cell surfaces by interaction of their sulfate groups, thereby blocking the viral adsorption process (Wang et al. 2012; Miao et al. 2004; Carlucci et al. 1997a).

*Inhibition of viral penetration and capsid uncoating:* For many animal viruses, the internalisation process involves endocytic uptake, cytoplasmic vesicular transport to endosomes or other intracellular organelles (Mercer et al. 2010). Uncoating may occur during or after internalisation. It is reported that SPs in particular can interfere in an allosteric manner with both the uncoating and internalisation processes. Carrageenans and the SP p-KG03, for example, are reported as acting at the uncoating and internalisation stages (Kim et al. 2012; Talarico and Damonte 2007).

*Inhibition of viral biosynthesis (transcription and replication):* Reports exist of low-molecular-weight SPs from marine organisms that are capable of inhibiting viral transcription and replication enzymes post internalisation into host cells. Examples are low-molecular-weight carrageenans (Talarico et al. 2011; Wang et al. 2011) and fucans—where sulfation and more specifically the spatial orientation of the anionic charges are crucial reportedly to the observed activity (Queiroz et al. 2008).

*Stimulation of host immune response:* The type-1 interferon system (IFN- $\alpha/\beta$  system) is the first line of defence following host cell exposure to a virus. In addition to this, the host NK cells and macrophages are also involved. Carrageenans are reported as likely possessing the ability to induce the synthesis of type-1 IFN and

upregulate macrophage and NK cellular activities (Yuan et al. 2006; Turner and Sonnenfeld 1979). Additionally, studies on SPMG indicated that it possessed significant ability to up regulate humoral and cellular immune processes, thereby inhibiting viral replication (Wang et al. 2012).

## 20.4 Overall Conclusions

It is recognised that the marine environment is a unique but currently an under-investigated resource in regard to identifying novel chemicals as potential therapeutic drugs. Therefore, any work focused on encouraging investigations into the marine environment are well founded. Antiviral glycans from the marine environment inherently possess (in most instances) attractive features, such low toxicity, broad spectrum of antiviral activity, wide acceptability and relatively low production costs.

It is evident from this review of antiviral glycans from marine organisms that the majority of glycans identified (~136 entries—total reports, where some of these refer to multiple glycans) to date come from the chromists, in particular brown and red algae. These glycans are indicated as being extensively unique (or not structurally defined) SPs. Interestingly, only seven monosaccharide or oligosaccharide glycosides have been identified as possessing antiviral activity (liouvillosides A and B, mirabamides A and D, and thalassiolins A–C) (Table 20.5).

For many of the antiviral glycans that have been ‘identified’, their unique, specific structures have not been determined. It is acknowledged that to carry this out is no simple task, due to the enormous complexity that exists potentially in glycan structures. Added to this, it is evident from the literature that many glycans have only been screened for antiviral activity *in vitro* against a single host cell line. Thus, it is evident that many identified glycans would have more extensive activity than has currently been identified and further *in vivo* studies would be pivotal for ongoing development as therapeutics. Additionally, the mechanisms of antiviral action have been examined in only a minority of cases, without mechanistic investigations. For many glycans, detailed molecular mechanisms remain unknown. Therefore, there are extensive avenues for further investigative studies that would likely reveal much useful information on understanding the molecular mechanisms by which specific glycans exert their antiviral activity. This information will provide significant insight into our ability to successfully target and inhibit viral diseases.



**Table 20.5** Number of literature reports of antiviral glycans from marine kingdoms

Marine kingdom	Phylum	Glycan reports
Bacteria (including blue-green algae) (Table 20.1)		7
Chromists (Table 20.2)	Ochrophyta (brown algae): Phaeophyceae	30
	Ochrophyta (brown algae): Eustigmatophyceae	1
	Miozoa (dinoflagellates): Dinophyceae	2
	Bacillariophyta (diatoms)	1
Fungi		0
Plantae (Table 20.3)	Chlorophyta (green algae)	20
	Rhodophyta (red algae)	57
	Tracheophyta (sea grass)	1
Animals (Table 20.4)	Porifera	2
	Sipuncula	1
	Mollusca	5
	Arthropoda	1
	Chordata	1
	Echinodermata	1

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# Chapter 21

## Cnidarian Jellyfish: Ecological Aspects, Nematocyst Isolation, and Treatment Methods of Sting



N. Killi and G. L. Mariottini

**Abstract** Cnidarians play an important role in ecosystem functioning, in the competition among species, and for possible utilization of several active compounds against cardiovascular, nervous, endocrine, immune, infective, and inflammatory disorders or having antitumoral properties, which have been extracted from these organisms. Nevertheless, notwithstanding these promising features, the main reason for which cnidarians are known is due to their venomousness as they have a serious impact on public health as well as in economy being able to affect some human activities. For this reason a preeminent subject of the research about cnidarians is the organization of proper systems and methods of care and treatment of stinging. This chapter aims to present the data about the morphological, ecological, toxicological, epidemiological, and therapeutic aspects regarding cnidarians with the purpose to summarize the existing knowledge and to stimulate future perspectives in the research on these organisms.

### 21.1 Ecological Aspects

#### 21.1.1 *Relationships Between Cnidarians and the Environment*

Many factors such as global warming, habitat losses, rising marine constructions, overfishing, eutrophication, bottom trawling, aquaculture activities, harmful algal blooms, hypoxia, and changes on biodiversity cause jellyfish blooms in the coastal environments (Arai 2001; Purcell 2005; Purcell et al. 2007; Graham and Bayha 2008; Halpern et al. 2008; Uye 2008; Richardson et al. 2009).

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Jellyfish cause to decrease zooplankton populations, fish eggs, and larvae by predation; therefore they are in competition with fish populations directly and indirectly (Purcell and Arai 2001; Arai 2005; Purcell 2005). Also, local blooms affect fishing activities by damaging nets and cause economic losses. Jellyfish blooms have negative impact on tourism and human health (Matsueda 1969; Graham et al. 2003).

Many studies showed that water temperature becomes warmer because of the global warming. Also, global climate change has been accepted as definite by IPCC (2014). Global warming and overfishing are primary triggers of increasing gelatinous organisms. Increasing water temperatures triggers the transition and increase in reproductive success of the jellyfish species (Purcell 2005; Boero et al. 2016). Warming marine waters is causing temperate species to move toward the poles and trophic species distribute through temperate waters (Boero et al. 2016). Moreover, warmer waters bind less oxygen than cold waters. Many jellyfish species could tolerate low oxygen levels such as *Chrysaora quinquecirrha* (Condon et al. 2001) and *Periphylla periphylla* (Arai 1997; Brueggeman 1998). Jellyfish species have the ability to survive at low resources and extreme parameters (Dawson and Hamner 2003). *C. quinquecirrha* can also tolerate low salinity levels (Condon et al. 2001). *Cotylorhiza erythraea* lives at high temperature and salinity levels. *Cotylorhiza tuberculata*, which is distributed in the Mediterranean Sea, the Red Sea, and the Atlantic Ocean (Mayer 1910; Kikinger 1992; Astorga et al. 2012), is affected by global warming, and ephyra production and survival of the polyps of this species increase at high temperatures (Galil et al. 2017).

Optimum temperature values of Mediterranean jellyfish are 12–28 °C. But, it was observed that some species could survive at 4 °C in winter and at 30 °C in summer (Boero et al. 2008). According to Gülşahin (2013) optimum temperature and salinity values for *Aurelia aurita* were 20.40 °C and 38.07 ppt in southwest coasts of Turkey. Also, these values were 23.63 °C, 37.97 ppt for *C. tuberculata* and 24.50 °C, 38.70 ppt for *Cassiopea andromeda* in that area. It was found that *Pelagia noctiluca* increased at 10–27 °C temperature and 35–38 ppt salinity in the Adriatic Sea (Purcell et al. 1999a). *C. quinquecirrha* produced more ephyra at 26–30 °C and 10–25 ppt in Chesapeake Bay (Purcell et al. 1999b; Brown et al. 2002). Dawson et al. (2001) recorded that optimal range of *Mastigias* sp. was the salinity of 25–40 ppt. This species needs zooxanthellae for strobilation, and zooxanthellae decreased at over 33 °C. Polyps of *Aurelia labiata* produced more ephyra at warmer temperatures, but strobilation was not observed at  $\leq 7$  °C (Widmer 2005). *Rhopilema esculentum* showed maximum strobilation at salinities of 10–24 ppt in Southeast Asia (Lu et al. 1989). *Koellikerina fasciculata* can survive less than 28 ppt salinity (Schuchert 2007). *Cassiopea xamachana* needs temperature over 20 °C for strobilation (Hofmann et al. 1996; Kitt and Costley 1998; Berryman 2005).

Also, diversity of the jellyfish species is increasing in the Mediterranean Sea with the opening of the Suez Canal in 1869. Many erythrean jellyfish species migrated into the Mediterranean Sea (Galil 1993). *Cassiopea andromeda*, *Phyllorhiza punctata*, *Rhopilema nomadica*, *Marivagia stellata*, and *Cotylorhiza erythraea* are lessepsian scyphozoans that entered via the Suez Canal (Galil et al. 1990, 2010, 2017).

*R. nomadica* reproduced rapidly and formed dense populations in the eastern Mediterranean Sea. Blooms of this species affected fishing activities and caused economic losses in Israel and Turkey (Galil et al. 1990; Turan et al. 2011). *Cotylorhiza erythraea* was recorded previously in Suez Canal (Stiasny 1920, 1921), the Red Sea (Vine 1986), and the Egypt coasts (Dar and El-Wahab 2005). In 2017 it was recorded in Israel, which is the first record from the Mediterranean Sea (Galil et al. 2017). *Marivagia stellata* was reported for the first time from the Mediterranean in Syria (Galil et al. 2010; Mamish et al. 2016). It was thought that this species entered the Mediterranean Sea via the currents bringing ephyra or ballast waters with polyps (Galil et al. 2010).

*Pelagia benovici* is an alien scyphozoan recorded in the Adriatic Sea for the first time. Native area of this species is not certain. This species showed bloom in the North Adriatic Sea. It was thought that *P. benovici* entered the Mediterranean Sea by shipping or aquaculture activities and could move to other areas of the Mediterranean (Piraino et al. 2014).

*Rhopilema esculentum* is one of the jellyfish which commonly caused sting in humans in Chinese coasts (Dong et al. 2010). Also, this species is consumed by people in Southeast Asia and cultured in China (Omori and Nakano 2001; You et al. 2007; Hamner and Dawson 2009). It has been observed that strobilation in this species occurred when the water temperature was over 13 °C. Early strobilation was seen at >20 °C (Dong et al. 2010). *Liriope tetraphylla* is euryhaline species distributed in temperate and subtropical waters (Furnestin 1959; Goy 1973). *Periphylla periphylla* is sensitive to light because of the pigments, which make the poison when exposed to light. Therefore, this species prefer deep waters (Brueggeman 1998; Jarms et al. 2002; Bamstedt et al. 2003; Dupont and Aksnes 2010).

### 21.1.2 Cnidaria Outbreaks

Degradation of the oceans caused decline in fish stocks, increasing hypoxia, eutrophication, and ocean acidification, and increasing jellyfish blooms (Jackson et al. 2001). It has been determined that jellyfish blooms increased significantly since the 1970s (Condon et al. 2013). But, the reasons of the jellyfish blooms are not fully explained.

Most of the jellyfish have two forms: polyp and medusa in their life cycle. Polyps produce ephyra larvae asexually by strobilation, and larvae grow to medusa. Medusae release eggs and sperms, and fertilized eggs develop to planula larvae. Then planulae settle on suitable substrate and develop to polyps which are sessile forms (Arai 1997; Boero et al. 2008). This complex metagenic life cycle of jellyfish increases their distribution and abundances.

Human activities such as overfishing, eutrophication caused by pollution, and increasing coastal constructions are the significant reasons of jellyfish blooms (Arai 2001; Purcell 2005; Purcell et al. 2007; Graham and Bayha 2008; Uye 2008;

Richardson et al. 2009). Habitat modifications allow to settle for the planula larvae and formation of polyps which are very resistant to environmental changes by producing scyphistomae. Duarte et al. (2013) observed that polyps settled on wide range of structures such as floating docks and buoys, pier columns, floating pontoons, artificial reefs, oyster shells, plastic bottles, collapsing nets, and shipwrecks.

Species of the genus *Cephea* commonly form blooms (Dawson and Hamner 2009; Hamner and Dawson 2009). Cruz-Rivera and El-Regal (2016) reported *C. cephea* blooms in Egypt coasts in September 2011. This species approached the abundance of 20 individual/m<sup>3</sup> in some reef areas. According to Dawson and Hamner (2009), *Cephea* species, which are in symbiotic relationship with zooxanthellae, do not show blooms unlike other *Cephea* species without zooxanthellae.

The giant jellyfish *Nemopilema nomurai* forms blooms in the Japan Sea and causes net clogging, stinging fishermen, and economic losses in fishery (Kawahara et al. 2006). *N. nomurai* shows blooms in summer and fall in China because of the Tsushima currents (Kawahara et al. 2006). Because of the increase of jellyfish blooms, the fish populations and thus fishing activities in China declined in the late 1990s (Cheng et al. 2004; Ding and Cheng 2007). Bloom of *N. nomurai* occurred in the East China Sea and reduced *Pseudosciaena polyactis* stocks (Ding and Cheng 2005, 2007).

*Cyanea nozaki* formed aggregations in the Bohai Sea in 2004 and decreased *R. esculentum* populations, which are important economically in China (Dong et al. 2010). *C. nozaki* prefers warm and high salinity waters and showed blooms at temperature between 23 and 26.8 °C in China (Zhou and Huang 1956; Lu et al. 2003; Ding and Cheng 2007). In 2003, bloom of this species reached 70–98% of the total fishery catch and caused economic losses by cutting fishing nets and consuming juvenile fish in Chinese seas (Zhou and Huang 1956; Peng and Zhang 1999; Zhong et al. 2003; Chen et al. 2007).

Also, *A. aurita* was another bloomed species in Chinese seas between 2004 and 2009 (Su 2007; Liu 2008; Lu 2009). These blooms caused to clog intakes of power plants in 2008 and 2009 (Liu 2008; Lu 2009). Substrate increase because of aquaculture activities caused *A. aurita* blooms in Taiwan (Lo et al. 2008). Wide tolerance in low oxygen levels of *A. aurita* allows it to live in eutrophic waters, which, due to their high nutrient values, provide food supply especially for zooplankton (Decker et al. 2004; Shoji et al. 2010). *A. aurita* shows blooms almost every year in May and June in İzmir Bay, Turkey. The bay is very eutrophic because of the domestic and industrial wastes (Gülşahin 2017), and these conditions are preferable for *A. aurita*, which thrives in eutrophic regions (Caddy and Griffiths 1990). Also, bloom of this species was observed in June 2017 in İzmir Bay (personal observation of the author) (Fig. 21.1).

*Pelagia noctiluca* blooms depend on ocean circulation patterns, eutrophication, and reduction of fish species, which consume zooplankton (UNEP 1984, 1991). This species formed great aggregations in the Mediterranean Sea. This blooms negatively affected fisheries and tourism (CIESM 2001). This species feeds on all types of zooplankton and eggs and larvae of nekton (Malej et al. 1993). According to Merceron et al. (1995), *P. noctiluca* damaged fish stocks in aquaculture because of

**Fig. 21.1** *A. aurita* bloom in İzmir Bay, Turkey in 2017 (Photograph by Nurçin Killi)



stinging the fish gills and caused fish mortalities in France. In October 2007, *P. noctiluca* bloom reached such a large number in Irish coast that it killed ~250,000 salmon (Doyle et al. 2008). From September to October 2007, abundance of *P. noctiluca* reached 2.8 individual/m<sup>3</sup> in the Irish coast. It is believed that bloom of this warm water jellyfish occurred because of the ocean warming in the Northeast Atlantic (Doyle et al. 2008). Bloom of *P. noctiluca* was reported in the Strait of Messina (Italy) in 1999. This bloom has been long term and affected tourism and fishing activities (La Spada et al. 2002). Also, *P. noctiluca* caused to decrease fish populations by consuming zooplankton and fish larvae (Larson 1987; Sabates et al. 2010; Gordoia et al. 2013). It has been observed that large ephyrae of *P. noctiluca* consumed a high number of Atlantic bluefin tuna eggs (Gordoia et al. 2013).

*Liriope tetraphylla* is commonly distributed in the Mediterranean Sea (Buecher et al. 1997). Yılmaz (2015) determined that maximum abundances of this species in the Marmara Sea in 2005 (255 individual/m<sup>3</sup>), 2006 (2978 individual/m<sup>3</sup>), and 2007 (2822 individual/m<sup>3</sup>) were much higher than that of the Mediterranean Sea. These blooms brought along mucilage accumulation and zooplankton decrease (Yılmaz 2015). Especially cladocerans were affected by *Liriope* blooms and showed significant decreases in abundance in 2006 and 2007.

### 21.1.3 Interactions with Other Organisms and Endosymbiosis

Some molluscans especially planktonic nudibranchs are living on medusa. The most famous example is the relationship between the cephalopod *Argonauta argo* and jellyfish *Phyllorhiza punctata*. *A. argo* settles on the exumbrella of the jellyfish and feeds on zooplankton captured by the jellyfish. Also, the cephalopod is protected by the jellyfish (Heeger et al. 1992). *Cunina* and *Pegantha* (Narcomedusae) polyps attach to other medusa and turn into juvenile medusae (Bouillon 1987). Some

trematodes and nematodes utilize jellyfish as second host (Marcogliese 1995, 2002). *Catostylus mosaicus* is the host of parasitic isopods *Cymodoce gaimardii*. Also, *Anemonactis clavus* which is a parasitic anemone lives on *C. mosaicus* but only between May and September (Browne et al. 2017).

Some copepod species are parasites of jellyfish. These parasites utilize the jellyfish for feeding, mating, and molting. *Cassiopea xamachana* is the host of ectoparasitic copepod *Sewellocchiron fidens* (Humes 1969). Also, some barnacles, isopods, amphipods, decapods, and ophiuroids have symbiotic or parasitic relationship with jellyfish (Ohtsuka et al. 2009). Amphipods use the jellyfish as nursery habitat. Thus, the jellyfish provides both a substrate and a food (Fleming et al. 2014).

Jellyfish and fish species are in competition with each other for food. Jellyfish consume zooplankton and affect fish populations indirectly. *Aequorea victoria*, which feeds on fish eggs and larvae, consumed high quantities of herring larvae and decreased fish population in British Columbia (Purcell and Arai 2001). *P. periphylla*, which consumes copepods, is in competition with fish species, especially *Maurolicus muelleri* and *Benthoosema glaciale* (Fosså 1992). These examples prove that jellyfish affect fish stocks, but unfortunately there is not much study on these effects. *C. quinquecirrha* preferred food were copepods, especially *Acartia tonsa* in Chesapeake Bay in 1987–1990. The quantity of copepods consumed by medusae increases with copepod's density, umbrella diameter, and temperature. When abundance of this species was at its maximum, the medusae consumed 42–96% d<sup>-1</sup> of the copepods. *C. quinquecirrha* controlled copepod populations by high predation rates in Chesapeake Bay (Purcell 1992).

On the other hand, jellyfish are utilized as a substrate, a refuge, or a food by fish species (Ohtsuka et al. 2009). The butterfish *Psenopsis anomala* feeds on jellyfish, and abundance of the fish increases when jellyfish bloom (Uye and Ueta 2004). *Chrysaora fulgida* is consumed by *Trachurus* species (Salvanes et al. 2011). *Mola mola* feeds on *C. quinquecirrha* (Houghton et al. 2006). *Siganus rivulatus* prefers *Cephea cephea* and *Netrostoma setouchianum* jellyfish (Bos et al. 2017; Cruz-Rivera and El-Regal 2016). *P. physalis* is consumed by some fish species and sand crabs *Portunus* sp. Other relations between some hydrozoan and scyphozoans are presented in Table 21.1.

Scyphozoan jellyfish are preferred in the diet of people in Thailand, Indonesia, Malaysia, the Philippines, Japan, and China (Hsieh et al. 2001). Rhizostome jellyfish, which have large umbrella, are preferred for human consumption. *R. esculentum* is one of the important edible jellyfish species with significant economic value in China (Omori and Nakano 2001). This species is cultured and exported as food in Chinese coasts (Omori and Nakano 2001; You et al. 2007; Hamner and Dawson 2009). *Rhopilema verrilli*, which can grow up to 51 cm, is used for food in Japan (Calder 1972). *C. cephea* is an edible jellyfish, which is preferred as food and cultured in Southeast Asia (Omori and Nakano 2001).

Jellyfish are more commonly cultured in China than fish and shrimp (Guan et al. 2004). Jellyfish are consumed commonly as salad in eastern Asia (Hsieh et al. 2001). Also, jellyfish are used in traditional Chinese medicine, and it was believed that



**Table 21.1** Relationship types between some medusae and fish

Medusae	Fish	Relationship	Literature
<i>Physalia physalis</i>	<i>Nomeus gronovii</i>	Commensalism	Arai (1988)
<i>Chrysaora melanaster</i>	<i>Theragra chalcogramma</i>	Commensalism	Brodeur (1998)
<i>Cyanea capillata</i>	<i>Merlangius merlangus</i>	Mutualism	Arai (1988)
<i>Rhizostoma octopus</i>	<i>Merlangius merlangus</i>	Mutualism	Arai (1988)
<i>Stygiomedusa gigantea</i>	<i>Thalassobathia pelagica</i>	Predation	Drazen and Robison (2004)
<i>Apolemia sp.</i>	<i>Leuroglossus stilbius</i>	Swimming association	Robison (1983)
<i>Apolemia sp.</i>	<i>Stenobranchius leucopsarus</i>	Swimming association	Robison (1983)
<i>Aurelia aurita</i>	<i>Psenopsis anomala</i>	Parasitism	Yasuda (2003)
<i>Aurelia aurita</i>	<i>Rudarius ercodes</i>	Parasitism	Yasuda (2003)
<i>Aurelia aurita</i>	<i>Decapterus maruadsi</i>	Parasitism	Yasuda (2003)
<i>Cyanea nozaki</i>	<i>Psenopsis anomala</i>	Swimming association	Yasuda (2003)

jellyfish treat arthritis, hypertension, back pain, ulcers, tracheitis, asthma, burns, fatigue, etc. (Wu 1955).

Scyphozoan blooms negatively affect human health, but crude venom of these organisms have cytotoxic and cytolytic activities, which can be used in biomedical applications and drugs. There are many studies of the effect of extracted jellyfish venoms on various types of cancer. This subject is discussed in detail in the following subchapters.

Several scyphozoan species especially *Cassiopea andromeda* and *Cassiopea xamachana* are in relation with endosymbiotic zooxanthellae (*Symbiodinium* sp.). Jellyfish gives CO<sub>2</sub> by respiration to zooxanthellae and receives photosynthetic carbon from algae (Verde and McCloskey 1998). Also, zooxanthellae remove the ammonia from the host tissues (Cates and McLaughlin 1976). Rahat and Adar (1980), Hofmann and Kremer (1981), and Hofmann et al. (1996) reported that symbiotic algae were not essential for strobilation in *C. andromeda*.

Most anthozoans also are in relationship with zooxanthellae. When zooxanthellae left or died, the reefs begin to bleach and even die. Coral bleaching is a major problem for coral reefs and even may cause coral death. Ocean warming stresses corals and causes bleaching, which is caused by disappearing of endosymbiotic algae (Eakin et al. 2010). In 2005, one of the largest coral bleaching was caused by 1.2 °C increase in ocean temperature in the Caribbean (Shein 2006).

### 21.1.4 Bioluminescence

Bioluminescence is the ability to produce visible light by living organisms. It is seen commonly in marine vertebrates and invertebrates such as dinoflagellates, jellyfish, starfish, crustaceans, cephalopods, worms, and some fish species. Bioluminescence is commonly used by deep-sea organisms for finding food, scaring predators, and attracting mates (Widder 2010). Bioluminescence is produced with the oxidation of luciferin molecule by catalyzing with an enzyme luciferase. Coelenterazine is a kind of luciferin which is used by Cnidarians (Haddock et al. 2009).

Some scyphozoans, hydrozoans, and anthozoans are bioluminescent organisms capable to emit light by mechanical stimulations. The scyphozoan jellyfish *P. noctiluca* is brilliantly luminescent (Widder 2002). This jellyfish emits light to be visible in the dark and when it is disturbed (Marino et al. 2008).

The genus *Atolla* is a bioluminescent scyphozoan, which in response to different stimuli produces flash, glow, or weak light or remains dark (Nicol 1958). *Atolla wyvillei* and *Atolla parva* are known to emit light (Nicol 1958; Widder et al. 1989). Herring and Widder (2004) observed emitting light by some coronatae medusa such as *Atolla wyvillei*, *Atolla vanhoeffeni*, *Atolla parva*, *Nausithoe rubra*, *Paraphyllina intermedia*, *Periphylopsis braueri*, and *Periphylla periphylla*. Dome, lappets, coronal groove, and tentacle bases of medusae produced wave of light which spread radially. But, it was determined that dome and tentacles of *Atolla* spp., in contrast to *P. periphylla*, lack luminous cells.

Davenport and Nicol (1955), Shimomura et al. (1962), and Morin and Hastings (1971a, b) reported the presence of green fluorescent protein (GFP) in *Aequorea victoria*, which emits green light. Another protein which was named “aequorin” was found in *A. aequorea* by Shimomura et al. (1962). Aequorin is a calcium-activated photoprotein. This protein is capable of emitting blue light by the presence of  $\text{Ca}^{2+}$ . Aequorin and GFP mechanisms work together in bioluminescence of *Aequorea* (Shimomura et al. 1962). GFP was also found in some hydrozoans (*Mitrocoma*, *Obelia*, *Clytia*) and anthozoans (*Acanthoptilum*, *Cavernularia*, *Renilla*, *Ptilosarcus*, *Pennatula*, *Stylatula*) (Hastings and Morin 1969; Wampler et al. 1971, 1973; Morin and Hastings 1971a, b; Cormier et al. 1973, 1974; Morin 1974). Photoproteins like aequorin are used by ctenophores, radiolarians, and hydromedusae. It was found that *A. victoria* specimens cultured with a luciferin-free diet do not emit light, in contrast to *A. victoria* collected in the wild.

Siphonophores *Nanomia bijuga* and *Nanomia cara* are also bioluminescent organisms (Barham 1963; Rogers et al. 1978). Morin (1976) recorded bioluminescence of some pennatulaceans (Anthozoa) *Ptilosarcus gurneyi*, *Acanthoptilum gracile*, *Stylatula elongata*, and *Renilla koellikeri*.

## 21.2 Cnidarian Stinging and Related Aspects

### 21.2.1 *Nematocytes and Nematocysts*

Cnidarians are well-known venomous animals, living mainly in the marine and less in the freshwater environment. All cnidarians are potentially venomous, thanks to their morphological and cytological features and to their specialized double-walled stinging capsules (sometimes named “organelles”), the nematocysts, included within the stinging cells, the nematocytes, which are cell type peculiar for this phylum; the nematocysts are secretory products of the Golgi apparatus (Özbek 2011; Beckmann and Özbek 2012; Marino et al. 2013). The venom potency and effects vary widely between species.

The first inclusive report about cnidarian nematocysts comes from Mariscal (1974) who reviewed all data previously available (see the references therein). According to Mariscal (1974), the best previous review on the subject was that by Weill (1934).

Nematocysts “are among the largest and most complex intracellular secretion products known” and vary widely in length, from about 3 to about 100  $\mu\text{m}$ , depending on the species (Mariscal 1974). Furthermore, in the same species, different nematocyst types and morphologies may coexist. A study of *Cassiopea andromeda* indicates that the size of nematocysts depends on the size of the whole organism (Gülşahin 2016). Similarly, it was reported that holotrichous isorhiza nematocysts of *P. noctiluca* are longer than nematocysts in other Scyphozoans (*Catostylus mosaicus* and *Phyllorhiza punctata*) (Peach and Pitt 2005; Mariottini et al. 2008).

The nematocysts store the venom and consist of a “container,” the capsule, and a thread, which under unstimulated condition is tightly wrapped and spiralized within the capsule. After stimulation the thread is extruded and acts as a syringe needle injecting the venom. The length and morphology of the extruded thread are very variable between species (Mariscal 1974). The thread may contain spines of different length and shape and its basal enlargement (the shaft) also contains spines of different shape and length, which are used for anchoring a prey. All these characters are important for nematocyst identification and for taxonomy (Mariottini and Pane 2010).

Some marine mollusks (e.g., nudibranchs and cephalopods) (Mariscal 1974; Mariottini and Pane 2010) and turbellarians (Greenwood 2009) can prey on Cnidaria and utilize their nematocysts as a defense tool (Karling 1966).

Tourists and sea workers can frequently stumble on free swimming or floating jellyfish or on benthic corals and anemones, which can cause local and/or systemic reaction ranging from simple skin rashes to serious atopic or anaphylactic phenomena. Cnidarian stinging has an effect similar to pricking, caused by the mechanical penetration of thread into the tissues, followed by the activation of the mast cells and the irritation of nerve endings (Theoharides 1983).

The nematocysts can be found in different parts of cnidarians’ body; more frequently they are located on tentacles and, in scyphozoans, also in oral arms, but

they can be also found over the whole body and on umbrella, as in *Pelagia noctiluca*, which is therefore an “all venomous jellyfish” (Mariottini et al. 2008). Nematocysts are often aggregated into groups and are provided with a setulous appendix—the cnidocil—which after stimulation induces the extrusion of the thread (Sarà 1991). In general, the discharge occurs under adequate physicochemical stimuli causing the injection of venom in the prey/attacker. Nematocyst discharge can occur also in “nonliving” specimens, for example, by handling stranded or dead organisms (Queruel et al. 2000).

The inhibition of nematocyst discharge from *Chrysaora quinquecirrha* and *Physalia physalis* tentacles was experimentally obtained in the early 1980s with the application of a baking soda slurry and vinegar, respectively (Burnett et al. 1983). In *Pelagia noctiluca* the nematocysts can be isolated and maintained in distilled water retaining their discharging capacity (Salleo et al. 1983). In contrast, the aqueous solutions with acidic pH (1.0–3.5) were shown to induce the collapse of undischarged nematocyst wall (Salleo et al. 1984a). The discharge of each stinging capsule can be independent or coordinated with the adjacent cells (Mariottini and Pane 2010). Nematocytes can be sensitized to mechanical discharge by vibrational frequencies and compounds such as mucins, *N*-acetylated sugars, proteins, and the prey which acts as a mechanical stimulus to induce the discharge of sensitized nematocytes (Cannon and Wagner 2003). Nematocysts are used only once and disintegrate after the discharge (Mariscal 1974).

Other studies showed that trypsin (Salleo et al. 1983), extremely acidic or alkaline pH values (<2 and >11) (Salleo et al. 1984a), vinegar (Fenner and Fitzpatrick 1986), some anions (from the most effective to the less effective:  $\Gamma^-$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ) (Salleo et al. 1984b), and thioglycolate which reduces –S–S– bridges (Salleo et al. 1986) play an important role in promoting the discharge of nematocysts. Some scientists reported that the ions could affect the conformation of proteins of the capsular wall/fluid (Salleo et al. 1984b), and studies confirmed this hypothesis by evaluating the discharging effectiveness of Hofmeister anions, which resulted in increasing potency from  $\text{SO}_4^{2-}$  to  $\text{SCN}^-$  (Salleo et al. 1984c).

Recent experimental data show that the discharge of nematocysts after mechanical stimulation may be countered by 1% lidocaine, 70% ethanol, 5% acetic acid, or 20% ammonia; these compounds are also able to impair the discharge response induced by chemosensitizers (*N*-acetylated sugars, aminoacids, proteins, and nucleotides) (Morabito et al. 2014a). The same research group showed that the treatment with 2–10 mM of heavy metals (zinc, cadmium, cobalt, and lanthanum) for 20 min affected the homeostasis of *Pelagia noctiluca* and the nematocysts discharge in excised oral arms, inhibiting the discharge response; the effect was dose-dependent and irreversible after treatment with lanthanum (Morabito et al. 2014b). Also Nordesjö (2016) in her experimental study did not observe discharge of nematocysts after addition of lidocaine.

New methods used ethanol to induce discharge of nematocysts of box jellyfish *Chironex fleckeri* and to recover the venom, which was used to study and define its proteome profile. The method used scanning electron microscopy and light

microscopy to examine nematocysts before and after ethanol treatment to verify nematocyst discharge (Jouiaei et al. 2015a).

Recent *in vitro* experiments about discharge of nematocysts from *Chrysaora chinensis* exposed to acetic acid, isopropyl alcohol, sodium bicarbonate, papain, heated water, and lidocaine showed that only acetic acid and isopropyl alcohol had induced discharge, while all other compounds were ineffective (DeClerck et al. 2016).

### 21.3 Toxicological and Epidemiological Aspects

During the last decades cnidarian outbreaks have been an emergent and global problem (Mariottini and Grice 2016). For example, repeated blooms of the strongly venomous jellyfish *Stomolophus meleagris* (e.g., *Nemopilema nomurai*) and *Cyanea nozaki* were observed in the China Sea causing threat to humans (Kang et al. 2014; Li et al. 2014, 2016).

Cnidarians, which are the predators of zooplankton and small fish, produce toxic substances useful for defense/offence purposes which characterize the physiology and the ecology of these organisms. The venom contained in the nematocysts is a complex mixture of (mainly) proteins used in nature for defense/offence and for prey capture (Lassen et al. 2011). Being composed mainly of proteins and peptides, cnidarian venoms can act as the antigens inducing immune defense, production of antibodies, and also activating the immunological “memory” (Mariottini and Pane 2010).

Recent studies in which proteins in crude tentacle extracts of jellyfish *Olindias sambaquiensis* (hydrozoa) were characterized demonstrated close analogies between nematocyst venom and snake venom, both containing orthologous enzymes (polypeptides with metalloproteinase, serine proteinase, and phospholipase A2 activities) (Knittel et al. 2016).

The site of synthesis of venoms in Cnidaria is not known, but it was proposed that they are synthesized within the tissues and subsequently transferred to the nematocysts (Allavena et al. 1998).

Cnidarian venoms can have an impact on functioning of the ecosystems and on the competition among species. This was also evidenced recently by studies evaluating the impact of jellyfish *Aurelia aurita* (Baxter et al. 2011) and *Pelagia noctiluca* (Bosch-Belmar et al. 2016) on fishes and the observed damage to gills. The venom of *Cassiopea andromeda* injected intramuscularly to *Cyprinus carpio* juveniles caused partial paralysis (Gülşahin 2016).

Fishing is affected because jellyfish are often caught into nets together with fish stinging the fishermen. Furthermore, the product of fishing can be greatly damaged when mixed with disaggregated jellyfish tissues. The jellyfish outbreaks also affect recreational activities; the presence of cnidarians, especially medusae, requires the management of public health, forcing the authorities to close wide coastal sea zones (Duarte et al. 2013; Leone et al. 2013; De Donno et al. 2014; Lucas et al. 2014).

The venom of cnidarians causes both local and systemic symptoms in humans, but the seriousness varies widely depending on the species involved. The serious threat to human health caused by jellyfish stinging along Asian and Australian coasts is well documented. It has been reported that the damage induced by cnidarian venom could be also caused by the pore formation mechanisms, oxidative stress (Morabito et al. 2012), or the inhibitory effect on mechanisms of ion transport across cell membrane (Morabito et al. 2014c).

Recent data show that *Pelagia noctiluca* is the most frequent and toxic jellyfish found in the sea zone around the Balearic Islands (Spain). The first dermoscopic data on *Pelagia noctiluca* stings in the island of Mallorca showed that 25 cases of stings were observed between 2009 and 2015. The cases showed a different shape of lesions, brown dots (observed in 84% of cases), with alterations of vessels that resulted in dotted (36% of cases) or reticular (16% of cases) lesions. A typical sign of *Pelagia noctiluca* sting is the “serpentine ulceration” described by Del Pozo et al. (2016). The authors propose the dermoscopy as a valuable tool for the diagnosis of jellyfish stings (Del Pozo et al. 2016).

In order to explain the mechanism responsible for the difference in pain caused by stings of harmful and harmless jellyfish, recent data indicate that it could be ascribed to the length of nematocyst tubule causing different epithelial damage (Kitatani et al. 2015) and that the “initial acute pain might be generated by penetration of the tubule, which stimulates pain receptor neurons, while the persistent pain might be caused by the injection of the venom into the epithelium” (Kitatani et al. 2015). These authors demonstrated that nematocyst tubules in harmful jellyfish species *Chrysaora pacifica*, *Carybdea brevipedalia*, and *Chironex yamaguchii* are long enough (>200  $\mu\text{m}$ ) to penetrate the human epidermis, stimulating the free nerve endings of pain receptor fibers. In contrast, the tubules of non-harmful species such as *Aurelia aurita* are shorter. Thus, the lengthy nematocysts tubules of dangerous jellyfish are able to cause acute and persistent pain and inflammation (Kitatani et al. 2015).

## 21.4 Cytotoxicity of Cnidarian Venoms

### 21.4.1 Hemolytic Effects

The cytotoxic properties of cnidarian venoms have been studied extensively during the last decades (Mariottini 2014; Mariottini and Pane 2014; Jouiaei et al. 2015b).

The hemolytic effects of cnidarian venoms and the associated hemolysins are known for decades (Crone and Keen 1969; Mariscal 1974; Brinkman and Burnell 2009). The hemolysins were discovered in some dangerous jellyfish, such as the box jellyfish (Crone and Keen 1969; Keen and Crone 1969; Keen 1972) and the Portuguese man of war (Tamkun and Hessinger 1981). However other hemolysis-inducing compounds are also present in the less dangerous cnidarians (Hessinger and Lenhoff 1973a, b; Macek and Lebez 1981, 1988; Turk and Macek 1986;

Cariello et al. 1988; Long and Burnett 1989). It has been shown that phospholipases play an important role in inducing hemolysis (Hessinger and Lenhoff 1976).

Hemolysins (peptides and proteins) from the sea anemones (Anthozoa) were classified into four polypeptide groups: I (5–8 kDa peptides), II (20 kDa actinoporins), III lethal 30–40 kDa cytolytic phospholipase A2, and IV metridiolysin from *Metridium senile* (80 kDa) (Anderluh and Maček 2002).

Other hemolytic toxins having phospholipase activity were isolated from *Stoichactis helianthus* (Avila et al. 1988), *Urticina piscivora* (Cline et al. 1995), and *Sagartia rosea* (Jiang et al. 2003). The crude venom from *Aiptasia mutabilis* contains hemolytic toxin whose activity can be counteracted by cations, mainly by  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$ , and  $\text{Cu}^{2+}$  (Marino et al. 2009a). In a recent study of the multigene pore-forming toxins, García-Linares et al. (2016) compared the hemolytic activity of equinatoxin II (EqII), fragaceatoxin C (FraC), sticholysin I (StnI), and sticholysin II (StnII). They observed that StnII was the most active and that in the presence of cholesterol StnII bound better to the membrane and produced faster calcein release. Rivera-de-Torre et al. (2017) studied the hemolytic properties of two wild-type and four mutant actinoporins from *Stichodactyla helianthus* and showed that the hemolysis rate against sheep erythrocytes, expressed as percentage of hemolysis per second, is protein concentration-dependent.

In octocorals the occurrence of hemolytic compounds was reported for eunicellin-type diterpenoids isolated from the mucus of *Litophyton* sp. (Miyamoto et al. 1994) and from the crude extract of *Sarcophyton trocheliophorum*. The hemolytic activity of these compounds increased in alkaline and neutral pH and was reduced at acidic pH and after repeated freezing-thawing cycles (Karthikayalu et al. 2010). Limited hemolysis was also induced by extracts from *Carijoa riisei* (Reimão et al. 2008).

Cubozoans possess lethal hemolytic proteins, CrTX-I, CrTX-II, and CrTX-III from *Carybdea rastonii* (Brinkman and Burnell 2009). Other hemolytic cytolysins are present in *Carybdea rastonii* (Othman et al. 1996), *Carybdea marsupialis* (Rottini et al. 1995), *Carybdea alata* (Chung et al. 2000), *Chiropsalmus quadrigatus* (Nagai et al. 2002), and *Chironex fleckeri* (Brinkman and Burnell 2007, 2008). Cubozoan hemolysins seem to act as a calcium-dependent vasoconstrictors, affecting the mechanisms of uptake and storage of noradrenaline (Azuma et al. 1986a, b, c). Recent data show that the venom from *Chironex fleckeri* induces concentration-dependent cytolysis of sheep erythrocytes (Ponce et al. 2015).

Modest hemolytic properties were demonstrated for extracts from *Rhizostoma pulmo* (Mazzei et al. 1995; Allavena et al. 1998), while stronger hemolysis was demonstrated for extracts from *Nemopilema nomurai* ( $\text{EC}_{50} = 151 \mu\text{g}/\text{mL}$  for dog erythrocytes) (Kang et al. 2009), *Cyanea capillata* (mainly extracts from specimens larger than 20 cm, in both mesenteric and fishing tentacles) (Helmholz et al. 2007, 2012), and *Cyanea lamarckii* (with stronger activity exerted by mesenteric tentacles) (Helmholz et al. 2007). The hemolysis caused by *Cyanea capillata* was subsequently observed to be a  $\text{Ca}^{2+}$ -dependent phenomenon (Wang et al. 2013).

Recent data show that *Nemopilema nomurai* nematocyst venom (HU50 value =  $75.4 \pm 1.0 \mu\text{g protein}/\text{mL}$ ) induced more pronounced hemolysis of sheep erythrocytes than *Cyanea nozaki* nematocyst venom; high concentrations of proteins

from *Cyanea nozaki* (up to 937.5 µg/mL) induced a maximum of  $62.5 \pm 0.5\%$  hemolysis. Preincubation of *Nemopilema nomurai* nematocyst venom with a metalloproteinase inhibitor caused a decrease in hemolysis to approximately 62%, which suggests that metalloproteinases participate in the hemolytic activity (Yue et al. 2017).

The crude venom from the dangerous Mediterranean jellyfish *Pelagia noctiluca* (the “mauve stinger”) has the hemolytic properties against chicken and rabbit but not against fish red blood cells (Marino et al. 2007). Further studies demonstrated that the hemolysis induced by *Pelagia noctiluca* stings is probably caused by a pore-forming mechanism (Marino et al. 2009b). Carbohydrates, cations, proteases, and antioxidants had protective antivenom activities (Marino et al. 2008).

The SDS-PAGE electrophoresis and high-performance liquid chromatography (HPLC) studies of the crude venom from *Pelagia noctiluca* showed that it contains at least four protein fractions, which induce lysosomal membrane destabilization in red blood cells of fishes *Carassius auratus* and *Liza aurata* (Maisano et al. 2013). The crude venom did not cause oxidative stress and affects sphingomyelin in red blood cell membrane (Maisano et al. 2013).

Recently, Morabito et al. (2014b) showed that the hemolysis of human erythrocytes induced by the venom of *Pelagia noctiluca* is significantly inhibited by a 20-min treatment with 2–10 mM heavy metals (zinc, cadmium, cobalt, and lanthanum). The venom from *Chrysaora quinquecirrha* was observed to induce concentration-dependent cytolysis of sheep erythrocytes (Ponce et al. 2015).

After intraperitoneal and subcutaneous injection, *Chrysaora* sp. venom induced hemolysis, nuclear abnormalities, and echinocytes (caused by phospholipase A2), as well as vascular dilation and release of inflammatory mediators in the zebra fish *Danio rerio* (Becerra Amezcua et al. 2016).

Hemolytic activity was demonstrated also for actinoporins, small 18.5 kDa pore-forming toxins, from the freshwater species *Hydra magnipapillata*. Hemolysis was induced by HALT-1 (hydra actinoporin-like toxin-1), and ten mutants of HALT-1 were constructed and tested for hemolytic activity on human erythrocytes; the substitution of amino acids and negatively charged residues in the N-terminal region of HALT-1 reduced the hemolysis (Liew et al. 2015).

### 21.4.2 Cytotoxicity of Cnidarian Extracts on Cultured Cells

With regard to the data published before 2014, we refer to a previous review (Mariottini and Pane 2014) which gives very extensive overview of cytotoxicity of cnidarian venoms against several types of cells. This review indicates that the soft corals (octocorals) are the main source of the cytotoxic compounds. A new cembranoid diterpene 7,8-epoxy-10-hydroxy-naphthenol acetate isolated from the soft coral *Nephthea* sp. showed recently anticancer activity against HeLa and MCF-7 cells (Ishii et al. 2016). Li et al. (2016) isolated seven new briarane diterpenoids (gemmacolides AZ–BF) from the gorgonian *Dichotella gemmacea*, which induced



growth inhibition of A549 and MG63 cells with IC<sub>50</sub> values varying from 13.7 to >37.8  $\mu$ M and from 11.4 to 72.0  $\mu$ M, respectively. Three peroxy sesquiterpenoids isolated from the soft coral *Sinularia* sp. induced cytotoxicity, apoptosis, and caspase 3/7 activity in HCT116 colon cancer cells which indicates that they have potential antitumor activity, via apoptosis pathway induced by the generation of reactive oxygen species (ROS) and the accumulation of intracellular H<sub>2</sub>O<sub>2</sub> (Miyazato et al. 2016).

Recently Ayyad et al. (2017) analyzed the structure and antitumor activity of a new steroid (3 $\beta$ -,5 $\alpha$ -,6 $\beta$ -,11 $\alpha$ -,20 $\beta$ -pentahydroxygorgosterol), new diterpenoid (xeniumbellal), and three known sesquiterpenes (aromadendrene, palustrol, and viridiflorol), extracted from the soft coral *Xenia umbellata*. They showed that only xeniumbellal had antitumor activity with LD<sub>50</sub> 0.57 mM against lymphoma cells.

Chao et al. (2017) obtained five new isoprenoids and ten known compounds from the ethyl acetate (EtOAc) extract of *Sarcophyton glaucum*. These compounds were assessed for cytotoxicity against human hepatocellular liver carcinoma cells (HepG2), human breast adenocarcinoma cells (MDA-MB-231), and human lung epithelial cells (A-549). Some compounds were also assessed against human acute lymphoblastic leukemia cells (MOLT-4), human T-cell lymphoblastic lymphoma cells (SUP-T1), and human histiocytic lymphoma cells (U-937). Sarcomilasterol (an already known compound) had cytotoxicity against MDA-MB-231, MOLT-4, SUP-T1, and U-937 cells (IC<sub>50</sub> values = 13.8, 6.7, 10.5, and 17.7  $\mu$ g/mL, respectively), and another known compound, sarcoaldesterol B, had cytotoxicity against HepG2, MDA-MB-231, and A-549 cells (IC<sub>50</sub> values = 9.7, 14.0, and 15.8  $\mu$ g/mL). All other compounds showed IC<sub>50</sub> values >20 mg/mL (Chao et al. 2017).

The crude extract from the soft coral *Cespitularia stolonifera* and two ceramides (2S, 3R-4E, 8E-2-(heptadecanoylamino)-heptadeca-4, 8-diene-1, 3-diol) and its derivative (2S, 3R-4E, 8E-2-(heptadecanoylamino)-heptadeca-4,8-diene-1, 3-diacetate) showed significant cytotoxic activity against A549 cells (lung cancer), and two steroids (24-methylcholesta-5, 24(28)-diene-3 $\beta$ -ol and 24-methylcholesta-5, 24(28)-diene-3 $\beta$ -acetate) exhibited significant cytotoxic activity against MCF-7 cells (breast cancer) (Elshamy et al. 2017).

Six gemmacolides (briaranes) and dichotellide O derived from octocorals were cytotoxic against A549 cells with IC<sub>50</sub> values of 28.3, 24.7, 34.1, 26.8, 25.8, 13.7, and 25.5 mM, respectively. Furthermore four of these briaranes and dichotellide O showed cytotoxicity also toward MG-63 cells (IC<sub>50</sub>s = 15.8, 11.4, 30.6, 34.8, and 36.8  $\mu$ M, respectively) (Su et al. 2017).

Vaikundamoorthy et al. (2016) isolated the compound 2-ethoxycarbonyl-2- $\beta$ -hydroxy-A-nor-cholest-5-ene-4one (ECHC) from butanol extracts of hexocoral *Acropora formosa*. This compound possesses antioxidative activity increasing ROS generation in human non-small cell lung cancer cells (A549), causes sensitization of mitochondrial membrane with consequent release of cytochrome C, decreases expression of antiapoptotic genes involved in cancer cell proliferation, invasion, and metastasis, increases expression of apoptotic genes responsible for cell cycle arrest and cell death, and upregulates tumor suppressor p53.

The anticancer activity of four low-molecular-weight proteins isolated from the sea anemone *Anemonia viridis* was studied using reversed-phase chromatography and PC3, PLC/PRF/5, and A375 human cancer cell lines; two fractions (III and IV) were cytotoxic to cells, while fractions I and II had very low toxicity but showed antiproliferative activities (Bulati et al. 2016). The recombinant polypeptide HCGS 1.20 derived from the sea anemone *Heteractis crispa* showed anti-inflammatory effect; it inhibits the increase of calcium ions caused by the histamine and decreases NO content in lipopolysaccharide-stimulated macrophages derived from mouse bone marrow (Sintsova et al. 2015).

New studies of the cytotoxicity of extracts/compounds from jellyfish show that the extract from an “apparent” innocuous species, the Mediterranean “fried egg jellyfish” *Cotylorhiza tuberculata*, showed biological activity against MCF-7 breast cancer cells and HEKa human epidermal keratinocytes. It has to be noted that this extract included not only polypeptides derived from jellyfish tissues but also photosynthetic pigments,  $\omega$ -3 and  $\omega$ -6 fatty acids, as well as polypeptides derived from algal symbionts. The extract had also antioxidative properties, affecting the viability of cells and the intercellular communication. MCF-7 cells were more sensitive than HEKa cells (Leone et al. 2013).

The venom from *Chrysaora quinquecirrha* shows concentration-dependent cytotoxicity against fish gill cells and rat cardiomyocytes, which exhibited morphological changes, clumping, and detachment from the substrate (Ponce et al. 2015; Ponce García 2017).

Recent transcriptomic and proteomic studies of *Chrysaora fuscescens* venom have identified 163 proteins. Twenty-seven of them were classified as “putative toxins” and grouped into six protein families (proteinases, venom allergens, C-type lectins, pore-forming toxins, glycoside hydrolases, and enzyme inhibitors). Other proteinases, lipases, and deoxyribonucleases were identified only in the venom transcriptome (Ponce et al. 2016).

The exposure of HEK 293 Phoenix mammalian cells to crude venom of *Pelagia noctiluca* was shown to alter ion conductance of plasma membrane; this activity influenced the homeostatic functions and the regulation and maintaining of cell volume, without influence on plasma membrane conductance of  $\text{Ca}^{2+}$  and  $\text{K}^{+}$ . The venom was observed to induce  $\text{NaCl}$  and water influx causing cell swelling which could be the reason of the known hemolytic and cytolytic activity of *P. noctiluca* venom (Morabito et al. 2017).

Cultured HaCaT (human keratinocyte) and NIH3T3 (mouse fibroblast) cells were utilized to evaluate the toxicity induced by *Nemopilema nomurai* venom. After treatment with venom, both cell lines showed cell viability decrease; this effect correlated with the induction of metalloproteinase-2 and metalloproteinase-9 (Kang et al. 2013).

Recent studies of the cardiac toxicity of *Nemopilema nomurai* venom on rat cardiomyocytes (cell line H9c2) showed that venom caused dose-dependent decrease of cell viability. Western blot analysis showed that the expression of myosin VII, annexin A2, aldose reductase, suppressor of cytokine signaling 1, and

calumenin which are known markers of cardiac dysfunctions was altered (Choudhary et al. 2015).

The venom from the cubozoan *Chironex fleckeri* induces concentration-dependent cytotoxicity with morphological changes, clumping, and detachment, against fish gill cells and rat cardiomyocytes; the cytotoxic activity of the venom from *C. fleckeri* was 12.2- (after 30-min exposure) and 35.7-fold (after 120-min exposure) higher than the venom from *C. quinquecirrha*. The authors suggest that the cytotoxic effects may be due to the activity of a group of toxic proteins previously identified in *C. fleckeri* and in other cubozoans (Ponce et al. 2015; Ponce García 2017).

Ten mutants of actinoporin-like toxin-1 (HALT-1) gene from *Hydra magnipapillata* were constructed and tested for their activity against HeLa cells. The cytotoxicity was reduced by substitution of amino acids and by negatively charged residues in the N-terminal region of HALT-1 (Liew et al. 2015).

## 21.5 Cnidarians as an Underexploited Rich Resource for New Therapeutics

Notwithstanding their well-known venomous characteristics, cnidarians have been repeatedly indicated as a source of natural bioactive compounds having possible utilization in synthesis, production, and development of new drugs or biomedical materials (Mariscal 1974; Ayed et al. 2012; Daly et al. 2014; Malve 2016; Mariottini and Grice 2016).

From the first discovery of bioactive substances in cnidarians (prostaglandins (15R)-PGA2 from *Plexaura homomalla*; palytoxin from *Palythoa toxica*; pseudopterosin, sarcodictyins, and eleutherobin) (Weinheimer and Spraggins 1969; Moore and Scheuer 1971; Kohl and Kerr 2003), a lot of other interesting compounds have been isolated from these organisms. Unfortunately, at present the practical utilization of cnidarian extracts and compounds isolated or synthesized on the basis of natural ones is still very scarce. Therefore, there is a great need to evaluate new compounds or reevaluate those already known for their potential to develop new drugs and new tools to fight diseases affecting humans and nonhuman beings.

## 21.6 Management and Treatment of Cnidarian Jellyfish Stings: Review Papers

The management of cnidarian sting has been the subject of several reviews.

According to Cegolon et al. (2013) and to Montgomery et al. (2016), the main issues in jellyfish envenomation are (1) how to prevent the discharge of unfired nematocysts and (2) how to fight the local and the systemic effects of venom. In their

review Cegolon et al. (2013) reported that hot water and ice packs and oral/topical analgesics are widely used to fight the pain caused by stings, while the prevention of discharge of unfired nematocysts is mainly obtained using vinegar. The review recommends to avoid the use of alcohol, methylated spirits, freshwater, and immobilization bandaging, all factors which can promote nematocyst discharge, and suggests the use of protective equipment for bathers in the areas at risk (Cegolon et al. 2013).

By examining seven trials assessing the treatment for *Physalia* sp., *Carukia* sp., and *Carybdea alata* stings in which 435 participants were enrolled, Li et al. (2013) reported that hot water immersion allowed major pain relief in comparison with ice packs, but these two treatments showed no statistically significant difference when dermatological outcomes were considered.

Montgomery et al. (2016) reported that oral analgesics, seawater, baking soda slurry, and hot water at temperature 42–45 °C inhibit nematocyst discharge and give pain relief, while the usefulness of vinegar depends on the stinger species.

According to Honeycutt et al. (2014), the pain can be fought with topical heat and oral analgesics, while a doubt about the use of parenteral analgesics, topical steroids, anesthetics, and antihistamines is reported; adhered tentacles are to be removed from the skin of stung subjects in order to prevent nematocyst discharge. This review states that the the vinegar should be used to prevent the discharge of nematocysts of *Chironex fleckeri*, *Physalia physalis*, and *Alatina alata*, but not to treat *Chrysaora quinquecirrha* and *Cyanea capillata* stinging whose nematocysts are better treated with baking soda. Stings from *Pelagia noctiluca* can be treated with magnesium sulfate.

According to Lakkis et al. (2015) the stung area should be washed with seawater and not with freshwater and immersed in vinegar but only if the stinger is *Carukia barnesi*, *Carybdea alata*, or *Pelagia noctiluca*; then tentacles are to be removed, and the stung area is to be immersed in hot water (not for *Chironex fleckeri* stings) and eventually treated with cold packs (only for *Chironex fleckeri*, *Pelagia noctiluca*, and *Physalia* sp.). Treatment with antihistamines, steroids, antibiotics, and immunomodulatory drugs should be considered.

A review about the treatments for jellyfish envenomation performed in North America and Hawaii showed variable response and conflicting results which, according to the authors, contribute “to considerable confusion about what treatment is warranted and efficacious” (Ward et al. 2012). The paper by Ward et al. (2012) suggests also that “vinegar causes pain exacerbation or nematocyst discharge in the majority of species,” except for *Physalia* sp. stings, and recommends removal of nematocysts, washing with salt water, and the utilization of hot water and topical lidocaine to treat pain. The statement about vinegar has been assertively confuted by Auerbach (2013) who, on the basis of his own experience, stated that mainly vinegar but also ammonia, isopropyl alcohol, or a combination of vinegar and isopropyl alcohol are to be used both for topical decontamination and for alleviating pain. This subject and the consequent debate will be reopened below in the section dedicated to cubozoans.

A review about the management of envenomations during pregnancy (Brown et al. 2013) reports only one case of serious envenomation by jellyfish which occurred in Australia and was treated with antivenom within 30 min from sting. The authors recommend to limit the exposure to jellyfish and to use clothing and proper lotions during pregnancy.

A recent review (Hornbeak and Auerbach 2017) summarizes the data about treatment of hydrozoans, scyphozoans, cubozoans, and sea anemone stings stating that acetic acid 5% (vinegar) is useful in all cases except for the treatment of *Physalia physalis* envenomation; corticosteroids and antihistamine are also suggested. A lidocaine-containing product is indicated for scyphozoan stings. The authors state that envenomation by box jellyfish requires specific antivenom administration to be repeated during the first hours after sting, while in Irukandji envenomation serum troponin and cardiac monitoring are to be considered; the use of  $\beta$ -adrenergic blockers is forbidden because it could contribute to myocardial ischemia (Hornbeak and Auerbach 2017). The suggestion to avoid vinegar when the exposure source is unknown is reported by Balhara and Stolbach (2014) in their review about envenomation in North America considering that *Physalia physalis* envenomations are common in the United States waters.

In summary, the treatment of cnidarian stinging is not well defined, as emphasized by Ostermayer and Koyfman (2015) who complain of different first-line treatment recommendations by international organizations.

## 21.7 Management of Stings by Hydrozoans

First aid for *Physalia* sp. stinging includes removal of tentacles from the skin by hand or by washing with seawater and hot (45 °C) water (preferred to ice pack) immersion limited to 20 min (Berling and Isbister 2015).

Wilcox et al. (2017) found only a small percentage (around 1.0%) of nematocyst discharge during stinging of *Physalia* sp., so that they tested various rinse solutions to determine their ability to affect discharging and found that vinegar (but only when undiluted) and a commercial spray product (Sting No More®) irreversibly inhibit nematocyst discharge. In contrast, alcohols, urine, baking soda, and shaving cream caused fast nematocyst discharge.

Kajfasz (2015) suggests to treat the sting by *Physalia physalis* by rinsing the affected part of the body with hot seawater or any warmed fluid, after removal of tentacles. One percent lidocaine acts as an anesthetic and prevents further nematocyst discharge, while ice packs, paracetamol, acetylsalicylic acid, or ibuprofen can be used to reduce pain, antihistamines to limit the allergic response, and hydrocortisone to reduce inflammation. They discourage to use alcohol (ethanol), methylated spirit, baking soda, diluted ammonia, vinegar (4–6% acetic acid), boric acid, lime juice, urine, and freshwater because they could induce nematocyst discharge.

Li et al. (2015) on the basis of the examination of guidelines and published results treated a 11-year-old girl stung by *Physalia* sp. with hot water immersion, while

Manabe et al. (2014) treated a case of allergic dermatitis caused by jellyfish suspected to be *Physalia physalis*, with topical steroids and anti-allergic drugs for a long period of time.

Ottuso (2013) states that “there is some controversy regarding the acute treatment of *Physalia* stings.” In fact, considering the literature he states that some authors indicate vinegar may cause further firing of nematocysts, but others recommend the prompt application of vinegar. Pain can be alleviated by immersion in hot water (45 °C), ice water packs, and seawater rinsing; in contrast, the freshwater must never be utilized because it is hypotonic and able to cause nematocyst firing. In proper cases, the use of topical and systemic steroids, antihistamines, and antibiotics is suggested (Ottuso 2013). Also for other hydroids, Ottuso (2013) recommends the same treatment as per *Physalia* injuries (ice packs, topical steroids, antihistamines, analgesics), but again the usefulness of vinegar is not clear. For lesions by true corals (such as *Millepora*), Ottuso (2013) recommends the removal of any residual and rinsing with cold seawater. Oral or topical antibiotics and tetanus toxoid should be considered for ulcerated or necrotic wounds.

Da Silva Barth et al. (2017) studied the properties of *Ipomoea pes-caprae* whose leaves are used in folk medicine to treat jellyfish stings. Experimental studies with the venom of *Physalia physalis* showed that in mice pretreated with plant extract, the effects of venom were significantly reduced.

## 21.8 Management of Stings by Scyphozoans

Many scyphozoans possess venomous properties. Scyphozoan stings are treated essentially as for hydrozoans (see above), tentacles must be removed from the skin, and a treatment with cold seawater and acetic acid application in order to inactivate the remaining nematocysts must be predisposed. In selected cases, the administration of analgesics is suggested, and the possibility of infections must be carefully considered. Larval forms of *Linuche unguiculata* (Coronatae) may cause eruptions which are treated with antihistamines and topical steroids (Ottuso 2013).

On the basis of experimental studies on in situ discharge of nematocysts, Morabito et al. (2014a) suggested that after stinging by *Pelagia noctiluca*, a prompt local treatment with lidocaine, acetic acid, ethanol, and ammonia may provide pain relief and reduce local and systemic adverse reactions.

A recent study involved 96 subjects stung by *Chrysaora chinensis* and treated with different methods (isopropyl alcohol, hot water, acetic acid, papain with meat tenderizer, lidocaine, sodium bicarbonate). Papain was effective in relieving pain in a relatively short time (30 min); erythema was reduced by sodium bicarbonate and papain (DeClerck et al. 2016).

Doyle et al. (2017) underline the absence of rigorous scientific support for medical treatment protocols and report that after stinging by *Cyanea capillata*, seawater rinsing, a method commonly recommended for tentacle removal, “induced significant increases in venom delivery, while rinsing with vinegar or” a commercial

spray (Sting No More®) “did not” (Doyle et al. 2017). The authors, on the basis of in vitro heat treatment of sheep erythrocytes, suggest to utilize heat and no ice pack, to treat stinging by *Cyanea capillata*. Metalloproteinase activity of tentacle extract from *Cyanea capillata* induced extensive hemorrhage and necrosis in rats (Wang et al. 2015), so the authors suggested the use of metalloproteinase inhibitors as therapeutic agents to treat hemorrhagic injuries induced by jellyfish stings.

An epidemiological study concerning the envenomation by *Rhopilema nomadica* suffered by children between 2010 and 2015 reports that treatments in the emergency department were focused on pain control, which was attempted using mainly nonsteroidal anti-inflammatory drugs and opiates; some cases required the use of antihistamines and topical corticosteroids (Glatstein et al. 2017).

Kang et al. (2013), on the basis of previous studies demonstrating that most of scyphozoan jellyfish venoms contain metalloproteinases (whose activity seems to be correlated with dermal inflammation and toxicity), after in vitro (already reported above in this chapter) and in vivo studies suggested the use of metalloproteinase inhibitors, such as tetracycline, as therapeutical tools in case of envenomation by *Nemopilema nomurai*. Notably, the pretreatment of venom with tetracycline countered dermal toxicity in rabbit skin (Kang et al. 2013).

Matrix metalloproteinase-14 and astacin-like metalloprotease toxin 3 precursors have been identified in proteins of venom from *Nemopilema nomurai* (Kang et al. 2014), and a polyclonal antibody against venom has been generated by immunization of rabbits with venom antigen and characterized for the neutralizing effect against the venom itself using tests on cells (Kang et al. 2014).

In a study carried out in the zone of Basra, Iraq, Al-Rubiay et al. (2009) described the local treatment (seawater, tap water, and ice) used by fishermen stung (mainly on the hands, arms, and legs) by *Rhizostoma* sp.; unfortunately, several subjects suffered from immediate and delayed skin reactions.

Ocular symptoms after stings of jellyfish suspected to be *Aurelia aurita*, characterized by pain, conjunctival injection, corneal lesion, and photophobia, were reported by Mao et al. (2016) and treated with irrigation of eyes with seawater or saline and with analgesics. Topical cycloplegics, steroids, antibiotics, and antihistamines were also used.

## 21.9 Management of Stings by Cubozoans

The treatment of cubozoan stings is a subject of primary importance for human health, due to the extremely dangerous consequences of lesions. It is known from experience and from literature that the stings of *Chironex fleckeri* can induce systemic envenoming and cardiac arrest and be life-threatening, therefore requiring early resuscitation (Berling and Isbister 2015). This subject has caused controversies about the suitability of some methods of treatment. The review by Ottuso (2013) emphasizes four main points: (1) the removal of residual tentacles adhering to the skin of stung people is mandatory and should be performed with gloved hands;

(2) 2–10% acetic acid is able to inhibit nematocyst discharge within 30–40 s and this occurs in all cubozoan nematocysts; (3) some data indicate that cold seawater or ice packs are useful in alleviating pain, but other data suggest hot water immersion to obtain the same result; and (4) the utilization of other procedures (utilization of urine, colas, alcohol, and administration of antihistamines) is discouraged because they could induce worsening of symptoms (Ottuso 2013).

The usefulness of vinegar has been the subject of debates between scientists after the publication of a paper (Welfare et al. 2014) aimed to verify the effect of vinegar on discharged nematocysts through a simulating system: tentacles of *Chironex fleckeri* were placed on amniotic membrane and subsequently electrically stimulated to induce the emission of venom which was collected before and after the application of vinegar and tested on human cardiomyocytes. The authors concluded that “vinegar promotes further discharge of venom from already discharged nematocysts” and advise against the use of vinegar as first aid for subjects stung by *C. fleckeri* (Welfare et al. 2014). Subsequently, Gibbs et al. (2014) criticize these recommendations stating that “it is unclear whether [the technique] correlates to what happens *in vivo* when someone is stung by a *Chironex* jellyfish” and defend the recommendation of the Australian Resuscitation Council affirming that vinegar is known “unequivocally” to inactivate undischarged nematocysts (around 80% of nematocysts employed in a stinging episode) in human skin and to prevent discharge. Also Yanagihara and Chen (2014) affirm that the dangerousness of the use of vinegar against *C. fleckeri* stings is not supported by funded data. Little et al. (2016) underscore that the Australian Resuscitation Council recommends the utilization of vinegar and ice as first aid for jellyfish stings, but they report the successful use of hot water to treat stinging by *Chironex fleckeri* and *Carukia barnesi* without resort to analgesics.

Horiike et al. (2015) complained about the lack of specific allergen and unconventional medical treatment after cnidarian stinging and reported that the most important toxic protein of *Chironex yamaguchii* venom (CqTX-A) is a heat-stable IgE-binding allergen. Commenting the results, the authors present the possibility to develop a specific venom immunotherapy.

Thaikruea and Siriariyaporn (2015) report the use of oral antibiotics after box jellyfish stinging in two patients; one of them was previously treated also with juice from the morning glory leaves, and subsequently with intravenous steroids, oral antihistamines, and oral analgesics, but the surgery was necessary to remove the necrotic tissue. The utilization of antibiotics is justified in case of large and unclean wounds and of secondary infections (Berling and Isbister 2015). Thaikruea and Siriariyaporn (2016) report that one fatal case of box jellyfish envenomation in the Gulf of Thailand received freshwater and ice packs as first aid treatment, while one out of six fatal cases received vinegar.

A comparison of the effects of hot water (45 °C) immersion and ice packs as remedy for *Chironex fleckeri* stings was reported by Isbister et al. (2017) who stated that the two treatments yielded similar results with hot water was better in decreasing pain.



Finally, it is to be remembered that antivenom for *C. fleckeri* stings is available and can be used in case of extensive lesions of the skin, severe pain, cardiac collapse, and decompensation with cardiac or pulmonary symptoms (Ottuso 2013; Berling and Isbister 2015). In the case of Irukandji syndrome, which is known to be connected with cubomedusae stinging and is characterized by generalized pain (requiring analgesia), autonomic excess, and, uncommonly, myocardial depression and pulmonary edema (Berling and Isbister 2015), the treatment of pain is to be performed with narcotic analgesics or magnesium sulfate infusion which is effective to treat sympathetic symptoms; in case of hypertension, the administration of an alpha-adrenergic receptor blocking agent may be useful (Ottuso 2013).

Berling and Isbister (2015) state that first aid for box jellyfish stings includes tentacle removal by hand or seawater washing and application of vinegar to prevent further discharge of venom. Pain should be treated with ice packs, oral analgesia, and, in selected cases, with titrated intravenous opioid analgesia. Hamann et al. (2014) reported the relief of pain caused by box jellyfish by using palm oil and lemon juice emulsion.

Yanagihara et al. (2016) performed experiments (ex vivo model) which involved a direct application of test solutions on live tentacles with the purpose to quantify the amount of spontaneous nematocyst discharge from freshly excised tentacles. By using this system, they evaluated the efficacy of some treatments: alcohols induced massive discharge; urine, freshwater, and carbonated soft drink (named by authors as “cola”) induced moderate discharge. Vinegar that the authors defined as “the 40-year field standard of first aid for the removal of adherent tentacles” completely inhibited discharge, but the better inhibition of firing and hemolysis was obtained with the application of a commercial formulation containing copper gluconate, magnesium sulfate, and urea (Yanagihara et al. 2016). In a subsequent paper, Yanagihara and Wilcox (2017) evaluated the inhibition of hemolysis by scraping or rinse solutions after cubozoan venom application, showing that hemolysis was significantly increased by scraping which causes discharge. Vinegar and heat inhibited effectively the increase of hemolysis caused by tentacles from the carybdeid cubozoan *Alatina alata*, but vinegar was less effective against the discharge of *Chironex fleckeri*. Rinsing with seawater (and also ice for *A. alata* stings) was not effective and increased venom load. The authors suggested to avoid scraping or seawater rinsing during the removal of cubozoan tentacles (Yanagihara and Wilcox 2017).

## 21.10 Treatment and Management of Stings by Undefined Cnidarians

Considering the possible secondary reactivations after a first stinging of an unknown cnidarian and to fight recurrent dermatitis, Asztalos et al. (2014) consider the use of immunomodulators (pimecrolimus, tacrolimus) and topical and local corticosteroid, as described for a patient originally treated with baking soda and vinegar.

Choong et al. (2015) described the need of surgical fasciotomy to fight swelling in a patient with Irukandji-like symptoms and previously treated with intramuscular antihistamine and oral antibiotics. Fu et al. (2014) described the case of a patient whose wound was treated with 8% acetic acid solution and topical steroids whose symptoms disappeared within 10 days and were resolved with moderate post-inflammatory hyperpigmentation.

Haddad (2013) stated that iced seawater or cold packs (not freshwater) has a potent analgesic effect and the application of vinegar is a good procedure for all cases of cnidarian stinging they treated but discourages the use of antihistamines and alcohol because they are not adequate.

In a study considering the hospitalizations in the Salento zone (Southern Italy), De Donno et al. (2014) reported that corticosteroids were the main drugs used, while ammonia was the main non-pharmacological treatment.

In the emergency department of Langkawi Hospital (Malaysia), Mohd Suan et al. (2016) reported the utilization of corticosteroids (hydrocortisone), antihistamines (Piriton), pain reliever 583, histamine H<sub>2</sub>-blocker (ranitidine), and vinegar.

A recent paper (Li et al. 2017) reports an allergic shock characterized by dizziness, pruritus, dyspnea, hypotension, and tachycardia and caused by cooked, salt preserved jellyfish ingestion in a young Chinese patient who was stung by an undetermined jellyfish 6 months before the reported episode. The treatment (intramuscular phenergan, normal saline, intravenous dexamethasone, and intravenous cimetidine) solved quickly the main clinical symptoms.

To treat epithelial keratitis and corneal edema consequent to eye stinging, Yao et al. (2016) utilized 3% NaCl eyedrops and 0.3% norfloxacin eyedrops; the treatment was effective.

## 21.11 Conclusions

The research on cnidarians has a preeminent importance from several points of view. The knowledge of morphological, biological, physiological, and ecological characteristics of these organisms is a key to the management of environmental threats and health and economic problems that they may pose.

Global warming triggers the blooms of jellyfish. Many jellyfish species begin to early strobilate at warmer temperatures (Hofmann et al. 1996; Kitt and Costley 1998; Purcell et al. 1999b; Brown et al. 2002; Berryman 2005; Widmer 2005). Increasing jellyfish aggregations are affected by anthropogenic factors such as overfishing, marine constructions, aquaculture activities, and eutrophication (Purcell et al. 2007). However, migration by the ballast waters, currents, or fouling cases cause to increase of jellyfish species. According to Boero (2013), lessepsian migrations of scyphozoan jellyfish have already continued. Cnidaria outbreaks may cause zooplankton and fish populations decrease, but the range of this decline is not been fully explored. Therefore, more researches are needed on this subject.

Cnidarian envenomations are a strong threat for public health causing “more deaths than shark attacks annually” (Wilcox and Yanagihara 2016); therefore, the prevention of stings and the removal of residual tentacles or cnidarian parts from human skin after stinging are the first measures to be taken. Many anecdotal and often unadequate popular treatments are reported, but a good knowledge about the mechanisms of toxicity and the measures to be taken, as well as univocal and data-funded first aid care, is mandatory. Controversies exist about the use of heat or cold applications and of vinegar (“vinegar yes” or “vinegar no”?) and acetic acid preparations, for which there is not agreement even though they are widely used with good results. Therefore, this is a research field with a “work in progress,” and a great impulse of the research is required with the perspective (1) to find and evaluate new compounds or new systems/methods of treatment and (2) to reevaluate the available systems/methods/compounds of treatment in order to reach a wide agreement between the researchers to give a good support to the operators in the field.

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# Chapter 22

## These Colors Don't Run: Regulation of Pigment—Biosynthesis in Echinoderms



Cristina Calestani and Gary M. Wessel

**Abstract** Pigment production is an important biological process throughout the tree of life. Some pigments function for collecting light energy, or for visual identification, while others have dramatic antimicrobial functions, or camouflage capabilities. The functions of these pigments and their biosynthesis are of great interest if only because of their diversity. The biochemistry of echinoderm pigmentation has been intensively studied for many years, and with more recent technologies, the origin and functions of these pigments are being exposed. Here we summarize the major pigment types in biology and emphasize the status of the field in echinoderms, taking full advantage of the new genomic and technologic resources for studying these important animals and their beautiful pigmentation.

**Keywords** Melanin · Carotenoids · Porphyrins · Quinones · Polyketides · Polyketide synthase · CRISPR/Cas9 · Echinoderm · Sea urchin

### 22.1 Introduction

Color is everywhere on our planet. Whether it is the sky, the mountains, the forest, or the caterpillar, color provides the signals for us to recognize and discriminate objects in our world. Color in biology is especially prevalent and perhaps a key feature for many organisms. The green, then red, then brown for the leaf of a maple tree, or the diverse skin tones of humans, color is everywhere in our biological world, and the regulation of pigment biosynthesis is key for this ubiquitous quality of organisms.

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For many organisms, pigments convey information for mating, camouflage, socialization, molecular protection, and many other functions.

Biological pigments have broad ranges of colors and a vast repertoire of pigment types with which to generate colors. In animals, the biosynthetic machinery for some pigments is inherently encoded within the genome of the organism, e.g., melanin in humans, whereas in other organisms, the color may come from a symbiont, e.g., corals, and their algae colleagues. Some pigments in animals are derived even from the diet, such as the dependence of the rich pink plumage in flamingos on carotenoids from their diet.

Here we consider pigments in echinoderms. These animals have been a rich source of pigment research for over 125 years (MacMunn 1883) and for many good reasons. One, echinoderms are enormously diverse in color with rich differences even within a species. Second, many echinoderm species are easy to harvest, and highly abundant, making deep biochemical analysis feasible. Third, echinoderms are basal branching deuterostomes whose sister taxa include chordates. Thus, many of the genes involved in development, in regeneration, and in pigment formation are more closely related to mammals, for example, than those of any other invertebrate. Thus, due to a rich literature in pigment research in echinoderms [see, e.g., Fox and Hopkins (1966)], now enhanced by tools of modern molecular biology, one can begin to parse out the regulatory mechanism and biochemical steps to elucidate pigment biosynthesis, and even function, in these animals.

## 22.2 Pigments for Consideration

### 22.2.1 *Melanins*

Melanins are widely present in plants, animals, fungi, and bacteria. Although its name is from the Greek meaning dark, or black, the many variations in melanin formation and modification result in a wide range of colors and properties. In echinoderms, melanin formation and distribution have been studied in several taxa, especially in sea urchins, sea cucumbers, and brittle stars (Fox and Hopkins 1966). Often associated with the melanin granules in echinoderms are fluorescent pigments, which are perhaps involved in the biosynthesis of melanin and/or melanin-containing granules (Fontaine 1962). Biosynthesis of the melanin pigment is initiated by oxidation of tyrosine, which is then polymerized into larger molecular structures that will be variably modified, depending on the cell type and organism studied. Melanin variants have been studied biochemically in echinoderm for many years, e.g., see review by (Fox and Hopkins 1966). In many animals, melanin is thought to be a block to UV radiation, and the appearance and distribution of melanin in human evolution are support for such a contention. Melanin is also involved in the innate immune system of many invertebrates in which invading microbes are coated with melanin, the so-called melanization reaction, which neutralizes the invader and aids in its destruction. The function of melanins in

echinoderms is not clear, but being rich in the integument of, e.g., sea cucumbers, suggests it may also function in blocking UV radiation. However, melanin has also been found in the amoebocytes of sea urchins, cells active in combating microbes located within the body cavity of the animal, suggesting a role instead (or in addition to) for protecting against pathogen infection (Jacobson and Millott 1953). Understanding the biosynthesis of this pigment is, however, complicated since the major enzyme responsible for converting tyrosine to DOPA, tyrosinase—a copper-containing phenol oxidase—is not detectable yet in any of the echinoderm transcriptomes/genomes listed ([echinobase.org](http://echinobase.org); Dec 2017). Perhaps this enzyme activity is accomplished by various different genes or is highly variable in sequence in certain taxa.

### 22.2.2 *Carotenoids*

Carotenoids are organic pigments responsible for the bright red, yellow, and orange colors that make many fruits and vegetables so recognizable. They are present in a wide variety of plants, algae, fungi, and bacteria and help the cell both absorb light energy and protect the chlorophyll pigment from photodamage. Several hundred carotenoids are known, all derivatives of tetraterpenes, which contain 40 carbon atoms derived from 8 polymerized isoprene units. This carotenoid building block is modified into hundreds of variants, which all have an absorption ranging from 400 to 550 nm (violet to green) and not in the yellow, orange, and red wavelengths. Animals benefit from carotenoids as vitamin precursors (vitamin A activity) and serve as an important antioxidant, yet these molecules must come from the diet of the animal since carotenoids are not generally produced in animals. The few exceptions to this rule are the spider mite and aphids, which appear to have acquired the genetic machinery to synthesize their own carotenoids as a result of horizontal gene transfer from a fungus (Moran and Jarvik 2010; Novakova and Moran 2012). Echinoderms do not have carotenoid biosynthetic genes but do have many varied types of carotenoid pigments in their adult body. These pigments are diet derived and then likely modified by the adult echinoderm in varied ways.

### 22.2.3 *Porphyrins*

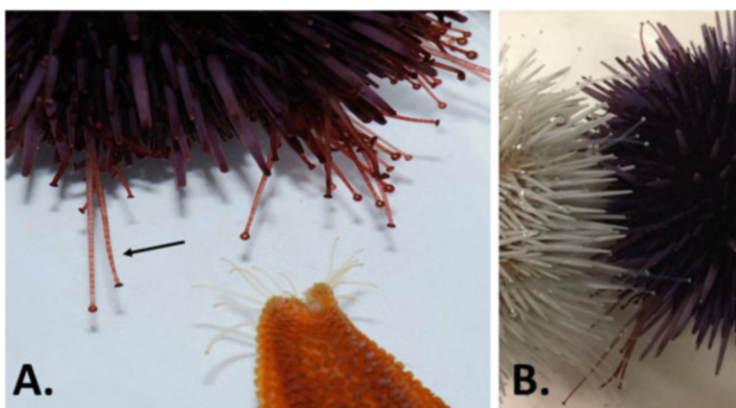
Porphyrins are heterocyclic organic molecules. Heme, from mammalian red blood cells, is the poster child of porphyrins. In animals, fungi, and protozoa, the commitment step of porphyrin biosynthesis is through aminolevulinic acid (ALA) synthase, which produces 5-aminolevulinic acid from glycine and succinyl-CoA. The ALA building blocks are then used by the cell to make the macrocyclic ring that becomes modified in many ways to make varied porphyrin pigments (and other organic compounds). Although many echinoderms have substantial levels of porphyrins in their integument, and animals are capable of biosynthesizing porphyrin pigments, it

is less clear in echinoderms which species biosynthesize their own tetrapyrrole rings and which species acquire these structures from their diet and only modify the parent molecule. Further, some echinoderms (holothurians) appear to use tetrapyrroles as a prosthetic for their respiratory molecules. The genomic resources now available in echinoderms and the well-known pathways for porphyrin biosynthesis should enable at least a cursory test of biosynthesis of this important pigment precursor. Indeed, a gene (SPU\_024016) was found in the sea urchin *Strongylocentrotus purpuratus* with strong identity (62% amino acid identity; e-164 significance) to the mouse gene encoding aminolevulinic acid synthase 2 (EDL07517) supporting a synthetic pathway at least in this sea urchin for the porphyrin biosynthesis [[echinobase.org](http://echinobase.org); Cameron et al. (2009)].

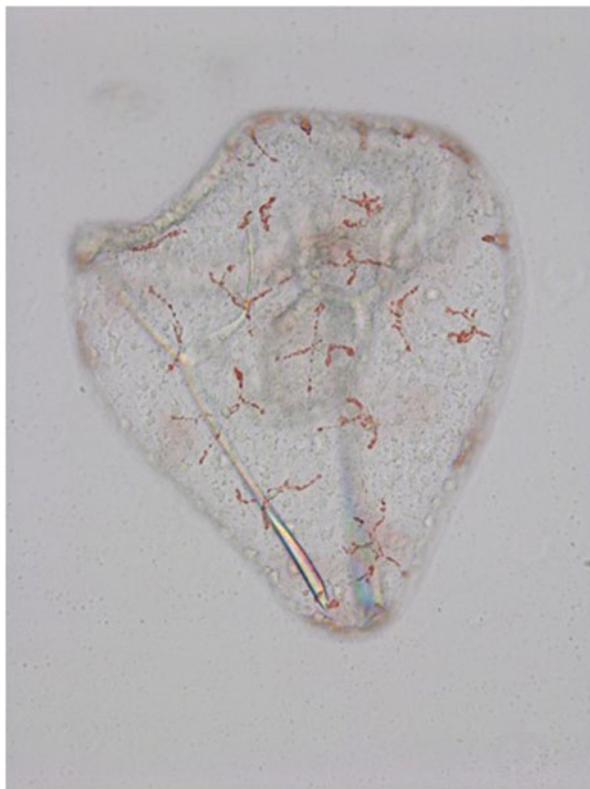
#### 22.2.4 Quinones

Quinones are a large family of reactive aromatic ring structures, of which naphthoquinones are a subgroup. These compounds are also a major family of pigments in echinoderms. Originally thought to be present naturally only in plants, the discovery of echinochrome in the coelomic fluid of sea urchins expanded that vocabulary. It is now known that naphthoquinones are prevalent in echinoderms with several major members having been identified and characterized from the larval pigment cells, test, spines, and tube feet in addition to the coelomocytes of sea urchins (Fox and Hopkins 1966; Figs. 22.1 and 22.2).

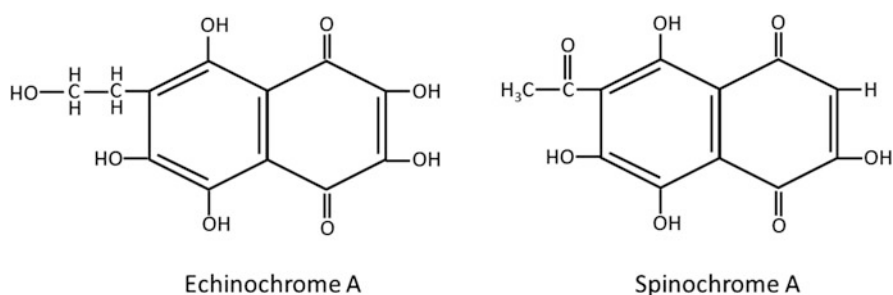
Within the family of quinones are the naphthoquinones, a group of heterocyclic polyketides that are highly modified into different pigment types. The sea urchin has



**Fig. 22.1** Pigmentation differences in echinoderms. (a) The sea urchin *Strongylocentrotus purpuratus* (purple) adjacent to the sea star *Patiria miniata* (bat star) and to (b) the sea urchin *Lytechinus variegatus*. Note the striped banding of pigment on the tube feet [arrow in (a)]. Photo in (a) complements of Adrian Reich



**Fig. 22.2** Sea urchin (*S. purpuratus*) pluteus larvae showing the reddish pigment cells embedded in the ectoderm. Pigment cells have a stellate shape, and they can extend and retract their pseudopods and move within the ectoderm



**Fig. 22.3** The chemical structure of two of the many polyketide pigments in echinoderms. From Fox and Hopkins (1966)

several closely related naphthoquinones, including echinochrome A and spinochromes A (Fig. 22.3). This review will now focus on the enzyme presumably responsible for the production of these polyketides, polyketide synthase (PKS).

## 22.3 What Is the Major Enzymatic Activity of Polyketide Synthases?

Polyketide synthases (PKSs) catalyze the synthesis of a wide variety of compounds ranging from antibiotics to plant pigments (Hopwood 1997, 2004; Staunton and Weissman 2001; Weissman 2015a, b). PKSs have been mainly studied in bacteria, fungi, and plants and produce compounds with antibiotic, mycotoxic, anti-inflammatory, and anticancer properties (Hopwood 2004). Plants polyketides have very diverse roles including flower pigmentation, UV and visible light responses, and symbiotic plant–pathogen interactions (Schroder et al. 1998; Winkel-Shirley 2002). Due to the vast array of biological activities of polyketide compounds and especially to their medicinal properties, research on the structural biology of PKSs has been growing. These studies led to the new field of genetic engineering and synthetic biology of PKSs to produce new compounds (e.g., Weissman 2015b, 2016).

The sea urchin Sp-PKS1 belongs to the type I class of PKSs (Castoe et al. 2007), and it is required for the larval echinochrome biosynthesis (Calestani et al. 2003). The major pigments of many echinoderms are echinochromes and spinochromes (Fig. 22.3), and these are naphthoquinone polyketides. The PKS encoded by XM\_788471.4 (Sp-PKS1) is a multifunctional enzyme that contains KS-AT-DH-MT-ER-KR-ACP domains most typical of a branched (methylated) saturated fatty acid synthase, although there is no TE-like domains in this protein. This PKS therefore most likely belongs to the class known as iterative PKSs, in which the product of one round of chain elongation and modification serves as the substrate for repeated rounds of the same set of elongation/modification reactions. There are no good algorithms to predict the length of the final chain or any deviations from the canonical methylation-ketoreduction-dehydration-enoyl reduction for any given cycle of elongation/modification.

### 22.3.1 How Is Echinochrome Synthesized?

Echinochrome seems to be produced from acetic acid molecules (Salaque et al. 1967), which are condensed and modified by Sp-PKS1. In addition to Sp-PKS1, a flavin monooxygenase (Sp-FMO1) appears to be involved in the oxygenation of the precursor of echinochrome since Sp-FMO1 knockdowns produced albino larvae (Calestani et al. 2003). A similar involvement in the oxygenation of phenols to quinone compounds has been observed in the biosynthesis of the antibiotic actinorhodin by *Streptomyces coelicolor* (Kendrew et al. 1997). Since polyketides are generally cytotoxic, most likely the echinochrome in the sea urchin larval pigment cells is either conjugated with some protein or other compound(s) to neutralize the toxicity and/or sequestered in vesicles, to protect the cell synthesizing the toxic compound.

### 22.3.2 *PKS Diversity in Sea Urchins*

Several PKSs have been identified in different sea urchin species and they are highly conserved. *Lytechinus variegatus* PKS (NCBI sequence ID DAA05847.1) has 92% amino acid sequence identity with Sp-PKS1; a partial sequence has been isolated from *Hemicentrotus pulcherrimus* (NCBI sequence ID BAQ21222.1), with a 99% amino acid sequence identity, from *Strongylocentrotus intermedius* (NCBI sequence ID AEL14404.1) with a 99% amino acid sequence identity and from *Paracentrotus lividus* (NCBI sequence ID SMC15637.1) with a 90% amino acid sequence identity. A second PKS has been identified in the sea urchin by genome and transcriptome sequencing, Sp-PKS2 (NCBI sequence ID NP\_001239013.1). Sp-PKS2 however has only 32% amino acid sequence identity with Sp-PKS1 (Castoe et al. 2007; Tu et al. 2014). Interestingly, *Sp-Pks2* is not expressed in pigment cells but in the skeletogenic cells of the sea urchin embryo and larva (Beeble and Calestani 2012). Sp-PKS2 is phylogenetically close to several bacterial PKSs and is composed of the conserved domains KS-AT-DH-ER-KR-PP-TE (Castoe et al. 2007). Recently it was discovered that Sp-PKS2 is required for skeletogenic development, specifically for the calcium carbonate mineralization process of the spicules (Hojo et al. 2015). A PKS homologous to Sp-PKS2 is also required for the initial step of biomineralization in the ear otolith of medaka (Hojo et al. 2015).

### 22.3.3 *Phylogeny of PKS*

The phylogenetic relationships of PKSs are quite complex. PKSs are present in bacteria, fungi, plants, and more animals than previously thought (Hojo et al. 2015). PKSs are related to fatty acid synthases (FAS), but they have a separate evolutionary origin (Castoe et al. 2007; Hojo et al. 2015). Sp-PKS1 and Sp-PKS2 are most closely related to PKSs from the lancelet *Branchiostoma floridae* and more distantly related to another animal PKS clade, which includes corals, hemichordates, reptiles, birds, fish, and marsupials (Hojo et al. 2015). A PKS, belonging to a separate animal clade, has been also identified in the protostome *Caenorhabditis elegans* but not in *Drosophila melanogaster* (Hojo et al. 2015; O'Brien et al. 2014). No PKS orthologs have been identified in humans or other mammals (Hojo et al. 2015). Therefore PKSs seem to have been lost from several animal lineages.

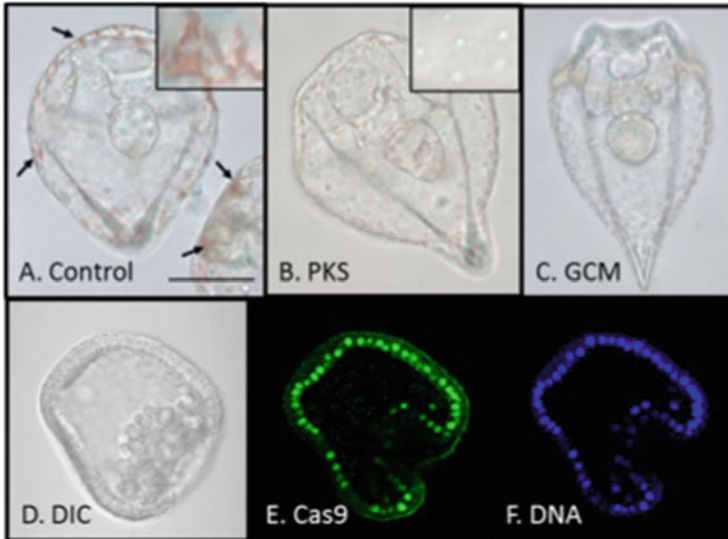
### 22.3.4 *The Gene Regulatory Network of PKS*

The *Sp-Pks1* gene is first activated in pigment cell precursors at the blastula stage (between 15 and 18 h post-fertilization in *S. purpuratus*), and its expression is maintained throughout larval development (Calestani et al. 2003). The specification process leading to the transcriptional activation of *Sp-Pks1* is regulated by a DELTA

(D)/NOTCH (N) signaling (Sherwood and McClay 1999; Sweet et al. 1999, 2002; McClay et al. 2000; Oliveri et al. 2002; Croce and McClay 2010; Materna and Davidson 2012). Previous studies demonstrated that the pigment cell gene regulatory network (GRN) including *Sp-Pks1* is quite shallow. In fact, *Sp-Pks1* is directly activated by the transcription factors glial cell missing (Sp-GCM), Sp-GATAE, and Kruppel-like (Sp-KRL; Calestani and Rogers 2010). Upstream, *Sp-Gcm* is directly activated by the N intracellular domain (Ransick et al. 2002; Ransick and Davidson 2006, 2012). *Sp-Krl* is D/N independent. *Sp-Pks1* expression is maintained by a positive feedback loop of *Sp-Gcm* throughout development (Ransick et al. 2002; Ransick and Davidson 2006), while *Sp-GataE* and *Sp-Krl* are most likely required only up to mesenchyme blastula–early gastrula stages (Calestani and Rogers 2010). In addition to that the activation of *Sp-Pks1* is locked-in by a triple positive feedback loop involving *Sp-Gcm*, *Sp-GataE* and *Sp-Six1/2*, and a feed-forward loop involving *Sp-Gcm* and *Sp-GataE* (Ransick and Davidson 2006, 2012; Calestani and Rogers 2010; Materna et al. 2013).

### 22.3.5 What Is the Function of the PKS-Derived Pigment in Sea Urchin?

Using two different approaches to reduce and/or eliminate PKS function, the resulting larvae are albino (Fig. 22.4; Calestani et al. 2003; Oulhen and Wessel 2016). They appear to still have pigment cells, but these cells are no longer pigmented. The albino animals still develop, swim, and feed—aside from color, the developing albino larvae are otherwise identical to the wild-type larvae (Fig. 22.4). Thus, PKS and the pigments resulting from its activity appear to not be involved in developmental signaling. The mechanisms of eliminating PKS activity have been demonstrated by both morpholino (phosphorodiamidate morpholino oligomer, MO) knockdown (Calestani et al. 2003), in which the MO is designed to block translation by sequence—specifically blocking ribosome scanning along the 5'UTR, and by CRISPR-Cas9 targeting the PKS upstream coding exons (Fig. 22.4; Oulhen and Wessel 2016). Both approaches gave the same result of albinism in the larvae. With such an easy visual indicator of pigment in larvae as a readout, the CRISPR/Cas9 system appears to be over 90% efficient for gene disruption. Thus, neither the pigment nor the PKS enzyme has any noticeable developmental role at least several days into the formation of the larvae. Echinochrome and related pigments may have roles in immune defense (Perry and Epel 1981; Service and Wardlaw 1984; Gibson and Burke 1987; Hibino et al. 2006; Kiselev et al. 2013; Ho et al. 2016). In fact, echinochrome showed antibiotic properties against six gram-positive and gram-negative marine bacteria (Service and Wardlaw 1984). In eggs echinochrome A is oxydated and released, especially after fertilization, producing H<sub>2</sub>O<sub>2</sub> potentially at sufficient levels for antimicrobial function (Perry and Epel 1981). Kiselev et al. (2013) tested the effects of sea urchin larvae exposure to several pathogenic marine bacteria and found that pigment cell number and *Sp-Pks1* expression were both increased as compared to untreated larvae. More recently Ho et al. (2016) showed



**Fig. 22.4** Sp-*PKS1*—knockdown of function by CRISPR/Cas9 (Oulhen and Wessel 2016) resulted in normal larvae except that they lacked pigment. Tests of the CRISPR/Cas-directed inactivation of genes involved in the synthesis of pigment: larvae contain over 100 pigment cells in their ectoderm [arrows in (a); inset is a magnification of the pigment (red) from control-injected embryos]. (b) CRISPR/Cas9-targeted gene inactivation of the enzyme responsible for the biosynthesis of pigment, polyketide synthase (Sp-*PKS1*) (Calestani et al. 2003; inset shows lack of pigment). (c) Knockdown of glial cell missing (Sp-GCM), the main transcription factor responsible for *Sp-Pks1* transcription (Ransick and Davidson 2012). Elimination of either target genes resulted in albinism in otherwise healthy, developing embryos. (d) Brightfield of E and F showing Cas9 immunolabeling enrichment in the nuclei of early embryos (e), highlighted by DAPI staining (f). Scale bar, 100  $\mu\text{m}$  [From Oulhen and Wessel (2016); with permission]

that pigment cells migrate to the gut and associate with other immune cells when the larvae are exposed to bacteria. Thus, an immune function for these pigments might be worthy for additional testing, especially using albinos as test subjects for pigment effect.

## 22.4 Concluding Statements

With the new technologies and genomic resources available, pigment function can now be effectively tested in echinoderms both *in vivo* and *in vitro*. With genetic knockouts feasible for *PKS*, one can test the developmental role, the ecological role, and use of these pigment-null animals in lineage analyses for rapid and easy visual identification of cellular fates and morphogenesis. While most efforts have been focused on the *PKS* gene activity and the polyketides produced, the presence, biosynthesis, and functions of other pigments, especially the porphyrins, for which defined genes are present in echinoderms, can now be pursued. The future looks colorful for these remarkable and brightly pigmented animals!



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**Part V**  
**Bioinformatics, Bioengineering**  
**and Information Processing**

# Chapter 23

## Reef-Building Corals as a Tool for Climate Change Research in the Genomics Era



Filipa Godoy-Vitorino and Carlos Toledo-Hernandez

**Abstract** Coral reef ecosystems are among the most biodiverse habitats in the marine realm. They not only contribute with a plethora of ecosystem services, but they also are beneficial to humankind via nurturing marine fisheries and sustaining recreational activities. We will discuss the biology of coral reefs and their ecophysiology including the complex bacterial microbiota associated with them.

**Keywords** Scleractinian corals · Coral microbiome · Holobiont · Climate change · Next-generation sequencing

### 23.1 Coral Biology: An Enigmatic Symbioses

Coral reef ecosystems are literally, massive deposits of calcium carbonate covering millions of square kilometers of shallow seafloor in tropical and subtropical regions. Yet, coral reefs only occupy between 0.1% and 0.5% of the world's ocean floor (Crossland et al. 1991; Moberg and Folke 1999). The main architects of these ecosystems are corals from the order Scleractinia, also known as reef-building corals. Scleractinian corals have existed for millions of years, as it is evidenced by their fossils, which dated them back to the mid Triassic, i.e., 240 million years ago (Budd et al. 2010; Shinzato et al. 2011). Most reef-building corals are modular animals, which by definition are animals composed of repeated building blocks derived by vegetative growth. In the case of corals, these repeated units are the polyps.

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Polyps are cylindrical-shaped extension of two single-cell thick epithelial layers: the epidermis derived from the ectoderm and the gastrodermis derived from the endoderm (Gattuso et al. 1999). At the base, polyps are supported by a calcium carbon skeletal cup, known as calix, formed by the calciblastic epithelium (Budd et al. 2010), whereas at the opposite end of the base is the oral opening, which connects the surrounding seawater with the gastrovascular cavity. Surrounding the oral opening are the tentacles, which vary in numbers and length depending on the coral species. Likewise, depending on the coral species and also on the size, a single coral may have from one or few polyps to several hundreds of polyps, which are interconnected via coelenteric canals but function as autonomous units (Tuomi and Vuorisalo 1989).

Coral polyps have multifunctional purposes. For instance, polyps are the structures used by scleractinian corals for heterotrophic feeding. In a healthy coral, heterotrophy could make up between 15 and 35% of the daily metabolic requirements (Grottoli et al. 2006; Houbreque and Ferrier-Pages 2009). By contrast, in bleached corals, i.e., corals that have lost their zooxanthellae or the photosynthetic apparatus of the zooxanthellae have been damaged, heterotrophic feeding makes up 100% (Grottoli et al. 2006; Houbreque and Ferrier-Pages 2009). Polyps are also the sexual reproductive structures of scleractinian corals. Throughout the life of certain coral species, polyps produce single sex gonads. Yet, in others, different polyps from a single colony may produce sperms or ova, i.e., be simultaneous hermaphrodites, or may produce gonads of a single sex during a reproductive season, shifting the gender of the gonads in the following reproductive season—i.e., be a sequential hermaphrodites (Okubo et al. 2013). Once produced, the gametes may be spawned from the polyps for external fertilization or brood, in which case fertilization and embryogenesis take place within the maternal polyps. The mature embryos, also known as planulae, are released from the polyps (Darling et al. 2012; Richmond and Hunter 1990).

Most reef-building corals are zooxanthelled, meaning that their gastrodermal tissue harbors photosynthetic dinoflagellates from the genus *Symbiodinium* (also known as zooxanthellae), in an obligatory mutualistic relationship. The density and biomass of zooxanthellae within scleractinian corals are highly variable, depending for the most part on water temperature cycles; they are the lowest during the periods of high water temperatures (i.e., summer) and pick during the winter when the water temperatures drop (Fitt et al. 2000). It is zooxanthellae that provide most of the coral's daily fixed carbon requirements for cellular respiration via the translocation of certain photosynthetic products such as sugars and amino acids to its coral host (Falkowski et al. 1984). Furthermore, zooxanthellae photosynthesis is thought to enhance coral calcification through the photosynthetic uptake of CO<sub>2</sub>, which increases the pH at the calciblastic epithelia facilitating the precipitation of CaCO<sub>3</sub> (Buchismaum Pearse and Muscatine 1971). However, even though photosynthesis and calcification occur simultaneously, photosynthesis is performed primarily within transparent gastrodermal cells of the oral opening of the polyp to facilitate the downwelling light, while calcification is performed at the calciblastic epithelium of the ectodermal cells at the aboral region of the polyp (Gattuso et al. 1999).

It is the intimate relationship between scleractinian corals and zooxanthellae that accounts for most of the primary production of reef ecosystems, although other photosynthetic organisms such as coralline rhodophytes and chlorophytes, as well as dinoflagellates, and invertebrates such as sponges, octocorals, and anemones among others also contribute to the high productivity (Knowlton 2001). It is the high primary productivity that supports the great biodiversity observed in coral reefs. In fact, among the realm of marine ecosystems, reef ecosystems are considered the most biologically diverse ecosystems per unit area, lodgings nearly a third of all described marine species (Knowlton et al. 2010; Shinzato et al. 2011). This makes the reefs a hotspot of biological diversity, only comparable to that of the rain forest. Yet, the great biodiversity of reefs is far from being associated with scleractinian corals alone, as only 656 scleractinian corals have been described thus far (Cairns 1999). Instead, the great diversity is due to the thousands of organisms associated with the myriad of niches provided by scleractinian corals (Godoy-Vitorino et al. 2017).

## 23.2 Microbial Evolution and the Coral Holobiont

From an evolutionary perspective, it is important to understand that the eukaryotic systems have coevolved with prokaryotic organisms. Planet Earth is ~4.5 Ga years old, as dated by our oldest rocks and minerals (Wilde et al. 2001), and early life in the Archaean Earth is thought to have evolved from a primordial soup. Microbes arose about 3.8 billion years ago (Mojzsis et al. 1996) from a single origin (Woese 1998). Sulfur-driven photosynthesis was found in Australian stromatolites (laminated, fossilized microbial communities within Precambrian carbonate sediments) at ~3.5 Ga, and *Proteobacteria* and protocyanobacteria are presumed to have used iron as reductant around 3.0 Ga. *Cyanobacteria* containing chlorophyll *a* and carrying out oxygenic photosynthesis appeared ~2.8 Ga (Olson 2006), and the great oxidation event of the atmosphere occurred some 2200–2400 Ma ago, according to the temporal distributions of red beds (Kappler et al. 2005). It is therefore reasonable to conclude that unicellular microbial systems are the genesis of all biological evolution. Evolution proceeded from its prokaryotic origin to eukaryotes (Williams et al. 2013) after the atmosphere's great oxidation event ~2.2 to 2.4 billion years ago (Schopf 2011). Archaea is a domain of life that evolved most likely after bacteria, about 2.7 billion years ago (Kappler et al. 2005). Recently, with the wealth of microbial diversity surveys due to increased sequencing depth, new archaeal lineages have emerged including the non-extremophilic archaeal taxa “DPANN” (e.g., Diapherotrites, Aenigmarchaeota, and Nanoarchaeota) and members of the TACK superphylum (Thaumarchaeota, Aigarchaeota, Crenarchaeota, and Korarchaeota), both close to the crenarchaeotes to which eukaryotes appear to be linked (Williams et al. 2013). Fungi arose after Archaea and also colonize many eukaryotic hosts. The Proterozoic eon, 2500 to 540 Ma ago, represents about 43% of Earth's history and includes the period of Earth's greatest changes, including the development of stromatolites in the Archaean, Proterozoic, and Early Paleozoic seas (Allwood et al. 2006).

These are calcareous sedimentary deposits composed by photosynthesizing cyanobacteria that are recognized as the oldest examples of reef-building organisms, and they have remained so until the early Cambrian (~520 mya) when more of the current reef complexity evolved (Laval et al. 2000; Wood 1998). Scleractinian corals appeared about 240 mya (Budd et al. 2010) and acquired photosymbiosis early in their history (~210 mya) (Wood 1998). Their evolution also included association with other microbial lineages. The host and its host-associated microbes—or microbiota (bacteria, archaea, protists, and fungi)—are termed the holobiont (Rosenberg and Zilber-Rosenberg 2013). The collective genomes of the host-associated microbes are known as the microbiome (Lederberg and McCray 2001), while the host genome plus the microbiome is termed the hologenome (Rosenberg and Zilber-Rosenberg 2016). Although the detailed evolutionary history of association between corals and their microbiomes is unclear, these microbes through their dynamic genetic diversity provide important functions such as nutrient cycling and antibacterial activity (Pereira et al. 2017; Kimes et al. 2010; Yokouchi et al. 2006). In fact, there is a three-way mutualistic benefit provided by bacteria, *Symbiodinium*, and the coral host (Nelson et al. 2013; Lesser et al. 2004). From now on, we will refer to corals as holobionts.

### 23.3 Climate Change and Coral Reefs

Coral reef ecosystems play a fundamental role in the sustainability of human society, due to the diverse array of goods, services, and ecological roles they provide. At the social–economic level, for instance, millions of dollars in annual revenues are obtained from recreational activities associated with coral reefs (Moberg and Folke 1999). Nearly 400 million people across the tropical coasts of the world depend, to some extent, on reef-associated organisms for their livelihood and protein intake (Ruiz-Diaz et al. 2016; Wilkinson 2004). In fact, coral reefs support nearly one quarter of the world’s marine fisheries (Shinzato et al. 2011). Likewise, reef-associated organisms are important sources of biologically active chemical compounds. For instance, anticancer, anti-inflammatory, antibiotic, and anticoagulant compounds have been isolated from several reef-associated organisms such as sponge, gorgonian corals, anemones, and algae among others (Higa et al. 2001; Rocha et al. 2011). Reefs also play an important role protecting the tropical shorelines from current and by dissipating wave energy during storm events (Sheppard et al. 2005). Reefs are also important players in several biogeochemical cycles. For instance, scleractinian corals and coralline algae are fixers of great amounts of calcium, which they precipitate in their skeleton (Ohde and Van Woesik 1999). The calcium carbonate skeleton of scleractinian corals are used as climate records to reconstruct climatic conditions from the past (Lough 2010). Likewise, corals and their microbial associates are important fixers of nitrogen, and in geological time, important fixers of carbon (Moberg and Folke 1999).

Despite their importance to human society and their persistence over geological scales, coral reefs are facing serious decline worldwide (Gardner et al. 2003). Overall, 11% of all coral reefs have been lost (Gardner et al. 2003). Furthermore, the average rate at which coral reefs are disappearing in the Indo-Pacific is 2% per year while at the Caribbean region is between 5.5% and 9.2% per year (Bruno and Selig 2007). This accelerated rate of coral reef loss is unprecedented according to the fossil record analyses. That is, in no other period, coral reefs have suffered such a dramatic decline. The reasons for these declines are variable, yet most of them are human derived. Destructive fishing methods, overexploitation of reef-related resources, increase in nutrient concentration, and water sedimentation due to deficient management plans in agricultural and urban upland areas are only some of the human activities related to the coral reef degradation (Hoegh-Guldberg and Bruno 2010). Diseases and bleaching are yet two of the most recent threats faced by coral reefs (Bridge et al. 2013). The earliest record of a coral disease was reported during the 1970s (Antonius 1973). Since then, over 35 coral diseases have been described. Some of these diseases such as the yellow-band disease (YBD) have a regional distribution, while others such as black band disease (BBD) have a worldwide distribution (Toledo-Hernández and Ruiz-Diaz 2014). In a similar way, the frequencies of coral bleaching events have been on the rise during the past few decades. Nearly 20 major bleaching events have been reported since 1979, with the 2016 bleaching event being the most devastating of all, at least in the Indo-Pacific waters (Hoegh-Guldberg et al. 2007).

The emergence of coral diseases and the increments of coral bleaching events both have been linked to stresses derived from the rising in seawater temperature. Most scleractinian corals live at the upper limits of their thermal tolerance, i.e., 28–30°C. Consequently, increases in water temperature over the annual average for prolonged periods could exert thermal stress on corals, potentially diminishing their immune system further inducing microbial dysbioses, diseases, bleaching, or both, with the ultimate consequence of massive coral mortalities, and/or a significant reduction of the coral colony growth and reproduction.

The occurrence of disease outbreaks and mass bleaching events is not regionally uniform, meaning that they could occur at a very localized scale, i.e., in a few individuals from the same reefs, or at a large geographical scale, and therefore is not easy to predict or explain. As mentioned earlier, coral bleaching and the potential disease outbreaks afterward are mostly linked to thermal anomalies. However, what makes these events hard to predict and explain is the fact that we are yet to know how much heat and for how long the thermal anomalies must prevail to cause physiological stress that drives bleaching or diseases (Baker et al. 2008). Nonetheless, several researcher groups have developed different indexes to hindcast and forecast bleaching episodes, most of which used above-average seawater temperature anomaly satellite records to alert about the possibility of bleaching (see Baker et al. 2008 for details).



### 23.3.1 *The Coral Holobiont as a Model for Climate Change*

The microbiota may be very slow at adapting to changes in the environment. That is, the stability of the bacterial structure, along with its carbon flux and other functional changes, is greatly impacted by sudden environmental changes, as demonstrated by a simulated climate change experiment of reciprocal soil transplant (Bond-Lamberty et al. 2016). Indeed, global climate change is having a great impact on oceanic waters with 0.4°C temperature increase and solar radiation due to depletion of the ozone layer (Levitus et al. 2009). A warmer climate may indicate loss in species and key functions and drive major disruptions in food webs and ecological breakdowns. Biological diseases as well as coral bleaching have been associated with increased water temperature (Bridge et al. 2013).

The way that corals may adapt to environmental change includes both genetic and nongenetic mechanisms (Webster and Reusch 2017). Genetic adaptation occurs at the population level, through genotype changes and natural selection (from DNA and genes to phenotype). The coral's adaptation includes somatic mutations in the coral colony (Schweinsberg et al. 2015) but also changes in the microbiome (the collective genomes of the microbiota) resulting in the holobiont phenotype's response to climate change. The coral adaptation to climate change is an evolutionary process that enhances the holobiont phenotypic plasticity and is mediated through the microbiome. In fact, the microbiota, which evolves at a greater speed than the host, can alter the holobiont phenotype in a relatively short period of time, i.e., from weeks to months (Elena and Lenski 2003). The microbiome–phenotype adaptation is vertically transmitted between corals, and this has been proposed as part of the hologenome theory of evolution (Rosenberg and Zilber-Rosenberg 2013).

The functional activities of the microbial symbionts vary within corals. The photosynthetic dinoflagellates *Symbiodinium* reside inside vacuoles of the polyp with which it acquires carbon (Muscatine et al. 2005). Bacterial symbionts have other nutritional pathways that benefit the host; in addition to carbon, nitrogen, sulfur, and phosphate, they produce vitamins, such as B<sub>12</sub> (necessary for the *Symbiodinium*) and secondary metabolites (Bourne et al. 2016; Agostini et al. 2009). Recent studies indicate that although the microbiota are species-specific, they share functions between different groups, a redundancy resulting from evolutionary convergence (Webster and Reusch 2017). For instance, in sponges, research has shown microbial functional equivalence in six phylogenetically divergent sponge species (Fan et al. 2012). The functional gene annotation of the sponge microbiomes based on comparisons with the clusters of orthologous group (COG) showed similar signatures for creatinine metabolism, denitrification, and stress resistance (Fan et al. 2012). This functional equivalence of the microbial communities revealed evolutionary convergence in the complex coevolution with the animal host (Fan et al. 2012). On the other hand, recent advances in metabolomics, a technique called meta-mass shift chemical (MeMSChem) profiling (which annotates molecules based on their mass shifts), show that highly taxonomically related genera of stony corals produced distinct patterns of metabolites (Hartmann et al. 2017).

Researchers found that despite high genomic similarity of the coral hosts, the same molecules acquire and lose chemical groups differently, depending on the bacterial taxa. In part, this suggests the capability of the coral holobiont to withstand stress and climate change, due to the high microbial biodiversity of coral reefs, which through different physiological pathways guarantees the production of distinct molecular repertoires in the holobionts (Hartmann et al. 2017). Another recent study on the effect of pH (acidification) and stress on the diversity of prokaryotic and eukaryotic microbial communities associated with the coral *Porites* spp., showed that endolithic communities inhabiting a naturally high pCO<sub>2</sub> reef are not significantly different from corals inhabiting other sites, suggesting that these endolithic microbiomes are well adapted to a wide pH range, being less disturbed by acidification than previously thought. The varied taxa of symbionts in *Porites* spp. included nitrogen-fixing bacteria (*Rhizobiales* and cyanobacteria), algicidal bacteria in the phylum *Bacteroidetes*, symbiotic bacteria in the family *Endozoicomoniacae*, and endolithic green algae (Marcelino et al. 2017).

### 23.4 High-Resolution Profiling of the Coral Biosphere

The coral microbiota is critical to the host's homeostasis, and consequently the host-microbiome unit is denominated as the coral "holobiont" (Thompson et al. 2014; Rosenberg and Zilber-Rosenberg 2013). With next-generation sequencing (NGS), an unprecedented scale of resolution surfaced that was previously unachievable by Sanger sequencing (Claesson et al. 2010). For example, via Sanger-based cloning and sequencing, researchers could characterize about 400 operational taxonomic units (OTUs) of which half were unclassified bacteria (associated with the Mediterranean coral *Oculina patagonica*) (Koren and Rosenberg 2006), while a very conserved NGS Illumina 16S analyses of the microbiota associated with *Acropora cervicornis* in the Caribbean found at least 800 OTUs mostly proteobacterial (Godoy-Vitorino et al. 2017). Another study on polluted corals off the coast of Florida found nearly 4000 OTUs (Staley et al. 2017), a number nearly impossible to get via cloning, unless the sample effort for the clone libraries was enormous. Indeed NGS technologies allowed the characterization of the microbiota in many coral species in health and disease and across geographies and also found coral-associated bacterial diversity to be between 100- and 1000-fold greater than that of the surrounding seawater (Rosenberg et al. 2007). Nonetheless, most of these many microbial reads were unclassified species, presenting a low or no match to 16S rRNA sequences in NCBI database (Rohwer et al. 2002; Frias-Lopez et al. 2008; Sun et al. 2016).

Coral biogeography across the globe indicates a dominance of *Proteobacteria* in the coral holobiont, which are ubiquitous bacteria of the marine environment and encompass diverse life forms, from symbiotic to free-living strains. A comparison of the microbiota from *Pocillopora verrucosa* and *Acropora hemprichii* in the Middle East, comparing locations with anthropogenic impacts with pristine areas, found significant dominance of *Proteobacteria*, and of these, *Vibrionaceae* were abundant in the impacted sites—a group that includes genus *Vibrio* with many pathogenic

strains (Ziegler et al. 2016). In the Atlantic, impacted reef genus *Mussismilia* from southern Brazil was dominated by *Vibrio*, while the core community was dominated by *Alpha*- and *Gammaproteobacteria* (Fernando et al. 2015). The proteobacterial dominant taxa were also found in the Indian Ocean associated with reef sponges (Java) (de Voogd et al. 2015). In five offshore reefs of the Northern Coral Sea of Australia, the core microbiome of coral species, separated by thousands of kilometers, contains very few universal bacterial phylotypes, and *Alphaproteobacteria* were only associated with the symbiotic microbiome, not being detected in the whole coral colony community (Ainsworth et al. 2015). In the Eastern Pacific, the microbiota associated with species of *Pocillopora* consisted of 38 phyla, with *Proteobacteria* being the most abundant phylum (within them *Alphaproteobacteria* and *Gammaproteobacteria*) (Hernandez-Zulueta et al. 2016). In the South China Sea, corals from the genera *Galaxea* and *Montipora* were dominated by *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Cyanobacteria* (Cai et al. 2018). In the Caribbean region (Florida, USA; San Salvador, The Bahamas), the microbiota of the octocoral *Erythropodium caribaeorum* was found to be dominated across sites by *Endozoicomonas* (*Gammaproteobacteria*) (McCauley et al. 2016). Off the coast of Puerto Rico, *Acropora cervicornis* corals inhabiting different water depths show prevalence of Rickettsiales-like bacterium, yet significant changes in less dominant taxa were observed at different water depths, including shallow-water corals inhabited by *Proteobacteria* (e.g., *Rhodobacteraceae* and *Serratia*), and the deeper-water corals by *Bradyrhizobium* and *Lactobacillus* (Godoy-Vitorino et al. 2017). Across all the geographical locations, proteobacterial taxa were dominant, in fact they account for the majority of the bacterial diversity in most of the marine environment (Rappé et al. 1997; Suzuki et al. 1997). Indeed, dominant proteobacterial groups have been found in the coral mucus, which also facilitated gene exchanges between members of the *Proteobacteria* through plasmids, indicating that the coral holobiont can indeed select and concentrate a specific diversity of plasmids to increase its fitness (Leite et al. 2018). Only few bioactive compounds have been described from *Proteobacteria* (Desriac et al. 2013); however, a potent anticancer (bryostatin) was biosynthetically produced by a proteobacterial endosymbiont of a bryozoan (Trindade-Silva et al. 2010). With the finesse and accuracy of current genomic techniques, we will certainly witness many new microbiome-based compounds associated with corals in the near future.

## 23.5 Brief Technical Guide to Microbiome Research

High-throughput sequencing in comparative metagenomics rose within the last 15 years due to the development of NGS methods such as pyrosequencing (Novais and Thorstenson 2011) and more recently Illumina sequencing (Caporaso et al. 2011) of 16S rDNA. This has increased drastically the number of survey studies that have advanced our understanding of the composition and function of microbial

communities in an array of different environments (Xu et al. 2007; Gilbert et al. 2010; Hug et al. 2016; Prescott et al. 2017; Ley et al. 2006; Ainsworth et al. 2015).

The precision of the taxonomic assignments in these 16S rDNA surveys is dependent on the size of the reads due to coverage and accuracy of the databases used. In fact, currently, the V6 region is considered to perform comparatively poorly to the preferred V4 (Liu et al. 2008). The V4 region is the most widely used for microbiome studies across the board and recognized as the most reliable subregion for representing the full-length 16S rRNA sequences with a superior phylogenetic resolution (Yang et al. 2016). Current analytical methods such as QIIME (Caporaso et al. 2010) or *mothur* (Schloss et al. 2009) include the capability of filtering chimeric sequences, as sequence artifacts can be generated when PCR amplification of the 16S region is incomplete and the partial sequences serve as primers that recombine with heterologous molecules giving rise to mixed reads. These reads and other unassigned taxa should be eliminated for the community analyses; otherwise, they may artificially inflate richness estimates and lead to wrong assignments. Databases for taxonomy assignment include the Greengenes 13\_8-based classifier that has been trained on the 515F/806R region of the 16S (McDonald et al. 2011; DeSantis et al. 2006), Silva-based classifier (ARB compatible) (Pruesse et al. 2007), and the RDP database (Cole et al. 2005). The new QIIME2 version of the QIIME pipeline ([qiime2.org](http://qiime2.org)) implements new algorithms through plug-ins that allow for taxonomic classification using DADA2 and Deblur, and now most taxonomic profiles can be assigned at the species level, with the former OTUs now named “sequence variants.” This is because the old 97% similarity has been shown to possibly represent different species. Most of these sequence variants in QIIME2 have species level assignments that can be given by any of the supported databases such as Greengenes, Silva, or others. In coral studies, the most widely used database for taxonomic assignment is the Greengenes database (Pootakham et al. 2017; Godoy-Vitorino et al. 2017). It is important to note that this 16S rDNA method only describes the bacteria and/or archaea in a given environment; for fungal microbiota, scientists rely on the ITS (Amend et al. 2012) and 18S rRNA genes (Putria et al. 2015).

Another method used to study the community composition is the PhyloChip, a DNA microarray that comprises 500,000 probes redundantly targeting 9000 operational taxonomic units whose sensitivity is nearly two orders of magnitude higher than that of a PCR clone library (Brodie et al. 2006), an approach that has also been used for coral profiling (Closek et al. 2014).

Fluorescence in situ hybridization (FISH) can also be used using group-specific 16S rRNA gene-targeted oligonucleotide probes for microbial community profiling including in the coral holobiont (Sharp et al. 2012; Brück et al. 2007). FISH-based counting is likely the most accurate way to determine the absolute abundances of microbial populations as it is not affected by the 16S gene copy number.

Shotgun metagenomics is another widely used microbiome method. It is the unrestricted sequencing of all the genomes of all organisms present in a sample, offering an unprecedented opportunity to acquire the gene catalogues of microbes offering a new vision on the functional possibilities of the microbes (Eisen 2007;

Handelsman et al. 1998). This technique offers resolution of the genomic composition of the communities and includes read preprocessing (QC), assembly, and gene prediction and annotation. Assemblies can be done with ARACHNE (Batzoglou et al. 2002), Phrap ([www.phrap.org](http://www.phrap.org)), or velvet (Namiki et al. 2012). Assemblies result in contigs and scaffolds, for gene prediction and gene annotation, and this can be done using Kraken (Wood and Salzberg 2014), Metagenomic Phylogenetic Analysis (MetaPhlAn) (Segata et al. 2012), HumanN (Abubucker et al. 2012), or Kaiju (Menzel et al. 2016). Protein annotations can be done with TIGRFAMs that has models for both domain and full-length proteins (Selengut et al. 2007), COGs (Tatusov et al. 1997) or PFAM (Finn et al. 2010) also allowing the annotation of full-length proteins. In metagenomic datasets, genes can come from different organisms. With enough sequence coverage, reads with same phylogenetic signal can be separated “binned.” Anvi’o (Eren et al. 2015) is an analysis and visualization platform for shotgun data, allowing for the taxonomic visualization of the metagenomes and the binning (separation) of the different genomes within each metagenome.

Single-cell sequencing is another powerful tool for characterizing rare cells/populations (Rinke et al. 2013). In fact, many new lineages have been characterized via single-cell approaches that have clarified the complexity of the tree of life (Hug et al. 2016). Methods include high-throughput single-cell flow sorting, whole-genome amplification, and SSU rRNA screening of single amplified genomes. Once assembled, the results should be validated to make sure they belong to one single taxa, using tetranucleotide frequency, BLAST (basic local alignment search tool), or even single copy marker gene analyses (Rinke et al. 2013).

Other approaches that complement the microbiome include characterization of the metabolic pathways, fluxes, and metabolite biomarkers through metabolomics. Mass spectrometry in combination with chromatography or nuclear magnetic resonance is used to resolve metabolic patterns and reveal the diversity of molecules available in a given habitat. The coral holobiont has been characterized through untargeted tandem mass spectrometry using the Global Natural Products Social Molecular Networking, revealing that different host phylogenetic groups had different metabolic profiles and found that fatty acid saturation flexibility in some species may help mitigate the damage of elevated temperatures that lead to bleaching in corals (Hartmann et al. 2017).

## 23.6 Concluding Remarks

Planet Earth’s climate has changed throughout history. Nonetheless, the warming trend we are now facing is of particular significance and is extremely likely to be the result of anthropogenic activities (Santer et al. 1996). The resulting effect in the ocean’s ecosystems is tremendous due to the rise in water temperature. The coral bleaching events, infectious disease outbreaks, and the carbon dioxide absorption into the ocean from the atmosphere, which decreases overall pH and may lead to

reduced calcification in the reef-building corals and alter the biochemical processes of the coral holobiont. Additionally, the rise in the sea level and the intensification of tropical storms and hurricanes, all of which dramatically alter the coral holobiont homeostasis, with great consequences for both the microbiome and for the immune stressed animal host, coral reefs service including the ecological and economical services will deteriorate causing unimaginable consequences to human society.

Many of these changes have already been reported, including mass loss in reef algae and runoff from land promoting pathogenic *Vibrio* and *Pseudovibrio* (Sheridan et al. 2014) and enteric pathogens (Sutherland et al. 2011). Massively, parallel sequencing of SSU rRNA gene amplicons has been proven to be a reliable technique that circumvents the time-consuming and biomarker-deficient culture-dependent methods, allowing the detection of diverse microbial communities and the early detection of pathogens and biomarker indicators of stress in the holobionts. The growing sequencing depth, accuracy, and the methodological sophistication behind microbiome studies are significant. The field of environmental metagenomics is expanding, due to the multiple reference sequence databases that allow biomarker detection at a greater speed and the increased accuracy of the available algorithms. This offers an unprecedented opportunity for data mining in microbiome symbioses to be used as a health assessment of the coral holobiont.

Possibly, we will be witnessing quick evolutionary changes in the coral holobiont, inherent to the plasticity and functional equivalence of some microbial populations, because of stress to the supraorganism. The changes could become long-term adaptive mechanisms and be fixed within the coral populations and lead to a new host–microbiome phenotype that will be able to withstand new drastic climate change-related stressors.

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## Chapter 24

# The Crown-of-Thorns Starfish: From Coral Reef Plague to Model System



Kenneth W. Baughman

**Abstract** *Acanthaster planci*, commonly known as the “crown-of-thorns starfish” (COTS) are famous for decimating coral reefs, yet the unique features of the COTS genome make the starfish a useful system for genomic and evolutionary developmental research. The COTS genome assembly is an order of magnitude more highly contiguous than other recently sequenced echinoderm genome assemblies. The high resolution of the COTS assembly is likely related to low heterozygosity resulting from historical population dynamics and possibly a recent population expansion. The high-resolution genome assembly is biologically meaningful, as confirmed by the discovery of several intact gene clusters. Therefore, the COTS genome is an ideal testing ground for new genomic technologies and bioinformatics tools.

## 24.1 Introduction

In this review, I summarize the current state of the COTS genome assembly, explore causes for the unique genomic structure of COTS, and discuss the kinds of inquiries that may be addressed using the COTS genome. The COTS genome and supporting materials can be downloaded from [http://marinegenomics.oist.jp/cots/viewer/info?project\\_id=46](http://marinegenomics.oist.jp/cots/viewer/info?project_id=46)

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## 24.2 The Remarkably High Resolution of the COTS Genome

The publication of the “crown-of-thorns starfish” (COTS) or *Acanthaster planci* “COTS-OKI V1” genome in 2017 (Hall et al. 2017) was notable for two reasons. First, the report leveraged genomics data to provide viable targets for mitigating the damage that COTS cause to coral reefs. Second, the COTS genome assembled remarkably well, likely due to the small size and low heterozygosity of the COTS genome. Recent updates to the COTS assembly have result in an n50 (which is the length of the scaffold that covers 50% of the total genomic length, when all scaffolds are sorted by length and added together) and total scaffold numbers that are ten times more contiguous than other publicly available echinoderm genomes (Table 24.1). Genome polishing methods including both the Dovetail Chicago plus HiRise service (Putnam et al. 2016) and the BioNano Irys device (Pendleton et al. 2015) reduced scaffold number in COTS from 730 to 103 scaffolds, approaching chromosomal level resolution utilizing only short-read, illumina-based sequencing. While the total gene number is roughly the same across all echinoderms (~25,000 gene models), the COTS genome is the shortest in length, at more than a half the size of the next smallest echinoderm genome; this shorter genome size likely contributed to the high quality of the COTS assembly.

Among echinoderms, COTS are a uniquely good system for genomics research. Large quantities of high-quality genomic DNA can be collected during the spawning season twice per year: once in the Northern Hemisphere and once in the Southern Hemisphere. On Okinawa, active local collection efforts (to remove COTS from targeted coral reefs) result in gram quantity of gonads per animal during spawning season, with on average hundreds of animals (~1–500 kg) available on a monthly basis. Such large quantities of high-quality genomic DNA make COTS amenable to biochemical or other reagent-limited methods.

## 24.3 The High Resolution of the COTS Genome Is Likely Due to Low Heterozygosity

Part of the initial design of the COTS sequencing project (Hall et al. 2017) was to compare two separate genome assemblies, one specimen from the Great Barrier Reef (“GBR”), Australia, and the other from Okinawa, Japan (“OKI”). Both genomes were sequenced, assembled, and annotated separately. While the hope was that comparison between the two genomes could provide insight into differences between aggregating and non-aggregating COTS, the impact of differences between COTS mitigation strategies in Japan and Australia, or possible cryptic speciation within the Pacific clade, the genomes were so highly similar that very few conclusions about differences were supported.

**Table 24.1** Genomic assembly statistics for published echinoderms, sorted by scaffold N50

Assembly—species name	Common name	GenBank/project #	Total length (Mb)	Scaffold number	Scaffold N50 (kb)	Gene models (#)	References
<i>Acanthaster planci</i> —hybrid	Crown-of-thorns starfish, Japan, Dovetail + BioNano	–	378	103	<b>5101</b>	–	(This report)
<i>Acanthaster planci</i> —dovetail	Crown-of-thorns starfish, Japan, Dovetail	–	384	730	<b>4440</b>	–	(This report)
<i>Acanthaster planci</i> —oki v1.0	Crown-of-thorns starfish, Japan	GCA_001949145.1	383	1765	<b>1521</b>	24,323	Hall et al. (2017)
<i>Acanthaster planci</i> —gbr v1.0	Crown-of-thorns starfish, Australia	GCA_001949165.1	373	3274	<b>916</b>	24,747	Hall et al. (2017)
<i>Apostichopus japonicus</i> v1.0	Japanese sea cucumber	GCA_001866495.1	805	3281	<b>487</b>	–	Zhang et al. (2017)
<i>Strongylocentrotus purpuratus</i> Spur_v4.2	Purple sea urchin	GCA_000002235.3	991	31,897	<b>420</b>	31,871	Cameron et al. (2015)
<i>Parastichopus parvimenis</i> Ppar v1.0	Warty sea cucumber	GCA_000934455.1	873	21,559	<b>89</b>	–	<a href="http://www.echinobase.org/">http://www.echinobase.org/</a>
<i>Ophiothrix spiculata</i> O. spiculata v1.0	Spiny brittle star	GCA_000969725.1	2764	75,696	<b>73</b>	–	<a href="http://www.echinobase.org/">http://www.echinobase.org/</a>
<i>Patiria miniata</i> Pmin v1.0	Bat star	GCA_000285935.1	811	60,183	<b>53</b>	29,697	Cameron et al. (2015)
<i>Lytechinus variegatus</i> Lvar2.2	Green sea urchin	GCA_000239495.2	1016	322,794	<b>46</b>	22,105	<a href="http://www.echinobase.org/">http://www.echinobase.org/</a>
<i>Eucladaris tribuloides</i> O. tribuloides v1.0	Slate pencil urchin	GCA_001188425.1	2187	637,071	<b>39</b>	–	<a href="http://www.echinobase.org/">http://www.echinobase.org/</a>
<i>Australostichopus mollis</i> v1.0	Brown sea cucumber	PRJEB10682	–	3,712,641	<b>0.626</b>	–	Long et al. (2016)
<i>Patriella regularis</i> v1.0	New Zealand cushion star	GCA_900067625.1	949	3,006,458	<b>0.557</b>	–	Long et al. (2016)
<i>Ophionereis fasciata</i> v1.0	Brittle star	GCA_900067615.1	1184	3,968,282	<b>0.484</b>	–	Long et al. (2016)

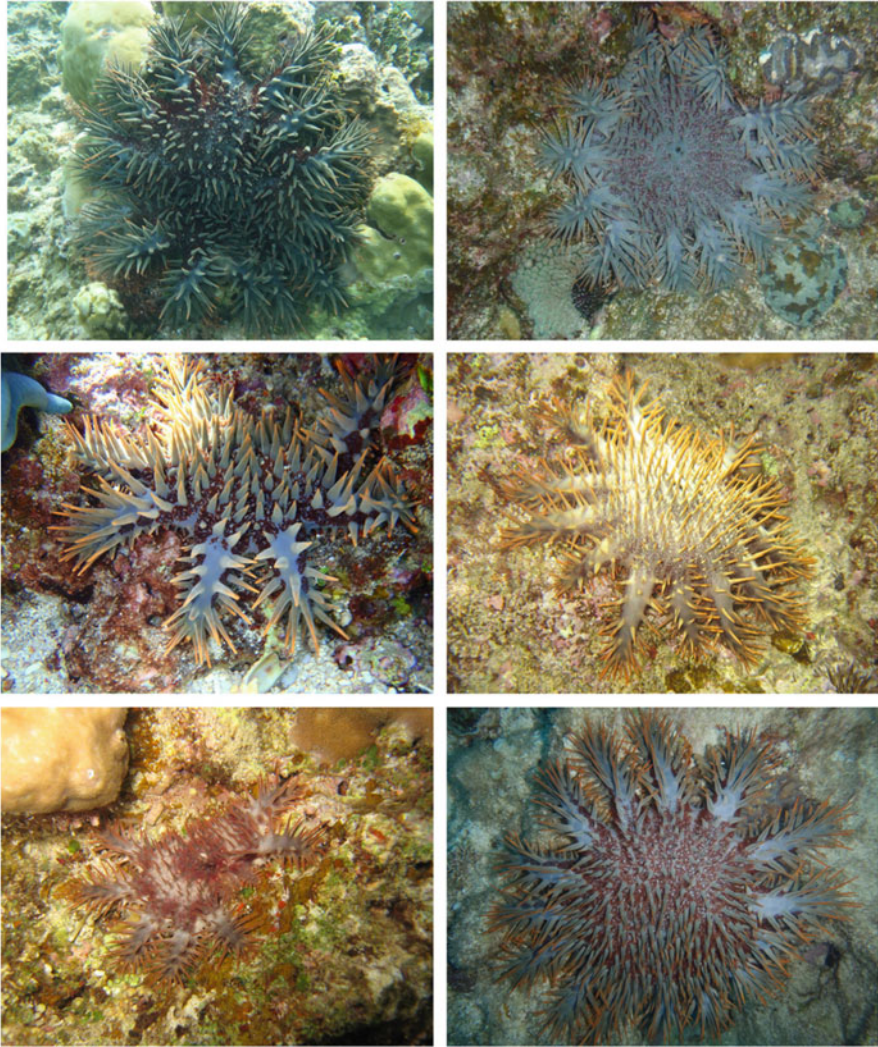


Genomic comparison between two geographically isolated specimens shows remarkably low divergence. Surprisingly, the overall genomic heterozygosity between the two specimens of 1.4% was lower than the gene model heterozygosity of 1.8% (Hall et al. 2017). This 0.4% difference between coding regions and the whole genome may reflect differences in RNAseq sampling but, regardless, highlights the overall similarity between the two genomes. Moreover, both genomes displayed unexpectedly low heterozygosity internally; SNP analysis resulted in a 0.9% internal heterozygosity rate. These results are consistent with a single Pacific COTS clade undergoing a recent population expansion and also provide a partial explanation for the high quality of the COTS genome assembly.

## 24.4 COTS and Echinoderms: General Biology

Echinoderms are among the most basal of the deuterostomes but until recently have lacked a high-resolution genome assembly. Conversely, the purple sea urchin *Strongylocentrotus purpuratus* was the second deuterostome genome sequenced, after *Homo sapiens* (Sea Urchin Genome Sequencing Consortium et al. 2006). This resource, along with the ample supply of embryos that urchins provide, resulted in an explosion of molecular embryology discoveries (McClay 2011), which in turn provided evidence for “gene regulatory networks” or GRNs (Peter and Davidson 2015). GRNs describe the cascading transcription factor interactions that are the control mechanism for spatial-temporal dynamics leading to the formation of boundaries and regions during development. More recent *S. purpuratus* assemblies continue to improve the resolution of the sea urchin genome (Cameron et al. 2015), but perhaps not to the resolution required to interrogate longer distance (~1–10 megabase) synteny and certainly not to the chromosomal level, which several other first-generation genome projects have attained.

The name *Acanthaster planci* was given by Linnaeus in 1758 (Haszpruner and Spies 2014). *Acanth-* can be translated as “thorn” and *aster* as “star,” while “*planci*” is derived from the same root word as “plankton,” presumably a reference to the slow motility of the starfish. COTS are in the phylum Echinodermata, the class Asterozoa, the order Valvulitida, and the family Acanthasteridae, shared with a sister species, *A. brevispinus*, to which COTS can hybridize (Lucas and Jones 1976). The common name for *A. planci* “crown-of-thorns” alludes to their venomous spines, which resemble the crown placed on Jesus’ head during his crucifixion (Fig. 24.1). Mature COTS can reach up to 1.2 m in diameter, weigh over 70 kg in mass, and have up to 23 arms (Moran 1988). Adult starfish are generally 25–40 cm in diameter and have 10–15 arms covered with 2–4 cm spines that range in color from orange or red to yellow (Fig. 24.1). The main body color is muted, generally brown, gray-green, or in some cases bluish or purplish. The spines are toxic to humans, and spine puncture results in rapid tissue inflammation, pain, up to a week of nausea and vomiting, and, in one case, death (Ihama et al. 2014).



**Fig. 24.1** Crown-of-thorns starfish (COTS). Taken at 2–30 m depth by the author, in Okinawa, Japan

Echinoderms share developmental traits with hemichordates, together forming the monophyletic clade “Ambulacraria.” Ambulacraria, along with Chordata, composes *Deuterostomia* (Satoh 2016). Sea urchins, in particular, have long served as a model system for studying deuterostome development and the evolution of the chordate body plan (Davidson 1997; Davidson and Erwin 2006; De Robertis 2008; Peter and Davidson 2010). Echinoderms share the first steps of embryological development with chordates. Echinoderm larvae are bilateral, but after metamorphosis adult echinoderms develop radial, generally pentameric, body plans. Thus,

any commonality between echinoderms, hemichordates, and chordates, either with regard to genomic organization and synteny or to developmental patterning, or indeed both, may have existed in the common ancestor of deuterostomes and bilateria itself (Holland et al. 2015; Lowe et al. 2015)

## 24.5 How COTS Damage Coral Reefs: COTS Aggregations

The historical population dynamics of COTS, along with their recent population expansion related to their role as a coral pest, may explain why the COTS genome is structurally divergent from other echinoderms. COTS are a primary cause of coral reef devastation across the Indo-Pacific region, due to localized population density fluctuations or “COTS aggregations” in which large groups of starfish move together during daylight, decimating reefs (Chesher 1969). COTS normally prey on corals but at much lower population densities and generally nocturnally (Moran 1988). COTS aggregations were initially reported in the late 1950s on Okinawa, Japan (Yamaguchi 1986). By the 1960s, several high-profile journal articles from across the Indo-Pacific reported the damage COTS aggregations were causing on coral reefs (Table 24.2). Interestingly, COTS became a centerpiece of the early environmental movement as an example of human intervention unbalancing nature and subsequently became a highly politicized and contentious scientific topic (Sapp 1999). Whereas the research of the 1960s and 1970s largely focused on characterizing the extent and possible causes for the COTS aggregations, by the 1980s, most COTS aggregations had subsided, and critics raised the idea that cyclicity of COTS population density was a natural phenomenon (Moore 1978).

Today, the consensus is that evidence exists for historical cyclicity of COTS populations, but the frequency of observed COTS aggregations over the past 50 years is much higher than reef recovery rates can sustain (De’ath et al. 2012). In short, geochemical analysis of reef front sediment (Walbran et al. 1989) and observed coral recovery rates both suggest COTS aggregations occur on the order of once per century, dramatically less frequently than the three, possibly four COTS population density increases observed on the Great Barrier Reef since the 1960s (Hall et al. 2017). Over the past 50 years, COTS have been the focus of more reef management efforts than any other marine species, as they are the most notorious controllable cause of coral reef devastation (Hall et al. 2017). More than half a century of COTS field sampling and population genetics provide a unique opportunity to explore population genomics. In other words, among deuterostomes, the existence of extensive field samples along with a high-resolution reference genome is unique and potentially useful.

**Table 24.2** High-profile COTS publications, 1969–1989

Article	Journal/date	Discipline
Chesher, R. H. Destruction of Pacific corals by the sea star <i>Acanthaster planci</i>	Science 165, 280–283 (1969)	Ecology
Barnes, D. J. Field and Laboratory Observations of the Crown-of-Thorns Starfish, <i>Acanthaster planci</i> : Locomotory Response of <i>Acanthaster planci</i> to Various Species of Coral	Nature 228, 342–344 (1970)	Behavior
Brauer, R. W., Jordan, M. R. & Barnes, D. J. Triggering of the stomach eversion reflex of <i>Acanthaster planci</i> by coral extracts	Nature 228, 344–346 (1970)	Behavior
Branham, J. M., Reed, S. A., Bailey, J. H. & Caperon, J. Coral-Eating Sea Stars <i>Acanthaster planci</i> in Hawaii	Science 172, 1155–1157 (1971)	Ecology
Henderson, J. A. & Lucas, J. S. Larval development and metamorphosis of <i>Acanthaster planci</i> (Asteroidea)	Nature 232, 655–657 (1971)	Rearing
Pearson, R. G. Changes in distribution of <i>Acanthaster planci</i> populations on the Great Barrier Reef	Nature 237, 175–176 (1972)	Ecology
Ormond, R. F. G. et al. Formation and Breakdown of Aggregations of the Crown-of-Thorns Starfish, <i>Acanthaster planci</i> (L.)	Nature 246, 167–169 (1973)	Behavior
Beach, D. H., Hanscomb, N. J. & Ormond, R. F. Spawning pheromone in crown-of-thorns starfish	Nature 254, 135–136 (1975)	Behavior
Moore, R. J. & Huxley, C. J. Aversive behavior of crown-of-thorns starfish to coral evoked by food-related chemicals	Nature 263, 407–409 (1976)	Behavior
Walbran, P. D., Henderson, R. A., Jull, A. J. & Head, M. J. Evidence from Sediments of Long-Term <i>Acanthaster planci</i> Predation on Corals of the Great Barrier Reef	Science 245, 847–850 (1989)	Geology/ ecology

## 24.6 The COTS Genome Assembly Is Biologically Significant: *Hox*, *ParaHox*, and Pharyngeal Gill Slit Clusters

Although the COTS genome assembled well from a statistical perspective, the discovery of three gene clusters that all recapitulated chordate gene order confirms the biological significance of the COTS genome assembly. Firstly, although several authors hypothesized that the unique *Hox* gene order in sea urchins was related to the pentaradial body plans of echinoderms (Mooi and David 2008), the COTS *Hox* and *ParaHox* clusters both resemble hemichordate and chordate clusters (Baughman et al. 2014). The publication of a high-quality holothurian genome (Zhang et al. 2017) confirms that the COTS *Hox* cluster shares with sea urchins the transposition of even-skipped (*Evx*), as well as posterior *Hox* reorganization, supporting an

evolutionary scenario for how shuffling of the Hox cluster in urchins may have arisen. Secondly, although extant echinoderms do not have pharyngeal gill slits, a cluster of transcription factors associated with gill slit formation was found in the COTS genome. This result supports an ancient origin for pharyngeal gill slits as a deuterostome-defining morphological feature (Simakov et al. 2015). These surprising discoveries confirm that the high resolution of the COTS genome is of biological significance.

## 24.7 COTS as Model System for the Study of Genomic Structure: 1-MA SBTN Example

The high quality and small size of the COTS genome assembly, relative to other echinoderms and marine invertebrate deuterostomes, suggest that COTS may be an ideal specimen for exploring genomic structure with novel technology and software. As an example, I mapped COTS candidate genes for the 1-methyladenine (1-MA)-mediated oocyte maturation pathway, using systems biology notation (Le Novère et al. 2009). 1-methyladenine (1-MA) is a hormone released by radial nerves, which induces female starfish to eject oocytes, which in turn causes the oocytes to initiate meiosis in preparation for fertilization via meiotic resumption (Kanatani 1964). Oocytes undergo a variety of cell signaling events upon 1-MA stimulation, many of which have been described in detail in the context of cell biology and cycle cell control (Kishimoto 2015).

“Figure 24.2: 1-MA oocyte resumption in SBTN notation” is a Systems Biology Graphical Notation (SBTN) approach to summarize the events of 1-MA signaling and map them to COTS gene models and RNA transcripts. The 1-MA oocyte meiotic resumption pathway was mapped in CellDesigner 4.3 (<http://www.celldesigner.org>) and converted to Systems Biology Graphical Notation (SBTN) via the conversion option in the CellDesigner 4.3 software (Funahashi et al. 2017). In cases where intermediate steps have been omitted, all displayed stoichiometry is correct. Table 24.3 includes Template IDs for all components in “Figure 24.2: 1-MA oocyte resumption in SBTN notation.” Table 24.4 includes Template PMIDs for all reactions in Fig. 24.2. Table 24.4 includes all reference papers used in “Figure 24.2: 1-MA oocyte resumption in SBTN notation.”

The reactions in Fig. 24.1 can be summarized as:

- Biosynthesis of 1-MA (Table 24.2: re7)
- Canonical G-protein-coupled receptor activation via PIP2 and PIP3 (re12, re13, re21, re22)
- Downstream signaling via PDK and Akt/PKB activation (re23, re27)
- Finally, cyclin activation (re28, re29, re30)

Three cellular components are defined: radial nerve cell, plasma membrane of the oocyte, and oocyte interior (including cytoplasm and nuclei). A COTS candidate

**Table 24.3** SBG N template IDs and associated COTS RNA transcripts

EPN name	EPN type	EPN ID	Synonyms	Organism	Paper reference	COTS genome scaffold #	COTS transcript ID
1-MeAde	Metabolite	CHEBI:18083		Methyladenine	Starfish	1. Shirai, H., Kanatani, H. & Taguchi, S. - 1-methyladenine biosynthesis in starfish ovary: action of gonad-stimulating hormone in methylation. Science 175, 1366–1368 (1972)	
Pi3K	Protein	GenBank	Z29090.1	Phosphatidylinositol 3-kinase	Human	Molecular Cloning, cDNA Sequence, and Chromosomal Localization of the Human Phosphatidylinositol 3-Kinase p110 $\alpha$ (PIK3CA) Gene Original Research Article Genomics, Volume 24, Issue 3, December 1994, Pages 472–477 Stefano Volinia, Ian Hiles, Elizabeth Ormondroyd, Dean Nizetic, Rachele Antonacci, Mariano Rocchi, Michael O. Waterfield	scaffold13 comp179390_c1_seq1

(continued)

Table 24.3 (continued)

EPN name	EPN type	EPN ID	Synonyms	Organism	Paper reference	COTS genome scaffold #	COTS transcript ID	COTS
Akt/ PKB	Protein	UniProt	Q95YJ0	Kinase Akt/PKB	Starfish	1. Okumura, E. et al. Akt inhibits Myt1 in the signaling pathway that leads to meiotic G2/M-phase transition. <i>Nat. Cell Biol.</i> 4, 111–116 (2002)	scaffold24	comp180351_c1_seq1
Myt1	Protein	UniProt	Q96YJ1	Inactivator of cyclin B-Cdc2	Starfish	1. Okumura, E. et al. Akt inhibits Myt1 in the signaling pathway that leads to meiotic G2/M-phase transition. <i>Nat. Cell Biol.</i> 4, 111–116 (2002)	scaffold469	comp174497_c0_seq2
Cdc2	Protein	UniProt	Q17066		Starfish	1. Okumura, E., Sekiai, T., Hisanaga, S.-I., Tachibana, K. & Kishimoto, T. Initial triggering of M-phase in starfish oocytes: a possible novel component of maturation-promoting factor besides cdc2 kinase. <i>J. Cell Biol.</i> 132, 125–135 (1996)	scaffold453	comp169732_c1_seq1

Cyclin B	Protein	UniProt	P90681	Starfish	Starfish	scaffold361	comp170404_c0_seq2
					I. Okumura, E., Sekiai, T., Hisanaga, S.-I., Tachibana, K. & Kishimoto, T. Initial triggering of M-phase in starfish oocytes: a possible novel component of maturation-promoting factor besides cdc2 kinase. <i>J. Cell Biol.</i> 132, 125–135 (1996)		
GSS	Protein	UniProt	C9K4X8	Starfish	Starfish	scaffold295	comp157746_c0_seq1
					I. Kanatani, H. Spawning of starfish: action of gamete-shedding substance obtained from radial nerves. <i>Science</i> 146, 1177–1179 (1964)		
ATP	Metabolite	(CHEBI:15422)		Starfish	Starfish		
					I. Mita, M., Yoshikuni, M. & Nagahama, Y. - 1-Methyladenine production from ATP by starfish ovarian follicle cells. <i>Biochim. Biophys. Acta</i> 1428, 13–20 (1999)		
PIP2	Metabolite	CHEBI:18348		Starfish	Starfish		
					I. Sadler, K. C. & Ruderman, J. V. Components of the		

(continued)



Table 24.3 (continued)

EPN name	EPN type	EPN ID	Synonyms	Organism	Paper reference	COTS genome scaffold #	COTS transcript ID
PIP3	Metabolite	CHEBI:16618		Phosphatidylinositol (3,4,5)-trisphosphate	Starfish	signaling pathway linking the 1-methyladenine receptor to MPF activation and maturation in starfish oocytes. <i>Developmental Biology</i> 197, 25–38 (1998)	
PDK1	Protein	UniProt	Q76BX2	Phosphoinositide-dependent kinase-1	Starfish	I. Hiraoka, D., Hori-Oshima, S. & Fukuhara, T. PDK1 is required for the hormonal signaling pathway leading to meiotic resumption in starfish oocytes. <i>Developmental ...</i> (2004)	scaffold292 comp174076_c1_seq1

PDK2	Protein	None (hypothetical protein)		3-phosphoinositide-dependent kinase-2	Starfish	Dong, L. Q. PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. <i>AJP: Endocrinology and Metabolism</i> <b>289</b> , E187–E196 (2005)		
sFTOR	Protein	UniProt	EZRWP8	Target of rapamycin	Starfish	I. Hiraoka, D., Okumura, E. & Kishimoto, T. Turn motif phosphorylation negatively regulates activation loop phosphorylation in Akt. <i>Oncogene</i> <b>30</b> , 4487–4497 (2011)	scaffold51	comp171410_c0_seq9
Cdc25	Protein	GenBank	AB076395.1		Starfish	I. Draetta, G. et al. Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. <i>Cell</i> <b>56</b> , 829–838 (1989)	scaffold1	comp171899_c0_seq2
I-MA GPCR	Protein	(Unknown)			Starfish	I. Sadler, K. C. & Ruderman, J. V. Components of the signaling pathway linking the I-methyladenine		

(continued)

Table 24.3 (continued)

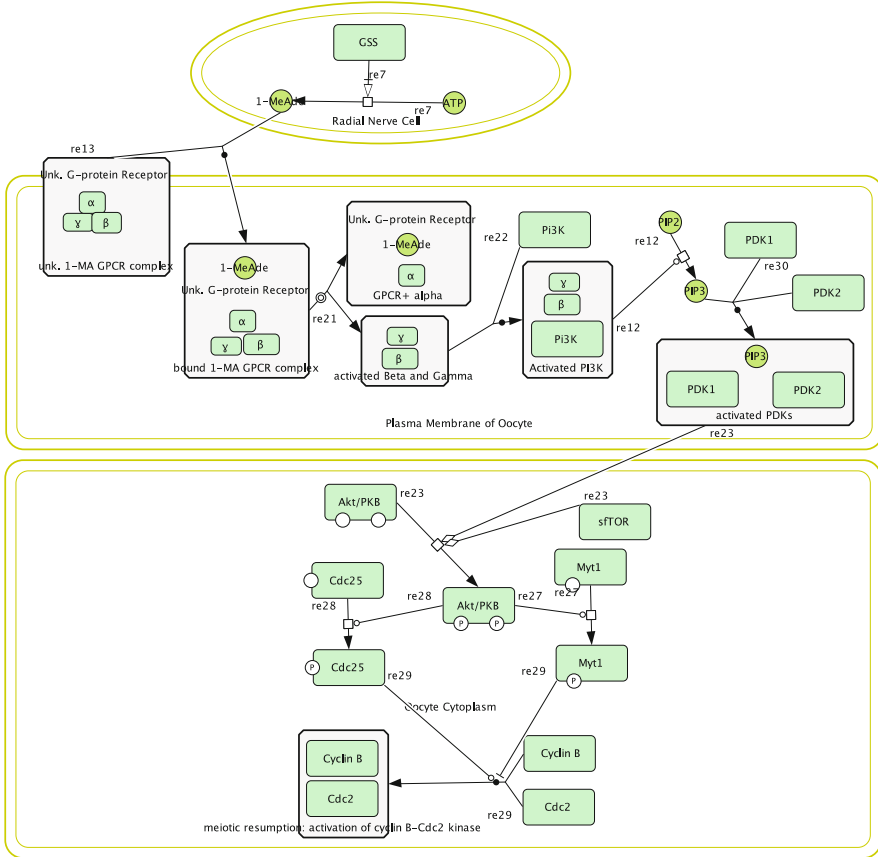
EPN name	EPN type	EPN ID	Synonyms	Organism	Paper reference	COTS genome scaffold #	COTS transcript ID	
G $\alpha$	Protein	UniProt	P30676	Guanine nucleotide-binding protein G (i) subunit alpha	Starfish	receptor to MPF activation and maturation in starfish oocytes. <i>Developmental Biology</i> 197, 25–38 (1998) I. Jaffe, L. A. Oocyte maturation in starfish is mediated by the beta gamma-subunit complex of a G-protein. <i>The Journal of Cell Biology</i> 121, 775–783 (1993)	scaffold321	comp163322_c0_seq1
G $\beta$	Protein	GenBank	AB894321.1	Guanine nucleotide-binding protein G (i) subunit beta	Starfish	Mita, M., Haraguchi, S., Watanabe, M., Takeshige, Y., Yamamoto, K. and Tsutsui, K. TITLE Involvement of Gs-alpha proteins in the action of relaxin-like gonadostimulating substance on starfish ovarian follicle cells/JOURNAL Unpublished	scaffold157	comp157533_c0_seq1

G $\gamma$	Protein	UniProt	Q568H1	Guanine nucleotide-binding protein G (i) subunit gamma	Zebrafish	I. Jaffe, L. A. Oocyte maturation in starfish is mediated by the beta gamma-subunit complex of a G-protein. The Journal of Cell Biology 121, 775–783 (1993)	scaffold56	comp174571_c1_seq1
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Table 24.4 1-MA reactants

Model ID#	Process type	Process	Modifier	EC	Modification type	Organism	Cell type	Subcellular location	PMID/lit ref.
id=re7	Metabolic reaction	ATP → 1-MeAde	GSS		Catalysis	Starfish	Radial nerve cell		I. Mita, M., Yoshikuni, M. & Nagahama, Y. 1-Methyladenine production from ATP by starfish ovarian follicle cells. Biochim. Biophys. Acta 1428, 13–20 (1999)
id=re12	Metabolic reaction	PIP2 → PIP3	PI3K, Gβ, Gγ		Catalysis	Starfish	Oocyte	Plasma membrane	I. Sadler, K. C. & Ruderman, J. V. Components of the signaling pathway linking the 1-methyladenine receptor to MPF activation and maturation in starfish oocytes. Developmental Biology 197, 25–38 (1998)
id=re13	Receptor/ligand binding	1-MeAde +GPCR			Binding	Starfish	Oocyte	Plasma membrane	I. Shirai, H., Kanatani, H. & Taguchi, S. 1-methyladenine biosynthesis in starfish ovary: action of gonad-stimulating hormone in methylation. Science 175, 1366–1368 (1972)
id=re21	Complex dissociation	GPCR complex → Gβ, Gγ			GPCR subunit activation	Starfish	Oocyte	Plasma membrane	I. Shirai, H., Kanatani, H. & Taguchi, S. 1-methyladenine biosynthesis in starfish ovary: action of gonad-stimulating hormone in methylation. Science 175, 1366–1368 (1972)
id=re22	Complex formation	Gβ, Gγ + PI3K			Activation	Starfish	Oocyte	Plasma membrane	I. Sadler, K. C. & Ruderman, J. V. Components of the signaling pathway linking the 1-methyladenine receptor to MPF activation and maturation in starfish oocytes. Developmental Biology 197, 25–38 (1998)

id=re23	Signaling	Akt → Akt (PP)	PDK1, PDK2, TOR		Phosphorylation	Starfish	Oocyte	Cytoplasm	1. Okumura, E. et al. Akt inhibits Myt1 in the signaling pathway that leads to meiotic G2/M-phase transition. <i>Nat. Cell Biol.</i> 4, 111–116 (2002)
id=re27	Signaling	Myt → Myt (P)	Akt (PP)		Phosphorylation	Starfish	Oocyte	Cytoplasm	1. Okumura, E. et al. Akt inhibits Myt1 in the signaling pathway that leads to meiotic G2/M-phase transition. <i>Nat. Cell Biol.</i> 4, 111–116 (2002)
id=re28	Signaling	Cdc25 → Cdc25 (P)	Akt (PP)		Phosphorylation	Starfish	Oocyte	Cytoplasm	1. Okumura, E., Sekiai, T., Hisanaga, S.-I., Tachibana, K. & Kishimoto, T. Initial triggering of M-phase in starfish oocytes: a possible novel component of maturation-promoting factor besides cdc2 kinase. <i>J. Cell Biol.</i> 132, 125–135 (1996)
id=re29	Complex formation	Cdc2 +CyclinB	Myt(-), Cdc25 (+)		Activation	Starfish	Oocyte	Plasma membrane	1. Draetta, G. et al. Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. <i>Cell</i> 56, 829–838 (1989)
id=re30	Complex formation	PIP2 +PDK1 +PDK2			Activation	Starfish	Oocyte	Plasma membrane	1. Hiraoka, D., Hori-Oshima, S. & Fukuhara, T. PDK1 is required for the hormonal signaling pathway leading to meiotic resumption in starfish oocytes. <i>Developmental ...</i> (2004)



**Fig. 24.2** 1-MA oocyte resumption in SBGN notation

gene model or transcript has been identified for each protein component of the pathway.

There are three observations that arise from viewing 1-MA oocyte resumption in SBGN notation. Firstly, the central role of Akt/PKB (Okumura et al. 2002) becomes much clearer. In contrast to other styles of signaling diagrams where this central role can only be alluded to, SBGN notation requires both validation via published results and accurate accounting of reactants and reactions, resulting in an easily viewable diagram, which is also biochemically robust (Le Novère et al. 2009). Secondly, an extensive review of the literature with regard to sFTOR and PDK2, coincidentally the most important contribution of a review by Kishimoto (Kishimoto 2011), suggests that while this association has been alluded to by several authors, the basic biochemistry of the reaction remains unknown. Thirdly, the 1-MA receptor remains unknown, though strong evidence indicates that it must be a G-protein-coupled receptor mediated (Jaffe 1993). These three observations could be made without

the use of SBGN notation, but the quantitative nature of SBGN allows for a higher level of confidence, while the simplified graphical nature of SBGN diagrams makes it easier to quickly assess complex signaling events. By connecting 1-MA signaling on the oocyte plasma membrane to the cyclin-dependent cell cycle resumption mechanisms in the nucleus in a quantitative manner, these results show one way that COTS genomic data can be connected to the extensive cell biology and cell cycle signaling literature.

## 24.8 COTS, GRNS, and KERNELS

Reflecting on the recent molecular embryology discoveries made in sea urchins, it is clear that we are beginning to understand the mechanisms that translate the necessary and sufficient information required to organize and grow complex body plans, from the genome (Peter and Davidson 2015). Additionally, comparison between how diverged species use the same genetic toolkits to organize similar body plans and novel structures has shed light on the molecular mechanics of evolution (Carroll et al. 2005). Thus, as the costs and technical challenges associated with sequencing and analyzing the reference genomes fall, comparison between the genomes of species that display divergent body plans, such as echinoderms, is one way to understand how control of body plan organization has evolved over geological time (Davidson and Erwin 2006). In contemplating the sea urchin GRN literature in the context of the collinearity mechanism of the Hox cluster, I am struck by the awkwardness of the lack of genomic coordinates in GRNs, which begs the question of the failure to find or identify additional Hox-like clusters. In other words, to date, the Hox cluster remains one of the only developmentally relevant gene clusters in which genomic structure correlates with gene function (collinearity). It appears that within the milieu of enhancers, transcription factor cascades, and GRN-defined tissue specification, there should be a roll for genomic order, synteny, collinearity, and perhaps even an “enhancer code.” That development is so precise, so timely, so reproducible, given the variability between the unique genomes that drive it, a missing layer of structure, of linguistics must surely exist.

Returning to the discovery of the Hox cluster provides a hint (Lewis 1978); perhaps genetic distance should be considered, rather than locations on static genomic scaffolds. Although a domain-based bioinformatics query might pull out the repetitive pattern of homeodomains found in the Hox cluster, the systematic mapping of the loci (in centimorgans) responsible for genetic phenotypes in *Drosophila* provided a functional consequence for the unique genomic organization of the Hox genes. While the longer scaffolds of the COTS genome suggest that pattern recognition efforts for genomic structures may be possible, comparison between individual COTS genomes mapped with GRNs could highlight those invariable regions most likely to be driving early developmental processes; the more COTS genomes that are compared, the higher resolution these regions should appear, and the more useful the genetic map that emerges. It is currently unclear if low-coverage



sequencing can recapitulate a true genetic map, though SNP analysis and large scaffolding methods including Dovetail and BioNano suggest that some patterns of genomic structure should be visible, at reasonable cost.

Classical taxonomy and embryology have provided insights by describing morphology and then categorizing specimens based on morphological similarities and differences. In the molecular era, identification and comparison of single genes, gene families, or gene clusters have furthered these relationships. Molecular phylogeny based on comparisons of coding regions now allows for definitive statements by revisiting classical relationships between and across taxa. But perhaps it is not these protein-coding regions that underlie the distinct morphology classical taxonomists have described. Maybe the genetic control systems, the toolkit genes, and the “programming” layer (in contrast to the “data” layer of protein coding genes), as depicted in GRNs, are what drive evolutionary divergence, speciation, and, more simply, body plan divergence (Kirschner et al. 2006; Peter and Davidson 2015).

The process of sequencing, annotating, and analyzing the COTS genome indicated that this intermediate layer of genomic information likely resides in the heuristics of transcriptional control mediated by transcription factors, akin to the collinearity of the Hox cluster, albeit on a grander scale. Put another way, the pluripotency of induced stem cells (iPS) via four transcription factors can be considered to undo a century of careful embryology and developmental biology. How meaningful is the developmental process? Four transcription factors function as a cellular reset button! In fact, iPS cells simply prove how robust the power of transcriptional control actually is; the question is the lack of a mechanism for precisely extracting relevant information from genomes, at the right time and place, in the right cell types. Creating a COTS genomic map (e.g., chromosome-level assembly) would allow for interrogation of genome structure across a population and perhaps provide a mapping of the genomic context for gene regulation and expression. In contrast to highly annotated model systems genomes, such as *D. melanogaster*, *C. elegans*, and especially *H. sapiens*, the lack of complexity in the COTS genome annotation may facilitate efforts to find patterns in genomic structure.

## 24.9 Conclusion

The COTS genome assembly is of remarkably high quality, likely related to low heterozygosity, resulting from recent population expansion in a single Pacific COTS clade. The low number and length of COTS genomic scaffolds are biologically significant, as confirmed by the discovery of several intact gene clusters. Aside from the potential utility that a COTS genome provides for the eminent threats COTS pose to coral reefs, the unique structure of the COTS genome due to its recent population history suggests that it is an important resource. COTS may be uniquely well suited for decoding how genomic information is extracted during development.

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# Chapter 25

## Structures and Composition of the Crab Carapace: An Archetypal Material in Biomimetic Mechanical Design



Parvez Alam

**Abstract** The structures and composition of crab carapaces are of interest to biomimetic designers and materials scientists as they are hierarchically optimised to dissipate fracture energies through molecular to macroscopic length scales. At each length scale, mechanical energy is absorbed and redirected, circumventing thus catastrophic fracture through the carapace cross-section on impact. The objective of this section is to elucidate the structural, chemical and compositional makeup of crab carapaces, to provide links between their architectures and mechanical properties, and to discuss highlight papers where attempts have been made to mimic the structure-property characteristics of crab carapaces in modern engineering composites.

### 25.1 Introduction

There are approximately 42,000 species of crustaceans, of which the vast majority inhabit aquatic environments. They are considered the *champions of biomineralisation* (Lowenstam and Weiner 1989), often constructing exoskeletal structures of similar strength to coral exoskeletons. As one of the oldest taxa from the arthropod phylum, crustaceans are the only major taxa from the Kingdom Animalia that make use of stable crystalline, stable amorphous and metastable amorphous polymorphs of calcium carbonate (Bentov et al. 2016; Addadi et al. 2003). The crustaceans include Branchiopoda (shrimps, clam shrimps and water fleas), Maxillopoda (barnacles and copepods), Ostracoda (seed shrimps) and Malacostraca, which include lobsters, shrimp, crayfish, mantis shrimps and crabs. Crabs are amongst the most well-known and documented members of any of the crustacean classes, often displaying very heavily biomineralised carapaces, which serve to protect their soft bodies from predatorial attack. With significant advances made in

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both optical and chemical characterisation techniques, crab exoskeletons have drawn considerable attention as their carapace structures tend to exhibit high stiffness, strength and fracture toughness values (Chen et al. 2008a). The carapace has many functions, the predominant of which is protection. Protection can extend as far as to allow the crab to blend in with its surroundings through decoration (Sanka et al. 2016; Sullivan et al. 2014), which is a carefully controlled growth process of surface architectures that may encourage microorganism or algal attachment.

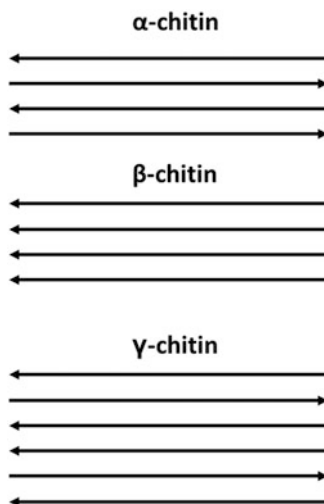
The crab carapace has recently been shown to have specialised architectures and materials that serve to optimise mechanical performance (Chen et al. 2008b). These structures are borne through cellular excretions that are a result of complex inter- and intracellular processes. The primary materials secreted through cellular activity are chitin, proteins and calcium carbonates. The crab exoskeleton exhibits varied properties and architectures that presumably form through the need to satisfy specific functional requirements. Beneath the crustacean exoskeleton is a layer of epithelial tissue known as a hypodermis (Travis 1963), which is responsible for secreting exoskeletal materials. The cuticle forms the major part of the crustacean exoskeleton and as such can be relatively thick at its premoult stage (just prior to exuviation) depending on the species. The cuticle is a layered, calcified structure, and its deposition initiates from the epidermal cells on the soft body of the crab. In order to grow, crustaceans moult, which is a five-stage event (Drach 1939) whereby the exoskeleton is replaced. At the intermoult stage, the carapace of a crab is in its fully deposited and mineralised form (Mangum et al. 1985). The intermoult stage is the stage whereby the crab is most effectively protected by its carapace from mechanical or impact damage and is the stage of primary interest to biomechanical and biomimetic researchers. This is because the fully mineralised form of the crab carapace is of high fracture toughness, yet the predominant materials that it is made of are inherently brittle.

The objective of this section is to detail the generic structures and architectures of crab carapaces, describe the materials that form them through cellular excretion, clarify the biomechanical benefit of such structures and materials and report on principles of biomimetic design that can be derived from the crab carapace.

## 25.2 Materials Secreted Through Cellular Activity

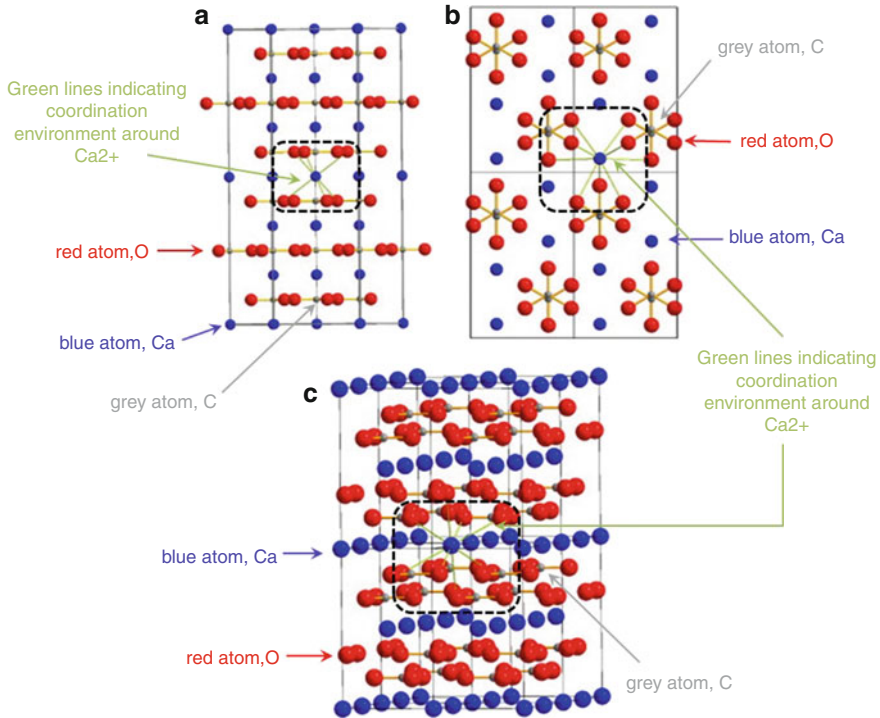
Chitin, proteins and calcium carbonates are the primary cellular-secreted materials making up the bulk of the crab carapace. Environmental parameters enable the deposition of microorganisms (including diatoms) to the carapace surface, adding to the global structure of the carapace (Sanka et al. 2016; Sullivan et al. 2014), though their mechanical contribution to the carapace is likely negligible. Both chitin and calcium carbonate exist in different polymorphs, which the crab exploits to generate specialised, function-specific architectures within its carapace. Chitin exists as three polymorphs,  $\alpha$ -chitin,  $\beta$ -chitin and  $\gamma$ -chitin. Whereas  $\alpha$ -chitin is an amorphous structure,  $\beta$ -chitin forms large networks of hydrogen bonds between relatively straight chains of *N*-acetylglucosamine molecules, causing it to be crystalline.

**Fig. 25.1** Organisation of different morphological states of chitin, from Alam (2015a, b), reproduced with permission of John Wiley & Sons, Inc.



$\gamma$ -chitin is a metastable state of chitin that can transition to either  $\alpha$ - or  $\beta$ -chitin conformations. Figure 25.1, from Alam (2015a), shows the fundamental organisation of both  $\alpha$ - and  $\beta$ -chitin polymorphs. The arrows in this figure indicate whether the chains of *N*-acetylglucosamine molecules are in an antiparallel arrangement (arrows in opposite directions,  $\alpha$ -chitin), a parallel arrangement (arrows in same direction,  $\beta$ -chitin) or a combination of both antiparallel and parallel arrangements ( $\gamma$ -chitin).

Calcium carbonate also exists in three polymorphic states, these being aragonite, calcite and vaterite. Whereas vaterite is a metastable phase of calcium carbonate, aragonite and calcite are stable crystalline polymorphs with distinct crystallographic faces [Fig. 25.2 (Heberling et al. 2014)]. The process of calcification of the crab carapace is an essentially intimate molecular-scale collaboration between both the organic and nucleating mineral phases. Chitin biopolymers (enveloped by protein) are initially deposited (Green and Neff 1972; Giraud-Guille 1984); after which there is an accumulation of calcium and carbonate ions (Falini et al. 1996). This consequently creates the necessary environment for the nucleation and subsequent growth of calcium carbonates. The calcium carbonate growth patterns tend to occur either dendritically (in a branching pattern) or through crystal-crystal fusion (Simkiss 1975). Calcification is generally thought to occur when  $\text{Ca}^{2+}$  and  $\text{CO}_3^{2-}$  ions accumulate in concentrations above their maximum solubility, setting off the process of  $\text{CaCO}_3$  crystallisation. Carapace constituents are borne of cellular secretions. Many of the mineral ions are retrieved through crab's blood. As such, it should come as no surprise that the blood of premoult crustaceans contains elevated levels of calcium ions when compared to intermoult or postmoult crustaceans (Greenway 1985). This is due to the crab's need to grow sufficient exoskeleton for protection during the early stages of carapace formation, which facilitates a more effective level of soft-tissue protection.



**Fig. 25.2** Crystal structures of (a) calcite, (b) aragonite and (c) vaterite (in orthorhombic  $Pbnm$  setting). Calcium (Ca), blue; oxygen (O), red; carbon (C), grey. Examples of each type of atom are indicated in the figure using arrows. Green lines indicate the coordination environment around the  $\text{Ca}^{2+}$  cations. The positions of these lines are indicated using green arrows and accompanying text in the figure. The coordination environment is highlighted using black broken-line boxes. Yellow lines indicate carbon-oxygen bonds. From Heberling et al. (2014), reproduced and modified with the permission of Elsevier

Crustacea are somewhat unique as they include large fractions of the amorphous phases of both calcium carbonate and calcium phosphate in their carapace structures (Addadi et al. 2003). This is different to other mineralising species, which use larger fractions of crystalline calcium carbonates such as calcite and aragonite. From a biomechanical perspective, amorphous calcium carbonates are lower in hardness than their crystalline polymorphs, so it logically follows that they should benefit crabs in some other way. One benefit could be perceived by their growth patterns. Since the minerals are amorphous, they have no distinct faces and as such, no direction-specific growth (Bentov et al. 2016). This allows for more complex shapes and growth patterns in the final macrostructure as amorphous calcium carbonate can take on essentially any shape without the crystallographic restrictions experienced by calcites and aragonites. A second benefit that can be derived from the use of amorphous calcium carbonate relates directly to fracture. Since there are no crystal cleavage planes in amorphous calcium carbonate, the material is less brittle than

calcite or aragonite (Aizenberg et al. 2002). Importantly, amorphous calcium carbonate is considered stable when containing bound water at a ratio of 1 mole of water per 1 mole of amorphous calcium carbonate (Grunenfelder et al. 2014a).

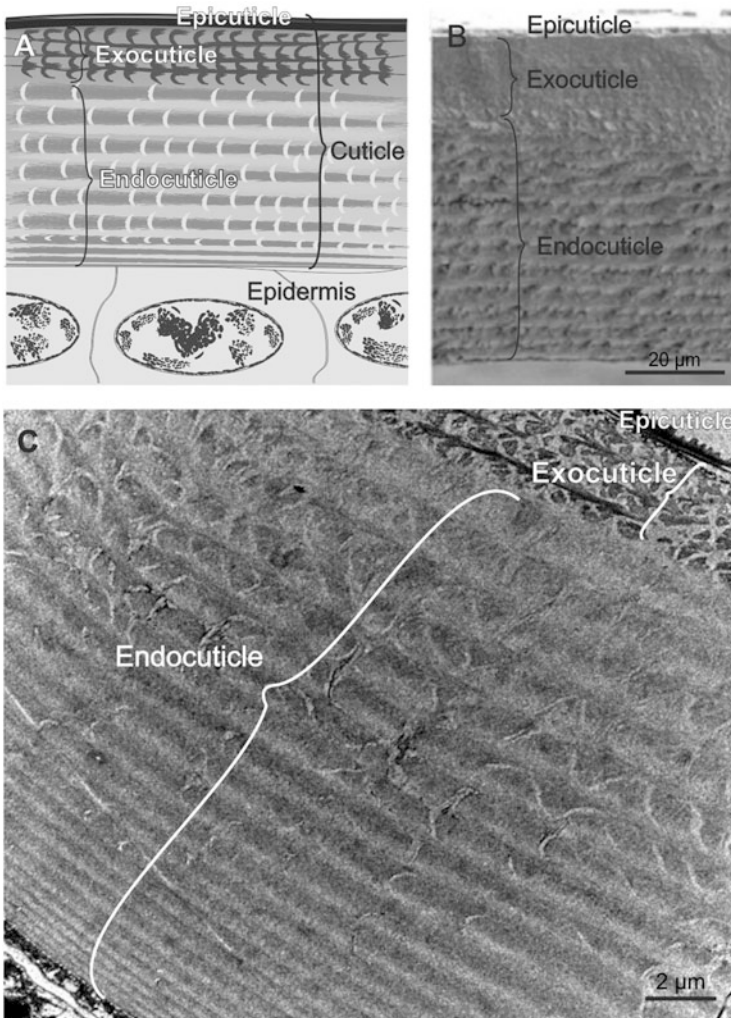
Another benefit in using amorphous calcium carbonate is that the material is able to more easily include a higher fraction of trace elements than its crystalline counterparts (Bentov and Erez 2006). This includes phosphate ions, which have the additional benefit of improving the elastic modulus and hardness of amorphous calcium carbonate (Currey et al. 1982; Bentov et al. 2012) while reducing its solubility (Bentov et al. 2016). By varying the ratio of phosphate to carbonate, crabs are able to improve the means by which mechanically graded structures are grown. Though much of the calcium carbonate phosphate construction is amorphous, Dillman (2005) has noted in at least the blue crab, *Callinectes sapidus*, that calcium carbonate phosphate forms acicular crystals. The inclusion of magnesium ions into biogenic amorphous calcium carbonate is believed to stabilise the calcium carbonate structure. This has been reported for both biogenic amorphous calcium carbonate and for structured (crystalline) calcite (Raz et al. 2003; Politi et al. 2010).

### 25.3 Fundamental Organisation of the Crab Carapace

The cross-section of a carapace reveals that there are three fundamental layers, each with its own structure and function. These are, from the outside of the carapace in, the epicuticle, the exocuticle and the endocuticle. The structures of each layer differ somewhat from each other, and this moreover changes throughout the mineralisation process, which is typically divided into intermoult, premoult and exuvia. Figure 25.3a–c compares the fundamental layers (epicuticle, exocuticle and endocuticle) of a crab carapace through (a) schematic drawing, (b) scanning electron micrograph (SEM) image and (c) transmission electron micrograph (TEM) image (Naleway et al. 2016; Mrak et al. 2017).

Hegdahl and co-workers compiled a series of papers on the morphological and structural differences in the different carapace layers, using *Cancer pagurus* L. as a model crab. The epicuticle was described as a bilayer structure comprising a superficial membrane and a thicker base layer (Hegdahl et al. 1977a). Interestingly, they noted spiny projections from the base of the epicuticle containing vertical columns or canals with crystals (Fig. 25.4). These spines presumably serve to at least improve epicuticular rigidity but may also enhance interlocking shear resistance between the two epicuticular layers, since they are the only mineralised regions of the epicuticle. Nevertheless, canals in crab exoskeletons are known stress concentrators (Chen et al. 2008a), and cracks tend to propagate through the material via these ‘weak holes’ in the structure. The exocuticle is considerably thicker than the epicuticle and is highly mineralised (Priester et al. 2005). Mineral crystal edge lengths are up to 300 nm, and the structure of the exocuticle is striated in an equatorial direction, while approximately hexagonally packed in a meridional direction (Hegdahl et al. 1977b). The thickest section of the crab carapace is the

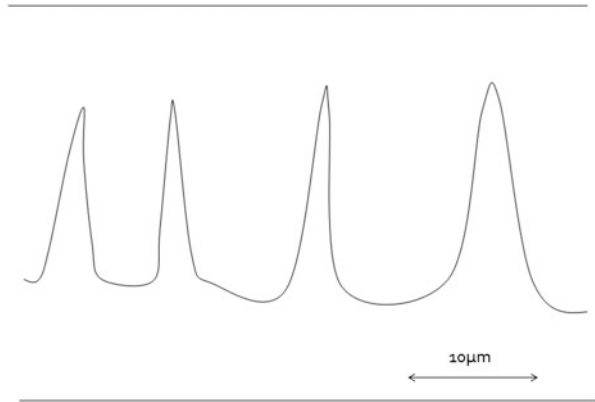




**Fig. 25.3** A general structure of the crustacean exoskeletal cuticle. (a) Schematic drawing of the cuticle architecture, showing the three principal horizontal layers: epicuticle, exocuticle and endocuticle. Epicuticle is the outermost thin layer. Exo- and endocuticle constitute the major part of the cuticle, and both consist of helicoidally arranged chitin-protein fibres, resulting in a lamellar appearance. (b) Ultrastructure of the exoskeletal cuticle in the adult decapod *Pachygrapsus crassipes*, imaging of the cross-fractured cuticle by scanning electron microscopy. (c) Ultrastructure of the tergite cuticle in the adult isopod *Porcellio scaber*, imaging of an ultrathin section by transmission electron microscopy. Figures (a) and (c) and caption from Mrak et al. (2017), Figure (b) from Naleway et al. (2016), reproduced with the permission of Elsevier

endocuticle (Priester et al. 2005), which can be considered thence, the primary protective layer of the crab carapace. The structure is fibrillar at its core (Hegdahl et al. 1977c) and arranged into lamellar arrays. On closer examination, these

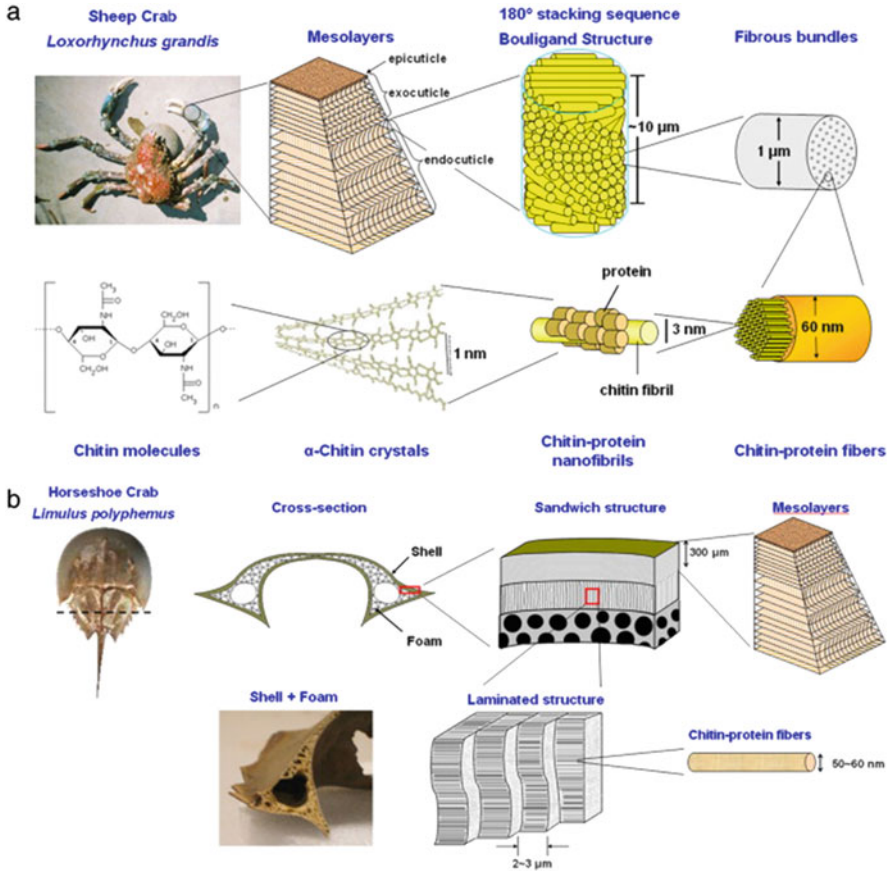
**Fig. 25.4** Schematic of spines of the epicuticle in a dorsoventral cross-section



structures exhibit a Bouligand arrangement (Bouligand 1965a, b, 1966a, b, 1970, 1971), which is essentially a helicoidal fibre pattern. Figure 25.5a from Chen et al. (2008b) illustrates both the hierarchical arrangements of the sheep crab *Loxorhynchus grandis* and identifies the level of hierarchy of the helicoidal Bouligand structure.

Considerable improvements in imaging technologies have enabled us to better understand such Bouligand-type structures and have also elucidated the crab carapace as a hierarchically organised structure (Fig. 25.5a). At every length scale, material architectures are optimised to resist damage. Each length scale has its own function at its local scale, but each scale concurrently works in parallel with higher- or lower-order scales to maximise damage tolerance.

Fundamentally, antiparallel arrangements of  $\alpha$ -chitin making up the chitin nanofibrils (ca. 3 nm in diameter and 300 nm in length) observed in Fig. 25.5a are composed of subunits of *N*-acetylglucosamine molecules. The antiparallel arrangement rigidifies the aligned chitin molecules through the creation of large networks of hydrogen bonds. A hydrogen bond is ordinarily very weak (5–20 kcal/mol), but when present in large numbers within a small confined volume, they significantly increase the load-resisting response of a material (Cranford and Buehler 2012). The chitin nanofibrils develop into orthorhombic symmetry crystallites, which are layered with proteins and collate to form organised fibres of both chitin and protein (ca. 60 nm in diameter). The protein can act as both a glue and an energy dissipater, providing connectedness between other nanofibres, as well as a degree of ductility to the rigidly bound chitin nanofibrils. As such, the chitin-protein combination has both high-energy-absorbing *and* high-energy-dissipating characteristics, which improves the global mechanical absorptive capacity of the chitin-protein composite. The final chitin-protein combination is a larger fibre that connects to other similar fibres in a Bouligand-type arrangement, which is then subject to mineralisation as described earlier. The Bouligand structure occurs when fibres stack helically. Why they stack helically has not as yet been exegetically documented, but a viable reason might begin at the molecular level with helical protein arrangements guiding helical growth



**Fig. 25.5** Hierarchical structure of (a) sheep crab (*Loxorhynchus grandis*) and (b) horseshoe crab (*Limulus polyphemus*) exoskeletons. Though both sheep crabs and horseshoe crabs are arthropods, the sheep crab is in the subphylum Crustacea, while the horseshoe crab is in the subphylum Chelicerata. From Chen et al. (2008b). Reproduced with the permission of Elsevier

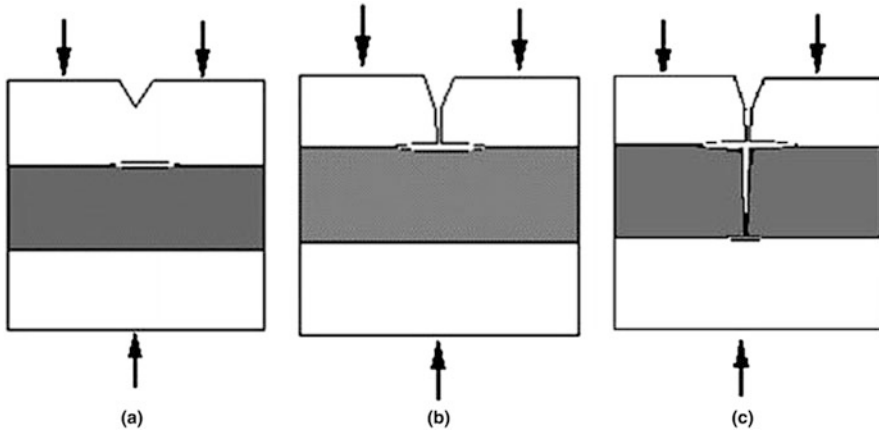
to the nano- and micro-levels and above. The extent of mineralisation will determine the mechanical properties of the material and will differ with respect to the moult stage. Interestingly, the growth orientations of calcite crystals relative to the direction of any equatorial Bouligand axis will affect the fracture and failure modes of the carapace. When cuticles lack vertical fibrils, the orientation of the calcite crystals parallel to the cleavage plane is problematic as it will more likely give rise to delamination fractures between the cuticle layers. Contrarily, if the c-axis of the calcite crystals grow parallel to the equatorial Bouligand axis, the theoretical likelihood of interlaminar fractures is greatly reduced. Nevertheless, c-axis growth is atypical and may result from the imposition of, and reaction to, mechanical loads (Fabritius et al. 2016).

Not all arthropod carapaces are organised into Bouligand-type architectures. The horseshoe crab (*Limulus polyphemus*) is a living fossil (>200 million years) exhibiting a vastly different structural assembly than Bouligand-type structured crabs such as the sheep crab (Chen et al. 2008a). Though both are arthropods, the horseshoe crab, unlike the sheep crab, is not a crustacean, but rather a chelicerate. Figure 25.5b from Chen et al. (2008b) shows the structure of the horseshoe crab as essentially a sandwich composite, with a foam-like, high-porosity structure overlaid by a dorsoventral axis laminated region made up of chitin fibres that lie perpendicular to the dorsoventral axis. This (chelicerate) crab structure shows a distinct orthotropic architecture with properties in three orthogonal axes. This is different to the Bouligand structures observed in (crustacean) crab carapaces, which exhibit different through-thickness and cross-thickness properties. The horseshoe fibrous architecture is arranged much like a cross-laminated fibre reinforced composite, which will typically have properties symmetrical about three orthogonal axes. The dorsal-most layer has a structure that is different to both the layers located beneath it (towards the ventral face of the carapace). The dorsal-most layer is composed of mesoscale lamina that is devoid of mineral presence.

## 25.4 Biomechanics of the Crab Carapace and Its Influence in Biomimetic Design

Ultimately, the crab carapace is a composite mix of both mineral, protein and chitin constituents. This fibre reinforced system enhances fracture toughness (Tai et al. 2007) by increasing the material's ability to absorb and redistribute mechanical energy. This can be achieved by mechanisms such as crack blunting (Fig. 25.6) where a propagating crack in a composite is blunted at the interface of a secondary material phase in the composite. Fracture energies will either dissipate entirely along the interface or will pile up at the interface until the secondary material phase fractures.

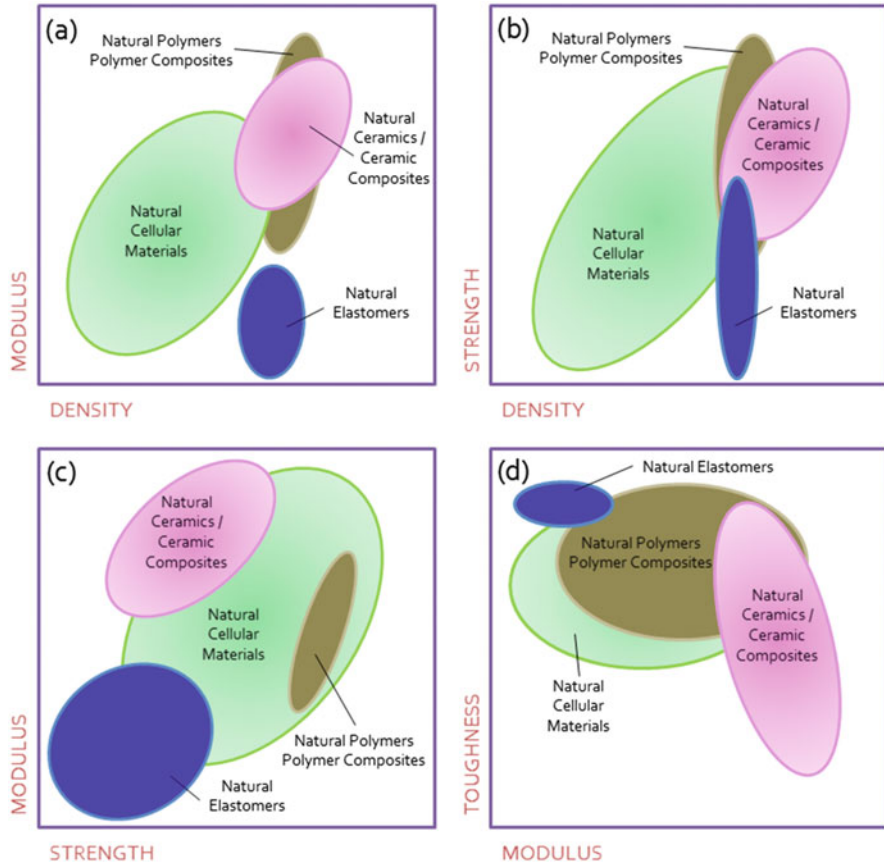
Crab exoskeletons with Bouligand-type arrangements can be loosely considered anisotropic materials with directional properties in the through thickness (meridional) and about the cross-thickness (equatorial) axis, which occurs due to the helical layup of the fibres. The exocuticle tends to be about twice or more in nanohardness as compared to the endocuticle. Moreover, the strengths and stiffnesses correlate to water presence. The study of Chen et al. (2008a) reports an almost threefold higher tensile strength of wet crab exoskeleton as compared to dry exoskeleton. This is ultimately because the water helps bind both polymeric and mineral phases more effectively by enabling electrostatic interactions, and as such the composite exoskeleton is able to function as a crack retarder. Importantly, the work of fracture measured by Chen and co-workers was 1.02 MPa in the wet state and only 0.11 MPa in the dry state, a value almost tenfold lower than in its wet state. In its dry state, the bound fibre composite system becomes discontinuous (Hepburn et al. 1975) and is unable to effectively work as a single unit in resisting and more



**Fig. 25.6** Schematic of crack propagation and blunting in a composite material: (a) delamination at interface prior to crack growth from the notch, (b) blunting of crack after crack propagation from the notch and (c) blunting of crack at the second interface after propagation through the composite layer. From Osman et al. (2008). Reproduced with the permission of Springer-Nature

importantly, redistributing mechanical load. Notably, dry crab exoskeleton is brittle and stiff, indicating that the properties of a crab exoskeleton function only as a composite system when wet. When dry, the properties of the stiff, brittle mineral components guide mechanical behaviour.

Figure 25.7 provides simplified Ashby charts for different natural materials, coupling a variety of material properties. The natural materials are classified here according to Wegst and Ashby (2004) such that natural ceramics and ceramic composites will generally include the mineralised materials such as crab carapace, bone, teeth, antler, mollusc shells and corals; natural polymers and polymer composites will generally include materials such as silks, cellulose, chitin and proteins such as collagen and keratin; and natural elastomers will include materials like resilin, elastin, skin, cartilage, abductin and artery, while natural cellular materials will include materials such as wood, cork, bamboo and spongy bone. In Fig. 25.7a the modulus (stiffness) of these different natural material classes is compared against density. It is interesting that natural cellular materials can have high stiffness even though their densities are low, and this is because they are able to effectively distribute mechanical energy over a large cross-sectional area through an interconnected network continuum (Erjavec 2011). The important aspect of cellular structures is that they *are* in continuum, as this enables an even distribution of force imposed upon the material. Certain natural polymers and polymer composites exhibit high moduli, even though they are relatively low-density materials. These would include mainly cellulose and cellulose-based fibres such as flax and hemp. Cellulose microfibrils have high modulus values, sometimes exceeding 140 GPa (Iwamoto et al. 2009; Hsieh et al. 2008), and it follows that derivative materials built up of cellulose microfibrils (such as flax and hemp) will reflect this modulus in their stiffness properties. Flax and hemp fibres also benefit their cellular structures comprising pore spaces with solid network continua. The



**Fig. 25.7** Simplified Ashby charts for different classes of natural materials showing in (a) modulus-density charts, (b) strength-density charts, (c) modulus-strength charts and (d) toughness-modulus charts

highest stiffness and highest density natural materials are the mineralised natural materials. This is expected as strong ionic bonds are very effective in resisting load within a range of recoverable deformation. In terms of strength (Fig. 25.7b), natural mineralised structures do well because they have hierarchical composite structures that spread load with considerable effectiveness. The strongest natural materials are nevertheless natural polymer composites such as silk. This material is a soft-matter polymer reinforced by crystalline matter which is held together by large networks of hydrogen bonds (Alam 2014b). The material has numerous mechanisms by which means it can circumvent failure including self-healing mechanisms, force redistribution mechanisms and unfolding mechanisms (over direct catastrophic failure). Cellular materials occupy a large range of strengths, and their corresponding densities typically increase with increasing strength. Figure 25.7c shows the modulus-strength plot where the characteristics of high strength and high modulus are most notably an

attribute of cellular materials. Toughness, or the ability to absorb mechanical energy and resist fracture, when compared against modulus, reveals that mineralised natural materials such as crustacean exoskeletons and mollusc shells are generally the stiffest *and* most damage-tolerant natural materials. It is this aspect of mineralised natural materials that is a function of their hierarchical structures (Fratzl and Weinkamer 2007; Alam 2014a), stick-slip mechanisms, which serve to hold and release concentrated stresses, thereby preventing catastrophic fracture (Alam and Alam 2017), and intricately organised composite architectures (Murr 2014).

A logical field in which crab microarchitectures could play a vital role is in composites technology. Composites are a class of material described as being made up of two or more materials with distinct interfaces between them. This class of material is exceptional as by combining different materials, a single material can be made with the properties and characteristics of each. Mechanically, one could combine high-strength but brittle fibres with soft low-strength but ductile polymers to achieve a single material with respectable strength and stretch properties, thereby circumventing the worry of experiencing a brittle failure or the inability to meet design requirements for loading. Due to that the growth, processing, manufacture and design of very complex natural materials are driven by environmentally unobtrusive, cellular processes; biomimetics has gained considerably more attention (Sarikaya 1994; Alam 2014a, 2015a, b; Gruber et al. 2011) in both academic and industrial circles. Numerous attempts have been made to recreate brick-and-mortar type structures that are typical to mineralising organisms containing brick-and-mortar structured abalone (mollusc) shell nacre (Finnemore et al. 2012; Espinosa et al. 2011; Wang et al. 2012). There are a few creditable works on the biomimicry of helicoidal structures, such as those observed in Bouligand-type arrangements. Cheng et al. (2011) researched the effects of helicoidal composites on the strength and stiffness of glass-fibre reinforced composites in flexion and in shear. Importantly, their work showed that biomimetic helicoidally arranged glass-fibre composites were superior in mechanical performance to isotropic control samples. The fracture toughness of composites is another important 'performance parameter' that materials scientists will regularly try to optimise. Fracture toughness has importance in retarding the early onset of failure in materials, which can be catastrophic in cases involving e.g. buildings or fast-moving vehicles. Chen et al. (2006) reported that a higher fracture toughness can be achieved by helicoidally arranging glass fibres in reinforced composites. In their work, they compared their helicoidal glass-fibre composites against unidirectionally aligned glass-fibre composites. Unidirectional glass-fibre composites normally have the highest mechanical properties in the fibre axis and the weakest in the transverse to fibre axis. By helicoidally arranging the fibres, they managed to increase the relative spread of mechanical energy within glass-fibre composites, without any great detriment to the stiffness and strength. The helicoidal structure has also been shown to improve the impact toughness of fibre reinforced composites using as little as one rotational angle in the helicoidal layup (Ravi-Chandar 2011, Apichattrabut and Ravi-Chandar 2006). Carbon-fibre reinforced plastics are important high-performance composites used in the aerospace

sector, an application where impact resistance (e.g. from bird-strike) is of high importance in the design process. Grunenfelder et al. (2014b) researched the application of helicoidal, Bouligand-type fibre arrangements on the impact resistance of carbon-fibre reinforced plastics. Grunenfelder and co-workers found that not only did impact damage tend to spread laterally across the composite perpendicular to the point of impact, but also that damage propagation through the thickness of the composite was less than for unidirectional carbon-fibre reinforced plastic controls. The damage through the thickness of their samples was also reported to lessen as a function of an increasing helicoidal rotational fibre angle.

## 25.5 Conclusions

The crab carapace is a highly evolved composite material with architectural arrangements and hierarchical structures that could benefit the manufacture of manmade composite materials. In this chapter, the complexity of crab carapace design is elucidated, and the biomechanical benefits are discussed. Biomimetic designers and engineers are still at the early stages of research and development and are often discovering new aspects of biological design that may be of benefit to the development of modern materials. Current manufacturing technologies do not make the mass production of biomimetic composites viable since they are inherently complex in geometry, material properties and arrangement. Nevertheless, engineering technologies are advancing at rapid rates with respect to need, and we can expect to see significant improvements in composite manufacturing methods as structural hierarchy in composite materials becomes more widely desired.

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# Chapter 26

## *Octopus vulgaris*: An Alternative in Evolution



Anna Di Cosmo, Valeria Maselli, and Gianluca Polese

**Abstract** *Octopus vulgaris* underwent a radical modification to cope with the benthic lifestyle. It diverged from other cephalopods in terms of body plan, anatomy, behavior, and intelligence. It independently evolved the largest and most complex nervous system and sophisticated behaviors among invertebrates in a separate evolutionary lineage. It is equipped with unusual traits that confer it an incredible evolutionary success: arms capable of a wide range of movements with no skeletal support; developed eyes with a complex visual behavior; vestibular system; primitive “hearing” system; chemoreceptors located in epidermis, suckers, and mouth; and a discrete olfactory organ. As if these were not enough, the occurrence of recently discovered adult neurogenesis and the high level of RNA editing give it a master key to face environmental challenges. Here we provide an overview of some of the winning evolutionary inventions that octopus puts in place such as the capacity to see color, smell by touch, edit own genes, and rejuvenate own brain.

**Keywords** *Octopus vulgaris* · Adult neurogenesis · Evolution · Chemoreception · RNA editing

### 26.1 Octopus: A Sophisticated Animal

Among cephalopods, *Octopus vulgaris* belongs to the behaviorally complex coleoids that include also squid and cuttlefish and the primitive nautiloids that play an important role in the community organization and function of the ecosystems they inhabit (Di Cosmo and Polese 2014). Cephalopods appeared about 530 million years ago in the late Cambrian (Kroger et al. 2011). Modern cephalopods include just two surviving genera of nautiloids that retain an external shell and soft body coleoids that colonized all oceans of the world except for the Black and Caspian seas, spreading

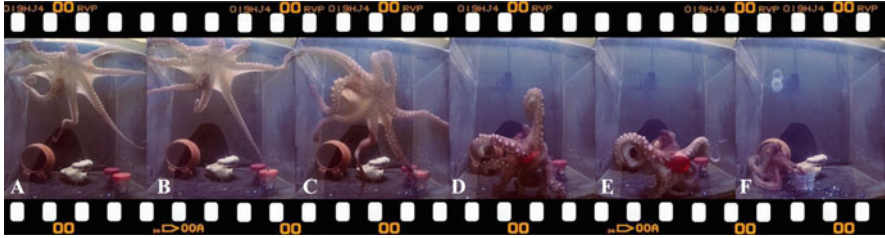
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from the surface waters down into the deep sea and occupying a wide range of ecological niches. But, what is the cause of such a success in evolution? The interesting Packard's theory (Packard 1972) of coleoid evolution gives an explanation how ancestral, external-shelled cephalopods were forced to reach deeper bathymetry by fish competition. The consequential exposure to the high pressure induced the loss of the external shell. Becoming a better swimmer, they could return to shallow waters and fight back their main competitors, fish (Tanner et al. 2017).

In octopus, the loss of the shell pushed them to adopt a benthic lifestyle and develop a diverse hunting strategy than squid and cuttlefish (Boal 1996; Di Cosmo and Polese 2014; Hanlon and Messenger 1996; Wells 1978). To cope with the benthic lifestyle, octopuses underwent a radical modification. They, in fact, diverged from their relatives in terms of body plan, anatomy, behaviors, and intelligence. Although descending from simple mollusks, they have independently evolved the largest and most complex nervous system and sophisticated behaviors among invertebrates in a separate evolutionary lineage (Moroz 2009; Moroz and Kohn 2016; Wanninger 2009; Winters et al. 2017). The octopus' nervous system size falls in the vertebrate range (Hochner et al. 2006; Shigeno and Ragsdale 2015) whereby its behavior and cognitive capabilities seem comparable with mammals rather than invertebrates (Vitti 2013; Young 1971). The octopus' nervous system is divided into three interconnected specialized anatomical parts with a functionally hierarchical organization—the central brain, the optic lobes, and the nervous system of the arms—which provides independent fine regulation of stereotyped movements (i.e., an amputated octopus arm is able to respond to stimuli in the same manner as an intact arm) (Altman 1971; Hochner 2012; Hochner et al. 2003; Rowell 1963; Sumbre et al. 2001; Zullo et al. 2009). In fact, octopus brain does not support somatotopy of the body and does not receive proprioceptive information about the arms (Graziadei 1971; Zullo et al. 2009).

Outside the brain capsule lie the optic lobes and the nervous system of the arms. The optic lobes contain around 160 million neurons. The arm nervous system contains about 300 million neurons (Sumbre et al. 2001, 2005, 2006). The central brain contains just 40 to 45 million neurons. Lying inside the cartilaginous brain capsule and surrounding the esophagus, the brain comprises 40 lobes that show a typical invertebrate organization with an inner neuropil surrounded by outer cell body layers. The evolution of octopus central brain occurred through shortening the connectives between ganglia and decreasing the distance between the integration centers to speed up the computational interactions among them (Budelmann 1995). The functions of the lobes have been deduced from the stimulation and lesion experiments (Boyle 1983; Wells 1978; Young 1971, 1995; Zullo et al. 2009). The lesions in the vertical lobe significantly impair the ability to learn avoidance, discrimination, and association tasks that require memory over periods of minutes. It has been shown that lesions to the vertical lobe abolish the consolidation of long-term memories (Boycott and Young 1956; Shomrat et al. 2008). Therefore, the vertical lobe and its input lobe, the superior frontal lobe, play an important role in learning and memory.



**Fig. 26.1** A challenge for *Octopus vulgaris*: (a) live prey in screw-drilled lid jars are offered to the octopus; (b) octopus touching a drilled lid to smell the prey; (c) octopus grabs one of the jars; (d) octopus starts to unscrew the lid; (e) octopus opens the first jar reaching the prey and at the same time starts to grab the second jar; (f) octopus playing with an empty jar after to have eaten both preys. Frames were collected during behavioral experiment performed at the Di Cosmo's cephalopod facility

A key role of cephalopod nervous system, as an internal correlation system, consists in decoding the signals from the sensory organs and selecting an appropriate reaction to the countless environmental stimuli, and in octopus, it provides the unusual capabilities to reach above the stereotyped behavioral pattern tuning their responses to different stimuli in real time (Godfrey-Smith 2013; Di Cosmo and Polese 2016; Norman et al. 2001). Cephalopods are equipped with arms capable of a wide range of movements with no skeletal support, allowing them to face and find solutions to different environmental challenges whenever they happen (Gutnick et al. 2011; Hochner 2013; Kuba et al. 2003; Mather 1991, 2008). These abilities are essential in exploring new environment, problem-solving, and play-like (Bertapelle et al. 2017; Kuba et al. 2003; Hanlon and Messenger 1996). Play-like meant a behavior that is incompletely functional, voluntary, modified from its regular form, repeated but not stereotypic, and initiated under stress-free conditions (Kuba et al. 2014).

In our problem-solving experiment (Fig. 26.1) when octopus learns how to reach food (Fig. 26.1d, e), after eating, under stress-free conditions, it shows the play-like behavior (Fig 26.1f; Kempermann et al. 2010; Bertapelle et al. 2017).

The best way to detect the environment is through sensory organs, and octopuses have sophisticated ones: developed eyes (Fig. 26.2) and complex visual behavior (Hanlon and Messenger 1996; Stubbs and Stubbs 2016), vestibular system, “lateral line analogue,” primitive “hearing” system (Hu et al. 2009; Hubbard 1960; Zhang et al. 2015; Budelmann and Williamson 1994; Williamson and Chrachri 2007), chemoreceptors located in the epidermis (Budelmann 1996), suckers and mouth (Anraku et al. 2005; Boyle 1983; Wells 1964, 1978), and the olfactory organ, a small pit of ciliated cells located on either side of the head, below the eyes, close to the mantle edge, recently described in *O. vulgaris* by our group (Di Cosmo and Polese 2017; Polese et al. 2015, 2016).

It has been found that octopus has high level of RNA editing that is uncommon among mollusks, including ancient nautiloids, and it might help in the evolutionary invention of soft body coleoids. The RNA editing occurs mostly within the nervous

**Fig. 26.2** Specimen of *Octopus vulgaris* in Di Cosmo's animal facilities: olfactory organs (**a**), eyes (**b**), and suckers (**c**)



system affecting, among others, proteins called protocadherins, which are involved in neural circuit plasticity (Liscovitch-Brauer et al. 2017).

## 26.2 Octopus Weird Pupil: How It Sees Colors

Any diver approaching an octopus in the sea or anyone keeping octopuses in a tank feels like being watched. The most prominent parts of the octopus' body are the eyes (Fig. 26.2). This is the main reason why starting from the first decade of the twentieth century studies on the cephalopods' vision began (Wells et al. 1965; Wells 1978; Wells and Young 1975). These studies resulted in a vast amount of literature on octopus' responses to figures and objects of different shape, size, and shades. There is no equivalent information about other important and evolutionary ancient sense such as the chemoreception (Di Cosmo and Polese 2017; Mollo et al. 2014, 2017). It is indisputable that the octopus eye shares analogies with the vertebrate eye, though two important features differentiate them: the retina is not inverted and it is structurally complex (Wells 1978). Octopus' eye is equipped with a cornea, an iris, and a lens. Because of its large size, the ability to survive long after dissection, the presence of only one type of receptor cells, and the very long optic nerves that connect the photoreceptors to the optic lobes (Grenacher 1884; Lenhossék 1894; Fröhlich 1913b), the octopus eye became an excellent model system to study the vision in cephalopods. Further studies have proven the ability of octopus to recognize the plane of polarized light based on rhabdomere dichroism (Moody and Parriss 1961; Moody and Robertson 1960; Wolken 1958; Young 1962). Only later it was shown

that, similar to vertebrates, the pigment present in the photoreceptors was rhodopsin (Hess 1902). This pigment showed two characteristics, the migration from an inner to an outer layer of photoreceptors and vice versa when exposed to different conditions of illumination and its conformational conversion into metarhodopsin, which is responsible for the phototransduction (Brown and Brown 1958; Hess 1905).

Physiological basis of color discrimination was assessed through electroretinogram experiments (Fröhlich 1913a) in which *O. vulgaris* retina reacted in a different way to different colors and brightness, letting octopus to distinguish red, yellow, green, and blue. Later behavioral experiments (Kühn 1930, 1950) supported the findings about cephalopods' color vision abilities. It is in the 1970s that, unfortunately, a misinterpretation of Kühn's experiments, together with some unexplained results, led John B. Messenger (1977) to come to the paradoxical conclusion that cephalopods were color blind. This assumption somehow blocked the work on cephalopods color perception for the next 40 years. The studies of the last decade, which demonstrate the ability of cephalopods to match with high fidelity their color to natural and artificial backgrounds (Akkaynak et al. 2013; Buresch et al. 2015; Chiao et al. 2011; Hanlon et al. 2013; Mäthger et al. 2010), revived and corroborated earlier Kühn's study (1950). Finally, we are faced with two fundamental questions: (1) how is it possible that an animal with just one type of photoreceptor can perfectly match its background; (2) if the cephalopods were color blind then why should they show such vivid chromatic patterns during mating if their conspecifics cannot discriminate them but it would make them visible to their predators, how would this be a selective advantage in the evolution?

A possible explanation for how cephalopods can see color lies in their pupil shape as proposed by Stubbs and Stubbs (2016): "The bizarre off-axis pupils of these animals can be understood as an adaptation that maximizes spectral information, even at the expense of image acuity." This is another clear example of convergent evolution that reaches the goal of color perception using a track totally different and independent from vertebrates with whom cephalopods rivaled for survival.

### 26.3 Octopus Smells by Touch: Its Chemical Sensing

The chemical environment of cephalopods also provides an important source of sensory inputs, especially for those that inhabit the light-limited conditions (Nilsson et al. 2012). Much of the stimulatory language that regulates cephalopod life consists of chemical cues that determine feeding, habitat, defense, and mating choice. Chemical cues could work in combination with visual signals, or alone, to inform cephalopods of ecological changes. In cephalopods, the known chemical sensory epithelia are buccal lips and mouth (Emery 1975), isolated sensory neurons all over the body surface (Baratte and Bonnaud 2009; Buresi et al. 2014), arm suckers (Graziadei 1962; Wells et al. 1965; Graziadei and Gagne 1976), and olfactory organs (von Kölliker 1844; Watkinson 1909; Von Zernoff 1869). The latter appear to act as



sophisticated organs (Polese et al. 2016) that play a crucial role in mate choice (Cummins et al. 2011; Di Cosmo and Polese 2014; Gilly and Lucero 1992; Lucero and Gilly 1995; Lucero et al. 1992, 2000; Mobley et al. 2007; Piper and Lucero 1999; Polese et al. 2015; Zatylny et al. 2000), predation (Boal et al. 2000), and food odor detection (Anraku et al. 2005; Basil et al. 2000; Boyle 1983; Ruth et al. 2002).

Octopus olfactory epithelia possess anatomical similarities to the olfactory systems of land-based animals, but the molecules perceived from a distant source are different because their water solubility is of importance. Consequentially, in the aquatic environment, the ecologically relevant odorants are water-soluble compounds such as salts, sugars, amino acids, amines, peptides, proteins, and functionalized hydrocarbons. To perceive this kind of molecules, the octopus' olfactory organs change shape from relaxed to erected posture, which allows the animal to orient itself to detect the spatial gradient of these chemical cues, helping in navigation and triggering spatial memories (Huffard 2013; Polese et al. 2016).

However, many insoluble molecules that are detected from distance on land, generally compounds with a molecular weight smaller than 300 Da (Touhara and Vosshall 2009; Mori et al. 2006; Mollo et al. 2017), must be perceived by touch in aquatic systems if they are not carried by micelles or rafts but just held or laid on a substrate. Insoluble compounds known as “odors” on the land have been isolated from marine invertebrates that use them as defensive chemical weapons (Mollo et al. 2014; Giordano et al. 2017), and octopus can “smell” them by touch (Fig. 26.1b) (Di Cosmo and Polese 2017). The demonstration of the presence of olfactory receptors on octopus' “gustatory systems” will corroborate our hypothesis. The evolution of chemotactile skill allows them to follow, or avoid, chemical trails adherent to the substrate by recognizing gradients of concentration using chemoreceptors located in the suckers' rim. However, taste and olfaction are part of a multimodal system of information transfer. The synchronous use and integration of different signals using different channels have the advantage of improving recognition, discrimination, and memory of inputs by the environment. A clear example is the reproductive behavior of *O. vulgaris* that is under the control of a complex set of signal molecules such as sex steroids, neuropeptides, and neurotransmitters that guide the behavior from the level of individuals in evaluating mates to stimulating or deterring copulation and to sperm-egg chemical signaling that promotes fertilization (Di Cosmo and Polese 2014, 2017). These signals are intercepted by the chemosensory organs and integrated in the central nervous system.

## 26.4 Octopus Edits Own Genes

Octopuses and other cephalopods seem increasing their population all over the world adapting themselves to different environmental habitats, responding rapidly to change or disturbance (Doubleday et al. 2016). Their great adaptability could be linked to another intriguing characteristic recently discovered in coleoids: the ability to “edit” some of their genes, changing RNA that encodes proteins (Liscovitch-Brauer et al. 2017). Commonly accepted paradigm is that RNA acts as a messenger,

passing instructions from DNA to protein builders in a cell. But it can happen that modified RNA, editing own genetic instructions, creates proteins that were not originally encoded in the DNA, allowing an organism to add new riffs to its basic genetic blueprint.

mRNA editing by adenosine deaminases acting on RNA (ADARs) was extensively recorded in coleoids' nervous tissue and hemolymph, contributing to their behavioral complexity and allowing dynamic control over proteins in response to different tasks or environmental conditions, such as temperature changes (Liscovitch-Brauer et al. 2017; Garrett and Rosenthal 2012).

Coleoids have tens of thousands of so-called recoding sites, and more than 60% of RNA transcripts are recoded by editing, incredibly higher than 1% of RNA editing observed in other animals, ranging from fruit flies to humans, in which the editing is confined mostly to the nonprotein-encoding RNAs. In coleoids' genes, DNA mutations are markedly depleted around recoding sites to preserve them, an innovative evolutionary trade-off to conserve RNA editing sites that cannot evolve fast.

The natural selection seems to have favored RNA editing in coleoids, even though it potentially slows the DNA-based evolution, because (1) it provides a faster mechanism to "try out" slightly different protein shapes and functions; (2) changes are transient; (3) editing could occur selectively, at specific tissues or conditions; and (4) it could be partial, with only a fraction of the reads edited, resulting in coexistence of both edited and non-edited forms of proteins.

An example of edited protein genetically expanded in octopus genome is protocadherins (Liscovitch-Brauer et al. 2017; Albertin et al. 2012). The octopus genome encodes 168 multi-exonic protocadherin genes, homophilic cell adhesion molecules important in controlling neural circuits and promoting nerve cell excitability. The expression of protocadherins in octopus neural tissues is consistent with a central role for these genes in establishing and maintaining cephalopod nervous system organization as they do in vertebrates, even if they evolved independently, offering a striking example of convergent evolution among vertebrates, squid, and octopus at molecular level (Liscovitch-Brauer et al. 2017; Albertin et al. 2012).

The study of the RNA editing in octopus could give new insights to the understanding of molecular neuroepigenetic mechanisms underlying the dynamic of reversible state-dependent modifications from cell to behavior (Fig. 26.1).

## 26.5 Octopus Rejuvenates Own Brain

The secret of octopus' rejuvenation lies in its adult neurogenesis. Octopus is able to adapt own behavior to infinite environmental situations that imply a high degree of structural and functional brain plasticity. This is none other than adult neurogenesis that is usually referred to the ability to add newborn cells to the existent circuits to reorganize brain connections (Lindsey and Tropepe 2006; Bertapelle et al. 2017). The observations of octopus' performance during behavioral experiments conducted in tanks enriched with cognitive and sensorial stimuli led us to conclusion that the

animals' capability to adapt their behavioral responses was due to this fascinating process. For a long time, the brain has been perceived to be a static organ, but the discovery of adult neurogenesis in most vertebrate species, including in humans, in insects and crustaceans (ectdysozoans), and among mollusks (lophotrochozoans) only in *O. vulgaris*, has changed this view. In our recent study, we showed, for the first time in lophotrochozoans, the presence of proliferating cells in the specific neurogenic zones of the adult *O. vulgaris* and how the enriched environment affects cell proliferation and synaptogenesis (Bertapelle et al. 2017).

The rationale for our experiments was to set ethological trials providing an enriched challenging environment. We altered the standard housing condition adding three objects as cognitive challenge. Octopuses were introduced to three plastic jars holding a live prey, closed with screw-drilled lids.

This “feeding challenge” represented the only feeding opportunity for those octopuses that were forced to learn how to unscrew the lids, open the jars, and reach the prey (Fig. 26.1).

To face this challenge, octopuses with several trials became able to solve the problem, and after the first success, they started to do it in shorter time. This acquired ability to solve a problem resulted from the newborn neurons and neuronal circuit reorganization, as it was already demonstrated in insects and mammals (Scotto-Lomassese et al. 2002; Lemaire et al. 2012; Cayre et al. 1996; Burghardt 2005).

The study of adult neurogenesis in different neurogenic regions from a system neuroscience perspective will pave the way to understand how it supports adaptive behavior.

## 26.6 Future of Research on Octopus

The extraordinary evolutionary convergence examples offered by *O. vulgaris* must be taken into account to avoid anthropocentric vision in future studies that can easily lead to a result misinterpretation.

An integrated approach among genomic, physiological, and behavioral studies to understand what were the strategic choices that octopus encountered during its evolution will be taken to study:

- The “smell by touch” hypothesis (Di Cosmo and Polese 2017) looking for olfactory receptors in the sucker rim (*in progress*)
- The alternative approaches to study octopus' nervous system, such as the establishment of protocol for neuron cell culture (Maselli et al. 2018) given that octopus has been declared a sentient animal (European Directive 2010/63 EU L276, the Italian DL. 4/03/2014, no. 26) (Fiorito et al. 2014; Polese et al. 2014)
- The functional characterization of individual neurons in octopus to address molecular and cellular mechanisms underlying complex and alternative brain evolution

Octopus reserves many surprises.

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## Chapter 27

# Vision Made Easy: Cubozoans Can Advance Our Understanding of Systems-Level Visual Information Processing



Jan Bielecki and Anders Garm

**Abstract** Animals relying on vision as their main sensory modality reserve a large part of their central nervous system to appropriately navigate their environment. In general, neural involvement correlates to the complexity of the visual system and behavioural repertoire. In humans, one third of the available neural capacity supports our single-chambered general-purpose eyes, whereas animals with less elaborate visual systems need less computational power, and generally have smaller brains, and thereby lack in visual behaviour. As a consequence, both traditional model animals (mice, zebrafish, and flies) and more experimentally tractable animals (*Hydra*, *Planaria*, and *C. elegans*) cannot contribute to our understanding of systems-level visual information processing—a Goldilocks case of too big and too small.

However, one animal, the box jellyfish *Tripedalia cystophora*, possesses a rather complex visual system, displays multiple visual behaviours, yet processes visual information by means of a relatively simple central nervous system. This—just right—model system could not only provide information on how visual stimuli are processed through distinct combinations of neural circuitry but also provide a processing algorithm for extracting specific information from a complex visual scene.

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## 27.1 Introduction

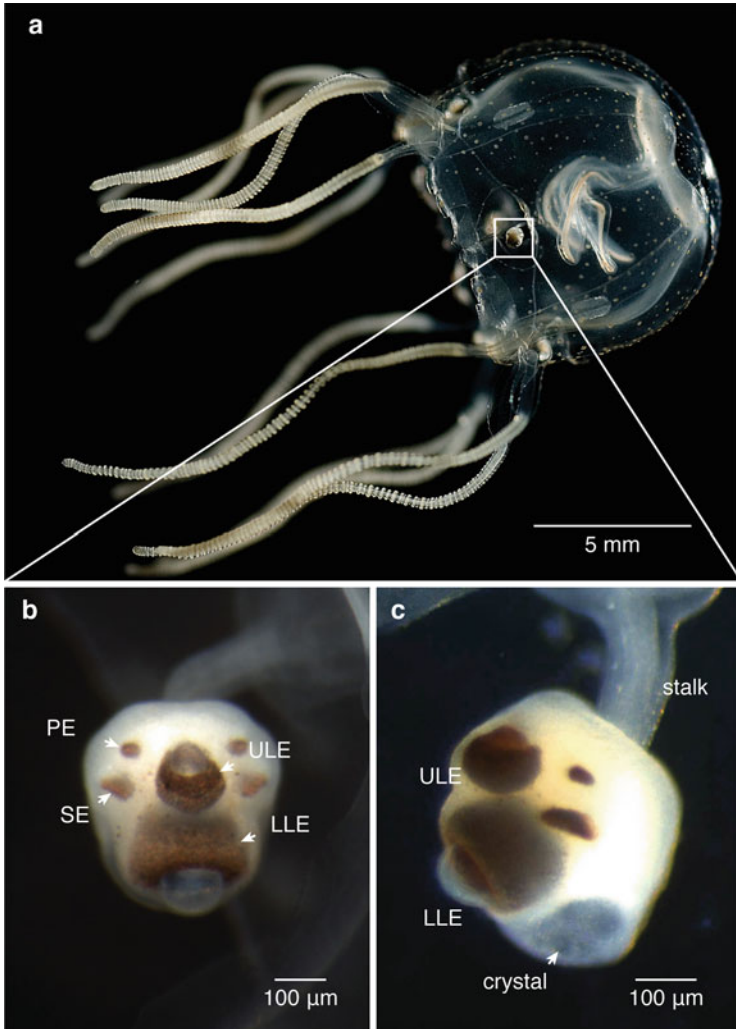
Choosing the right model system is a multifaceted Goldilocks conundrum: it has to be complex enough to answer the questions at hand, and putative future questions, yet simple enough for the resident researcher to understand. We propose that the cubozoan jellyfish *Tripedalia cystophora* is emerging as a close to ideal model to understand visual information processing in detail and with cellular resolution (Fig. 27.1). This animal possesses camera-type eyes structurally similar to vertebrate eyes and has multiple distinct visual behaviours and an experimentally tractable central nervous system (CNS).

So far, our understanding of visual information processing by interconnected neurons in the brain has been hampered by challenges inherent to the model systems currently available—too large and complex to understand in detail. Mice are the preferred model animals of medical and pharmaceutical research, and they admittedly possess a number of advantages, not least the evolutionary proximity to humans that allows for a direct comparison. Unfortunately, being a mammal, mice have huge brains comprising approximately 75 million neurons, interconnected by 1 billion synapses. In the cortex alone, mice have about 4 million neurons (Oh et al. 2014). But the greatest challenge is that the myelinated neurons render the brain opaque and that limits optical penetration to a few 100  $\mu\text{m}$ . So even though the past two decades have given us wonderful advances in fluorescent tools and microscopy techniques, enabling simultaneous observation of large populations of neurons, our understanding of neuronal response to visual stimuli is largely limited to the outermost layer of the visual cortex.

Other conventional models have more tractable nervous systems, but even the brain of a 6-day-old zebrafish larvae comprises about 100,000 neurons (the adult has 10 million), and fruit flies have 250,000 brain neurons. While these systems seem more attractive, they are still too large to retrieve specific neuro-neuronal responses to a particular visual stimulus.

The much simpler model systems (*C. elegans*, *Planaria*, and *Hydra*) can be disregarded for the lack of image-forming eyes and distinct visual behaviour, and therefore they cannot help us understand biological image analysis.

In contrast, the cubozoan system offers a unique opportunity to provide answers to how complex image analysis could possibly be conducted by interconnected neurons, and with the small transparent CNS of *Tripedalia cystophora*, we can monitor an entire working CNS in response to specific visual stimuli. *T. cystophora* comes with its own set of challenges, though. The animals are highly sensitive to variations in osmolarity, temperature, oxygen tension, and water composition. But in our opinion, the possible gain in understanding greatly surpasses the disadvantages of working with this type of fragile marine organism, and hopefully the scientific community will soon realise the huge potential of these stunning mangrove-dwelling animals.

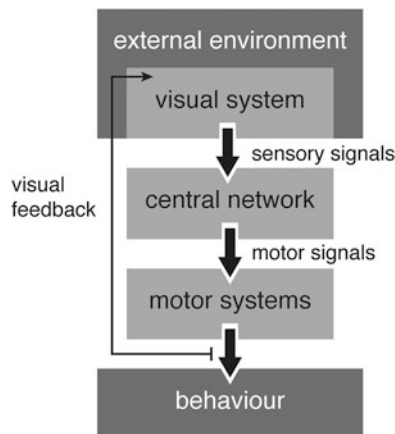


**Fig. 27.1** Visual ecology of the box jellyfish *Tripedalia cystophora*. Box jellyfish are agile swimmers and predominantly guided by visual input. A sensory structure (rhopalium) is located on each side of the medusoid bell; (a) each of the rhopalia carries six eyes of four morphological types (*PE* pit eyes, *SE* slit eyes, *ULE* upper lens eye, and *LLE* lower lens eye), two of which are similar in structure to vertebrate eyes, (b) (front view) and (c) (side view). The transparent rhopalium also contains the processing neural network (CNS) of the animal. Merely 1000 processing neurons are available for analysing the visual information received by the eyes and relay motor signals to the bell musculature, yet *T. cystophora* display several robust visual behaviours (see Fig. 27.7). (a) and (c) modified after Bielecki et al. (2013b)

## 27.2 External Environment

The present account will attempt to elucidate the neural involvement in image analysis based on principles from neuroethology (Fig. 27.2) by guiding the reader through the neuronal processes from the environmental cues to the behavioural output. Neuroethology is the evolutionary and comparative approach to the study of animal behaviour and the underlying mechanistic control by the nervous system. This implies correlating sensory cues from the habitat to specific behavioural responses performed by the animal. *T. cystophora* is thought to possess chemo- and mechanoreceptors (Skogh et al. 2006), but vision is putatively the predominant behavioural modulator in box jellyfish. We must therefore specifically evaluate the visual environment of *T. cystophora*.

*T. cystophora* inhabits mangrove swamps in the Caribbean, where it can be found between the prop roots of *Rhizophora mangle* trees. The jellyfish must navigate the maze of roots to avoid collision thereby damaging their bell. The epidermis of the bell is merely one cell layer thick, and abrasion could prove fatal due to bacterial infection. Intuitively it would be wiser for *T. cystophora* to avoid the mangrove altogether, but sunlight penetrates the foliage and between the roots to create light shafts, where positive phototactic copepods congregate in high densities, and it is of course in the interest of *T. cystophora* to position itself here to prey on the abundant crustaceans. In congruity with the described habitat, *T. cystophora* performs at least four distinct, different, and visually modulated behaviours: obstacle avoidance



**Fig. 27.2** Principles of visual neuroethology. The specific task of a **visual system** is to evaluate electromagnetic radiation with wavelengths in the detectable spectrum from the **external environment**. And subsequently transform the radiation into **sensory signals**, which can be processed by the neurons in the **central network** (CNS). Relevant sensory information will generate **motor signals** that are relayed to the effectors of the **motor system** and thereby regulate the **behaviour** of the animal. Own movements cause a significant disturbance of the recorded image in a visual system and must be considered. A **visual feedback** loop ensures that motor system-generated inconsistencies can be adjusted for or, in some cases, actively utilised by the visual system

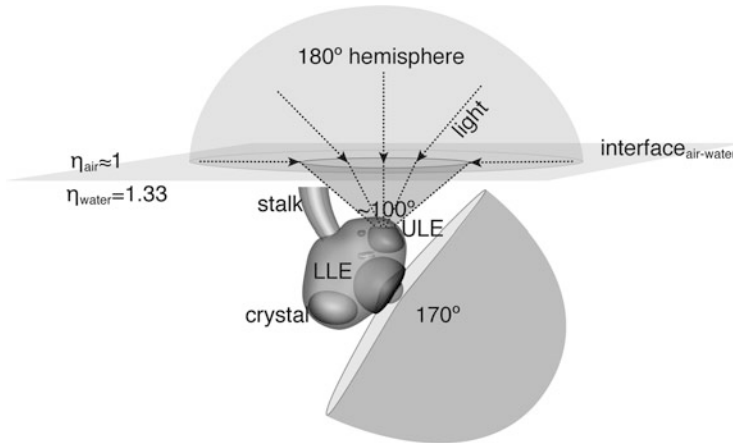
(Garm et al. 2007c, 2013), prey location (Buskey 2003), long-distance navigation (Garm et al. 2011), and diurnal activity (Garm et al. 2012). The first three are the best studied and therefore the ones considered here.

## 27.3 Box Jellyfish Visual Ecology

Vision is based on the ability to perceive electromagnetic radiation or photons within a range of detectable wavelengths, which is transformed into neuronal relevant electrical signals in the photoreceptors. Two main classes of photoreceptors in image-forming eyes are found throughout the Metazoa: ciliated and rhabdomic. The classification is based on the membrane folding strategy of the outer segment, the photoabsorption part of the photoreceptors. Membrane folding greatly expands the light-sensitive area making the photoreceptor more sensitive to light. In rhabdomic photoreceptors, the cell membrane itself is extended by microvilli, whereas the ciliary receptors have a cilium extending from the cell, and the membrane of this cilium is then extended in microvilli (Land and Nilsson 2012). Both types of photoreceptors can be found throughout the Metazoa, and, contrary to the majority of invertebrates, vision in cubomedusae is mediated by ciliated photoreceptors.

### 27.3.1 Visual System Morphology

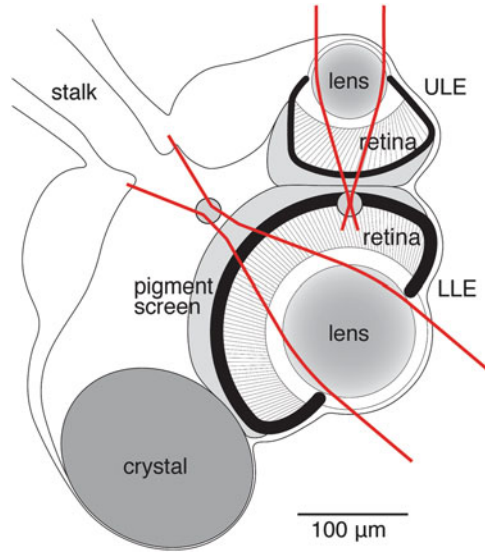
Within Cnidarians, cubozoans have the most elaborate visual system comprising 24 eyes located on 4 sensory structures, called rhopalia. In addition to four smaller eyes (two pit eyes and two slit eyes), each rhopalium carries two camera-type lens eyes, morphologically similar to vertebrate eyes (Fig. 27.1b and c). Among the four rhopalia, the visual field of cubozoans spans a complete sphere around the animal, but it is clearly divided between the upper lens eyes (ULE), directed upwards with a visual field of just less than 100°, and the lower lens eyes (LLE), which are directed downwards into the water with a visual field of 170° (Fig. 27.3). The smaller eyes share large parts of the visual field with the lens eyes; the pit eyes are directed upwards and the slit eyes into the water. Their function is not yet understood and therefore not considered here (cf. Garm and Bielecki 2008). The division of the visual field between the eyes is accomplished by the unique morphology of the rhopalium. It is suspended from the bell by a stalk and weighted by a heavy calcium sulphate crystal at its distal end, which ensures a constant vertical orientation of the rhopalium regardless of the orientation of the animal (Fig. 27.3; Garm et al. 2011).



**Fig. 27.3** Visual fields of the lens eyes. The rhopalium is suspended from the side of the bell by a flexible **stalk** and weighed down by a heavy **crystal** at the distal end. This morphological specialisation ensures a constant orientation of the rhopalium regardless of the orientation of the animal itself. This also ensures a strict division of the visual fields between the upper and lower lens eye. The upper lens eye (*ULE*) peers upwards, observing terrestrial cues through Snell's window; the lower lens eye (*LLE*) is directed into the water below the animal. Snell's window compresses the **180° hemisphere** above water into a 97° cone under water (*dashed lines*). The visual fields of the upper and lower lens eyes are **100°** and **170°**, respectively. The refractive indices ( $\eta$ ) of air and water are indicated on the **air–water interface**

### 27.3.2 Optics

The quality of a visual system is determined by the sensitivity to light and the spatio-temporal resolution of the images produced. Usually the two are at an inverse relationship, but the evolution of lenses dramatically improved sensitivity by focusing the light onto the retinal photoreceptors. The acuity of the retinal image is determined by the number of photoreceptors and the angular width of their receptive field—smaller angle, higher acuity. The upper and lower lens eyes have nearly spherical lenses with graded refractive indices, which in the case of the upper lens eye can produce near aberration-free images (Nilsson et al. 2005). The lower lens eye has less than perfect optics, but the lenses of both eyes are capable of producing receptive fields of less than 1°. However, the retina in both the ULE and LLE is displaced in regard to the focal plane of the lenses causing a severe under-focus (Fig. 27.4). This is thought to eliminate high spatial frequencies (small details) from the retinal image, which diminish the needed processing capacity significantly. The displaced retina decreases the spatial resolution of the eyes to 10–20°, depending on the location of the photoreceptors in the retina, restricting the animals to see large objects in their field of view and not, e.g. their copepod prey (Nilsson et al. 2005). It has been suggested that box jellyfish uses matched filters to optimise the processing capacity and increase the signal-to-noise ratio in their sensory systems (Wehner 1987; Bielecki et al. 2013a). Matched filters are used to extract a known signal,



**Fig. 27.4** Retinal displacement in the lens eyes of *Tripedalia cystophora*. Sagittal transection of the rhopalium reveals the components of the upper and lower lens eyes (ULE and LLE), respectively. The **retinas** are shielded from false light by a **pigment screen**. The spherical **lenses** of the upper and lower lens eyes have graded refractive indices and are capable of producing near aberration-free images. However, the retinas are displaced in relation to the focal point (in *light grey circles*) of the incident light (*red traces*). This displacement results in a much wider acceptance angle of the individual photoreceptors—10–20° dependent on the location on the retina. Modified after Nilsson et al. (2005)

whenever present, from an unknown signal. By disregarding redundant sensory information, the nervous system can process the presence and absence of the known signal rather than evaluate the entire unknown signal. This way the box jellyfish visual system can extract specific information from a complex visual scene with the limited neural capacity available to the animal (Wehner 1987; Bielecki et al. 2013a).

The iris of the LLE can contract in response to changing light intensities. This regulates the intensity of incident light in the LLE. In contrast, the iris of the ULE does not contract (Nilsson et al. 2005). The functional significance of regulating incident light intensities in the LLE but not in the ULE is not fully understood.

### 27.3.3 Opsins

Light has four characteristics: colour, intensity, direction, and polarity. The wavelength determines the perceived colour, and the amount of photons received within a given time defines the intensity. The visual neuroethology is of course based on what



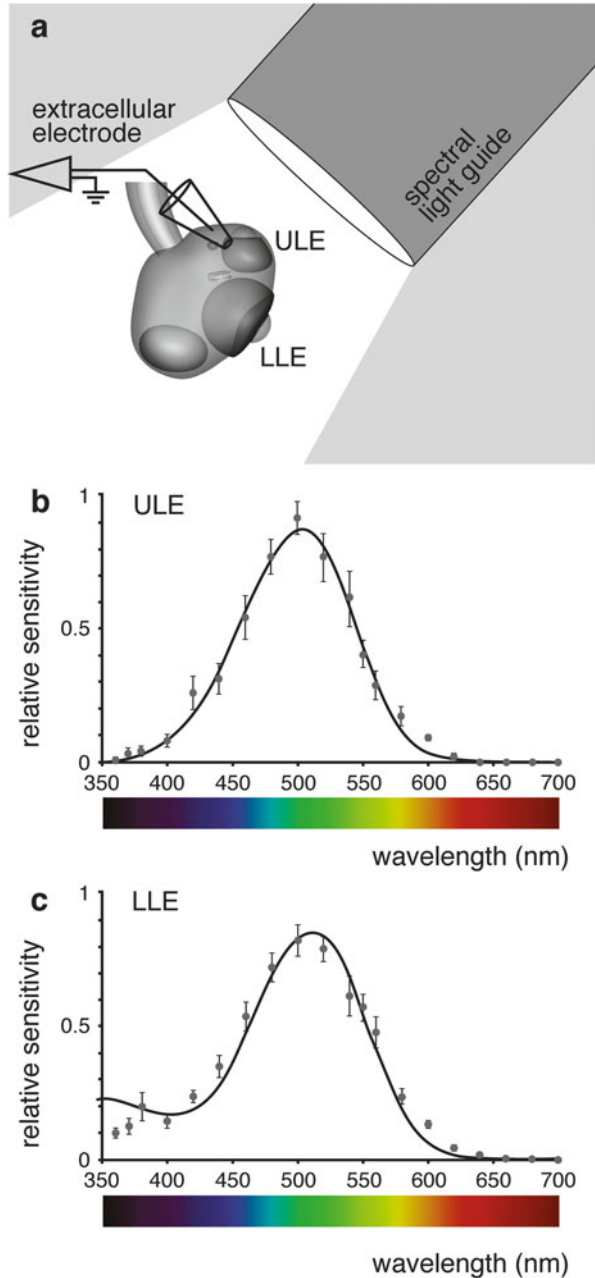
the animal actually sees, and the signal transmission starts with the photoreceptive opsin located in the retinal photoreceptors. Opsins are transmembrane receptors binding an 11-*cis*-retinal molecule that changes configuration to all-*trans*-retinal in response to a photon, or quanta of photons, inducing an electrical response in the cell, and opsins are in this way light sensitive. Ciliary opsins (c-opsins) in general signal through a G protein and cyclic nucleotide (cNMP) pathway to hyperpolarise the photoreceptor membrane (Nilsson 2004; Kozmik et al. 2008; Koyanagi et al. 2008). Opsins have been categorised into eight groups by the amino acid sequence, which also corresponds to the type of G protein involved in the signal transduction pathway (Terakita 2005; Plachetzki et al. 2007; Suga et al. 2008), and one type of c-opsin, cnid-ops, was found exclusively in cnidarians (Plachetzki et al. 2007). The opsins expressed in box jellyfish lens eyes were demonstrated to utilise a G<sub>s</sub>-cAMP phototransduction cascade (Koyanagi et al. 2008).

Opsin photon catch makes photoreceptors light sensitive, but additionally, opsins have peak responses to a given colour and are thereby tuned to a narrow range of wavelengths. In *T. cystophora* lens eyes, opsin responses peak in the blue-green spectrum (approximately 510 nm) and then progressively diminish to wavelengths diverging from this optimum (Garm et al. 2007a; Fig. 27.5). Single opsins expressed in the lens eyes, common among all the examined cubozoans, therefore suggest that the cubozoans are colour-blind and have peak sensitivity in the blue or blue-green spectrum of visible light (Garm et al. 2007a; Koyanagi et al. 2008). Curiously, Kozmik et al. (2008) found a blue 470 nm opsin in the *T. cystophora* rhopalial transcriptome and, by immunohistochemical staining, placed it in the retinas of the lens eyes. Since the electrophysiological evidence discourages dichromatic systems in the cubozoans (Garm et al. 2007a), these results are seemingly contradictory. However, we found the 470 nm opsin to be an extraocular opsin expressed in the neuronal mass of cells that comprise the neuropil of the rhopalium (Bielecki et al. 2014), where it presumably is involved in controlling the diurnal activity pattern (Garm et al. 2012).

### 27.3.4 Spatio-Temporal Properties

About 400 and 600 photoreceptors make up the retinas of the *T. cystophora* upper and lower lens eyes, respectively. The spatio-temporal resolution of the lens eyes has been established (Nilsson et al. 2005; Coates et al. 2006; Garm et al. 2007a; O'Connor et al. 2010). The temporal resolution of the lens eye receptors has been investigated both indirectly (half width and time to peak of the response) and directly (flicker fusion frequency—fff) from ERG recordings (Garm and Ekström 2010). The receptors were found to have minimum half widths of approximately 30 ms from the lens eyes (Garm et al. 2007a) and flicker fusion frequencies of 10 and 8 Hz for the upper and lower lens eyes in *T. cystophora*, respectively (O'Connor et al. 2010).

**Fig. 27.5** Spectral sensitivity of the *Tripedalia cystophora* lens eyes. ERGs from the lens eyes (**extracellular electrode**) when exposed to narrow bandwidth light throughout the visible spectrum (350–700 nm) (**spectral light guide**) revealed that the box jellyfish have monochromatic vision with peak sensitivity in the blue-green spectrum, approximately 510 nm (506 nm for the upper lens eye (**b**, *ULE*) and 508 for the lower lens eye (**c**, *LLE*)). The average spectral sensitivity of each measured wavelength (grey circles  $\pm$  SEM) in (**b**) and (**c**) was fitted to the absorption curve of single-opsin models (Govardovskii et al. 2000). (**b**) and (**c**) modified after Garm et al. (2007a)



### 27.3.5 *Directional Vision*

In most image-forming eyes, the photoreceptors are shielded by pigments to avoid light entering the eye from undesired directions. In mammalian eyes, these pigment screens are formed by specialised pigment cells encasing the photoreceptors in a dark orb. This adds architectural strain on the mammalian eye since all supportive cells (bipolar, amacrine, and ganglion cells) must also be located within the pigment screen, and the retina is therefore considered inverted. As a result light has to penetrate supportive cells and the cell bodies of the photoreceptors to strike the outer segment membranes. In cubozoan retinas, pigment granules are located within the photoreceptors themselves (Yamasu and Yoshida 1976; Bielecki et al. 2014), the retina of *T. cystophora* lens eyes is everted, and light strikes the outer segments directly. The photoreceptors articulate directly on putative second-order neurons, presumably the retinal-associated neurons described by Skogh et al. (2006).

## 27.4 Central Network

In model animals with large complex nervous systems, it is not only the sheer size and vast amount of synaptic connections that is an obstacle to understand neuronal image analysis in detail. Recent advances into the plasticity of complex nervous systems show that astrocytes modulate neuronal response and performance (Takano et al. 2007; Iliff et al. 2012; De Pittà et al. 2012; Volterra et al. 2014) and therefore play a significant role in nervous system plasticity. Needless to say, if the goal is to understand direct neuro-neuronal activity in response to a specific visual stimulus, highly plastic systems pose a much greater challenge. Astrocytes have never been observed in the cubozoan nervous system, indicating very limited plasticity. In fact, both behavioural and electrophysiological responses to specific visual stimuli are robust and consistent between individual preparations (Garm et al. 2007c, 2011; Garm and Bielecki 2008; Garm and Mori 2009; Petie et al. 2011, 2013).

### 27.4.1 *Rhopalial Nervous System*

Retinal images must be analysed by the nervous system for an animal to utilise the visual information for behavioural guidance (Fig 27.2). In many animals, the computational network resides within a centrally located brain. The central nervous system of cubozoans comprises a ring nerve and the rhopalial nervous system (RNS), where the latter is thought to be the centre for visual information processing and the ring nerve is involved in inter-rhopalial communication (Garm et al. 2006, 2007b). Each rhopalium contains about 1000 neurons available for the sensory information processing (retinal-associated, flank, and giant neurons), which must

accommodate all sensory systems (in addition to the eyes, the rhopalial system also includes a number of putative chemo- and mechanoreceptors (Skogh et al. 2006)). These three types were classified based on morphological characteristics and are located in different areas of the rhopalium. A large part of the volume of the rhopalium between the epidermis and gastrodermis is filled with a neuropil made up of neurites. Large numbers of bi- and unidirectional synapses have been found here using ultrastructural techniques, but due to the complex 3D structure, it is not possible to follow the neurites at any considerable length (Skogh et al. 2006). The neural architecture, or connectome, has not yet been described in detail, but ultrastructural and immunohistochemical studies have hinted at the morphology and physiology (Garm et al. 2006; Skogh et al. 2006; Gray et al. 2009; Parkefelt and Ekström 2009). Electrical synapses have never been associated with the cubozoan nervous system (Mackie et al. 1984), but chemical synapses are plentiful (Anderson and Grünert 1988; Skogh et al. 2006; Gray et al. 2009). This suggests that signal transduction and processing involve chemical synapses, and we made an effort to identify the involved neuroactive substances in visual processing of *T. cystophora*. We found that FMRFamide, serotonin, and dopamine all had inhibitory effect on the pacemaker signal frequency (Bielecki et al. 2013b), which is consistent with earlier behavioural and immunohistochemical studies (Plickert and Schneider 2004; Kass-Simon and Pierobon 2007; Parkefelt and Ekström 2009). Comparing our findings to the morphological data led us to suggest that putative second-order neurons and computational units use FMRFamide as transmitter, serotonin is involved at the retinal level, and this neuropil relies on dopamine to control the diurnal rhythm of the animal (Bielecki et al. 2013b).

### 27.4.2 Central Pattern Generators

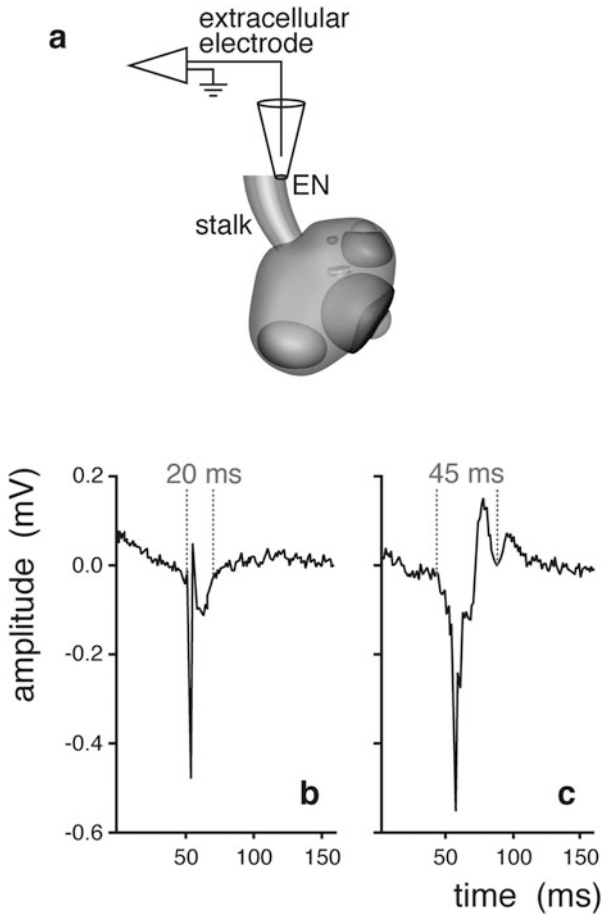
The processing of sensory information, or “decision-making” (cf. Schall (2001)), is necessary to relay the correct motor signals to the musculature for the animal to appropriately navigate the immediate environment (Fig. 27.2; Briggman et al. 2005; Ijspeert 2008; Satterlie 2011). One of the major behavioural control systems in cubozoans is the swim pacemakers, which set the swim pace of the medusa (Satterlie 1979, 2002). Pacemakers are cells that produce a repetitive rhythmic output, and they control a wide variety of behavioural activities across the Metazoa, such as breathing, chewing, digestion, and locomotion (Delcomyn 1980; Ijspeert 2008). The pacemaker activity can be modified by both intrinsic and extrinsic neuromodulators (Morgan et al. 2000; Marder et al. 2005; Nusbaum and Blitz 2012). In the cubomedusae, pacemaker cells are located in the rhopalia, and the pacemaker signals control the discrete swim contractions in a one-to-one manner (Satterlie 1979). There is evidence for age-related pacemaker signal frequency in cubomedusae (Shorten et al. 2005), but the base frequency in the adult *T. cystophora* is approximately 1 Hz, corresponding to leisurely swimming activity by which the animal maintains the position in the water column and searches for food sources.

Pacemaker responses diverge dependent on whether a visual stimulus is presented to the entire rhopalium or the lens eyes individually. In the context of the visual environment of *T. cystophora*, a sharp change in light intensity presented to the entire rhopalium resembles a sudden change in ambient light intensity. Contrary, stimulating the eyes individually implies a specific and directional change in the visual environment of the animal. A light-on stimulus presented to an entire rhopalium produces a distinct arrest of swim pacemaker signals for about 10–20 s, after which the signals return to the base frequency of approximately 1 Hz. Conversely, a light-off stimulus results in a steep and instantaneous increase in pacemaker frequency (Garm and Bielecki 2008). Similarly, a light-on stimulus decreases pacemaker signal frequency when presented to the lower lens eye, and light-off increases pacemaker signal frequency (Garm and Mori 2009). Contrary, stimulating the upper lens eye causes the opposite response: instantaneous increased signal frequency to light-on and decreased to light-off stimulus (Garm and Mori 2009). Additionally, the pit eyes and the neuropil are light sensitive and capable of modulating the pacemaker frequency. The pit eyes are located laterally on either side of the upper lens eye, also directed upwards, and with overlapping visual fields (Garm and Bielecki 2008). The neuropil is not directionally screened from incident light but expresses a 470 nm opsin, indicating extraocular light detection with peak sensitivity in the blue spectrum (Skogh et al. 2006; Bielecki et al. 2014). When the pit eye and neuropil are stimulated individually, they respond similar to the upper lens eye: increased pacemaker signal frequency in response to light-on stimulus and decreased frequency to light-off (Garm and Mori 2009). Curiously, both the pit eye and neuropil respond slower to the light-on stimulus than to the upper lens eye. They do not reach peak response until 10–20 s after the light-on stimulus and show a strong pacemaker signal inhibition in response to the light-off stimulus (Garm and Mori 2009). The extraocular opsin found in the neuropil has peak absorbance in the blue spectrum of visible light (470 nm). This is consistent with opsins controlling diurnal rhythm (Levy et al. 2007), and the neuropil could be the modulator of diurnal activity in *T. cystophora* (Garm et al. 2012). It would also explain the slower response time when stimulating the neuropil individually. The function of the pit eye is not yet understood (Garm and Bielecki 2008; Garm and Mori 2009).

The identical response patterns when stimulating the lower lens eye individually and the entire rhopalium indicate an override mechanism that favours the response in the lower lens eye over the other eyes of the rhopalium (Garm and Bielecki 2008; Garm and Mori 2009).

Even though their neural output is well described (Satterlie 1979; Garm and Bielecki 2008), the pacemaker cells themselves have not yet been positively identified. Excision experiments place them at the proximal part of the rhopalium near the insertion of the rhopalial stalk (Yatsu 1917), where Skogh et al. (2006) found approximately 30 giant neurons on each side of the rhopalium. These putative pacemakers have several afferent synapses indicating that a number of input channels articulate to one or a limited number of pacemaker cells producing a number of pacemaker subsystems (Skogh et al. 2006). These subsystems probably have neuromodulatory effect on each other to produce the complex neural output

necessary for each of the specialised behavioural tasks. The pacemaker cells subsequently transmit their complex signal to the ring nerve through the bidirectional epidermal stalk nerve (Fig. 27.6; Garm et al. 2006; Garm and Bielecki 2008). Only pacemaker signals elicit swim contractions, but a wide variety of signals can be recorded from the stalk. The pacemaker signal has a distinct different electrical signature compared to other more action potential-like afferent signals (Fig. 27.6; Bielecki et al. 2013b). Little is known about the action potential-like signals beyond



**Fig. 27.6** Neuronal signals from the rhopalial nervous system. Various neuronal signals can be recorded from the epidermal stalk nerve (a, EN) by an **extracellular** suction **electrode**. Some signals resemble action potentials in duration and electrical signature (b), but the specific function remains uncertain. However, the more complex pacemaker signal (c) controls the bell contractions in a 1:1 manner and thereby the behaviour of the animal. The duration and electrical signature diverge significantly from action potentials, and where an action potential has a duration of approximately 20 ms and originates from one cell (b), the pacemaker signal has a duration of 45 ms and a profile indicating multiple cell origin (c). It is thereby possible to predict the behaviour of the animal in response to visual stimuli just by assessing the occurrence of pacemaker signals. Modified after Bielecki et al. (2013b)

the epidermal stalk nerve, and only the pacemaker signals will be discussed in this account (Petie et al. 2011).

### 27.4.3 Motor Systems

Once the visual information has been filtered, processed, and modulated to a neural output, it is finally transmitted to the effectors, which are normally the muscular system (Fig. 27.2). The ring nerve is located orally on the bell and is responsible for transmitting neural signals between rhopalia (Satterlie 1979; Garm et al. 2007b). The ring nerve directly innervates the velarium, which controls directional swimming, and connects to a nerve net that extends to cover the entire bell (Garm et al. 2007b). It is suggested that the motor signal is first transmitted to the ring nerve and then to the muscular tissue since numerous neurons extend from the ring nerve to the muscular tissue. Innervation of the velarial opening is especially prominent, but nerve net neurons from the bell also articulate on the ring nerve (Satterlie 2002). Excision experiments showed that even with a severed ring nerve, the animal could still perform swim contractions (Satterlie 1979); however, it must be assumed that the ring nerve aids in propagating the nervous signal and contributing to contraction synchrony since the nerve net neurons articulate on the ring nerve (Satterlie 2002). Such a system is known from the hydromedusa *Aglantha digitale*, where special action potentials propagate in the ring nerve which influence the synaptic transmission time and thereby allow for synchronous bell contractions (Mackie and Meech 1995a, 1995b).

In *T. cystophora*, rhopalial pacemaker signals produce a swim contraction in a 1:1 manner, but the animal possesses four rhopalia, and a higher level of motor signal control is necessary to prevent multiple inputs to the motor effectors simultaneously (Stöckl et al. 2011). Since the rhopalia are interconnected through the diffuse bipolar nerve net of the bell and the ring nerve (Satterlie 1979, 2011; Garm et al. 2007b), it has been suggested that the driving rhopalium inhibits the others by hyperpolarizing the pacemaker cells of subordinate rhopalia (Satterlie 1979; Stöckl et al. 2011). The role as driving rhopalium alternates and is determined by which rhopalial pacemaker is currently modulated by visual input (Satterlie 1979; Petie et al. 2011; Stöckl et al. 2011).

In accordance with earlier studies on the cubozoan nervous system (Plickert and Schneider 2004; Kass-Simon and Pierobon 2007), we could merely induce inhibition of the swim pacemaker system of *T. cystophora* by bath-applying neuroactive substances (Bielecki et al. 2013b). Thus, it is attractive to propose a rhopalial pacemaker system that comprises an intrinsic signal frequency of about 3.5 to 4 Hz, which is downregulated according to the sensory input to the given rhopalium and the neighbouring rhopalia. The suggested intrinsic frequency is based on recorded maximum frequencies and the fact that one bell contraction has duration of 200–250 ms (Petie et al. 2011; Bielecki et al. 2013a) making a higher frequency unlikely. Directional swimming is accomplished by asymmetrical swim contractions and directional constriction of the velarial opening to produce a jet of water that propels the animal in the right direction (Petie et al. 2011).

## 27.5 Integrative Approach to Behaviour

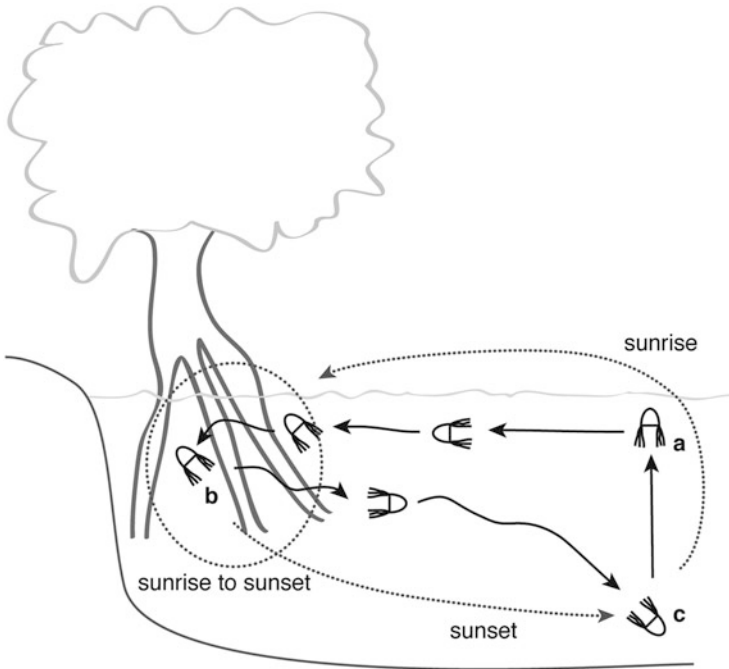
For an integrative approach, all activity of an animal must be viewed in this context and correlated to the restraints of the sensory habitat. In the case of *Tripedalia cystophora*, three behaviours justify these considerations and will be discussed here: long-distance navigation, light shaft detection, and obstacle avoidance. All these behaviours are integrated parts of the diurnal activity of the animal, and all are tractable for systems-level understanding of visual information processing (Fig. 27.7): robust, repeatable, and instantaneous in response to visual stimulation. In addition, *T. cystophora* displays diurnal activity. In response to diminishing ambient light, the activity of *T. cystophora* decreases, and the animal seeks the bottom of the creek where it presumably attaches to the sea grass (Fig. 27.7 position c) (Garm et al. 2012). When the light returns to the habitat, the activity of *T. cystophora* increases, and the animal returns to the daytime behavioural repertoire. However, the behavioural response to diminishing light is slow, possibly controlled by the extraocular opsin found in the neuropil (Garm et al. 2012; Bielecki et al. 2014), and cannot be used for visual information processing purposes.

### 27.5.1 Long-Distance Navigation

At sunrise, when the light conditions enable visual orientation, *T. cystophora* rise to the surface water layer of the mangrove/creek habitat (Fig. 27.7 position a). Since the murky water does not offer any directional cues, the animals are reliant on the ability to detect the mangrove canopy for long-distance navigation (Garm et al. 2011; Fig. 27.8). This behaviour is supported by the upper lens eye, which scans Snell's window for the contrast line between the bright open sky and the darker canopy (Garm et al. 2011; Bielecki et al. 2013a). Snell's window is a phenomenon produced by the difference in refractive indices of air and water causing the visual input from the hemisphere above water to compress into a cone of about  $97^\circ$  under water (Fig. 27.3). The mangrove creeks are affected by tidal currents that could carry animals into the middle of the creek and onwards to the open sea. If this happens, the animal will be unable to find food and, since all the other animals of the species are located under the mangrove canopy, will be far in between reproductive opportunities. This way long-distance navigation behaviour serves the animal to remain within the habitat and to avoid starvation.

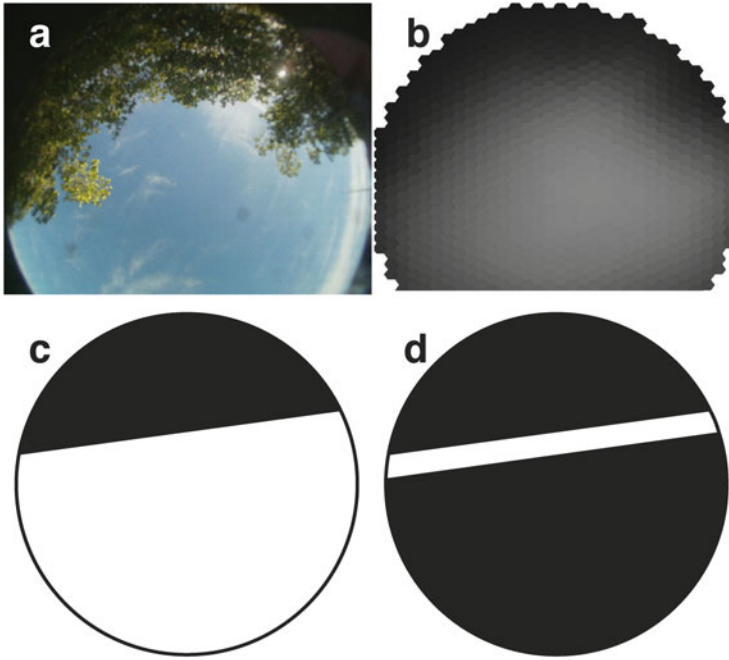
An additional challenge to the long-distance behaviour comes from the fact that all known photoreceptor cells adapt to constant light stimulus (Matthews et al. 1988; Fain et al. 2001), and without countermeasures an animal would be unable to detect stationary objects in its visual field (Land 1999). To avoid this photoadaptation, a strategy must be integrated into any visual system that refreshes the retinal image congruent to the spatio-temporal resolution. In humans and primates, a series of muscular-controlled fixational eye movements (microsaccades, ocular drift, and





**Fig. 27.7** Behaviour of *Tripedalia cystophora*. The mangrove-dwelling box jellyfish display several robust visual behaviours: long-distance navigation, light shaft detection, obstacle avoidance, and diurnal activity. At sunrise the animal rises to the surface (position **a**), scans Snell's window (see Fig. 27.3) for the contrast line between the canopy and the open sky, and swims under the canopy for the duration of the day (**sunrise to sunset** dashed circle, see Fig 27.8). During the day *T. cystophora* passively hunts copepods in light shafts created by sunlight penetrating the foliage of the mangrove trees (position **b**, see Fig. 27.9), all the while avoiding contact with the prop roots penetrating their marine habitat (**sunrise to sunset** dashed circle, see Fig. 27.10). At sunset when light conditions no longer support visual behaviour, the activity of *T. cystophora* significantly diminishes, and the animal seeks to the bottom of the creek (position **c**) where it attaches itself to sea grass (**sunset** dashed line). The described behaviours, long-distance navigation, light shaft detection, and obstacle avoidance, can be used for exploring neuro-neuronal response to visual stimuli since they are robust, instantaneous, and repeatable. Diurnal activity has a much slower response to changing light conditions, and is presumably controlled by extraocular opsins, making it less suitable for vision experiments

ocular microtremors) prevent adaptation of the photoreceptors (Land 1999). Box jellyfish do not have muscles associated with their visual system, and the animals are too slow to refresh the retinal image by swim speed alone. Instead cubomedusae refresh the retinal image of the upper lens eye by utilising their swim contractions. The contractions cause the rhopalium to swing within the rhopalial niche where it is situated, and the amplitude of the swing matches the acceptance angle of the individual photoreceptors in the upper lens eye. The consequent displacement of the retinal image causes a contrast line between light and dark to shift to an adjacent



**Fig. 27.8** Long-distance navigation. At the water surface (Fig. 27.7 pos. **a**), *Tripedalia cystophora* peers through Snell's window to locate the mangrove canopy (**a**) (see Fig. 27.3). The upper lens eye is severely under focused due to the displaced retina, but the canopy can still be discerned with box jellyfish acuity (**b**). *T. cystophora* scans the visual field for the contrast line between the canopy and the open sky (**c**) to assess the direction and the distance to their habitat under the trees. An additional challenge must be met, since all known photoreceptors adapt to constant light conditions and the swim speed of the animal alone is insufficient to refresh the retinal image. However, the bell contractions cause the rhopalium to swing within its niche, and the amplitude was shown to match the acceptance angle of the individual photoreceptors. The canopy-sky contrast line would then shift to an adjacent row of photoreceptors, creating a local light-on stimulus. The contrast line will then light up on the retina as a bright line on a dark (adapted) background (**d**). **a** and **b** modified after Garm et al. (2011)

line of photoreceptors (Fig. 27.8c and d; Bielecki et al. 2013a). This creates a local light-on stimulus on the affected receptors of the upper lens eye and results in an increased in pacemaker signal frequency (Fig 27.8d; Garm and Mori 2009). The animal will thereby increase swim contractions and swim in the direction of the canopy cover until the contrast line is located on the peripheral photoreceptors of the retina in the upper lens eye (Garm et al. 2011).

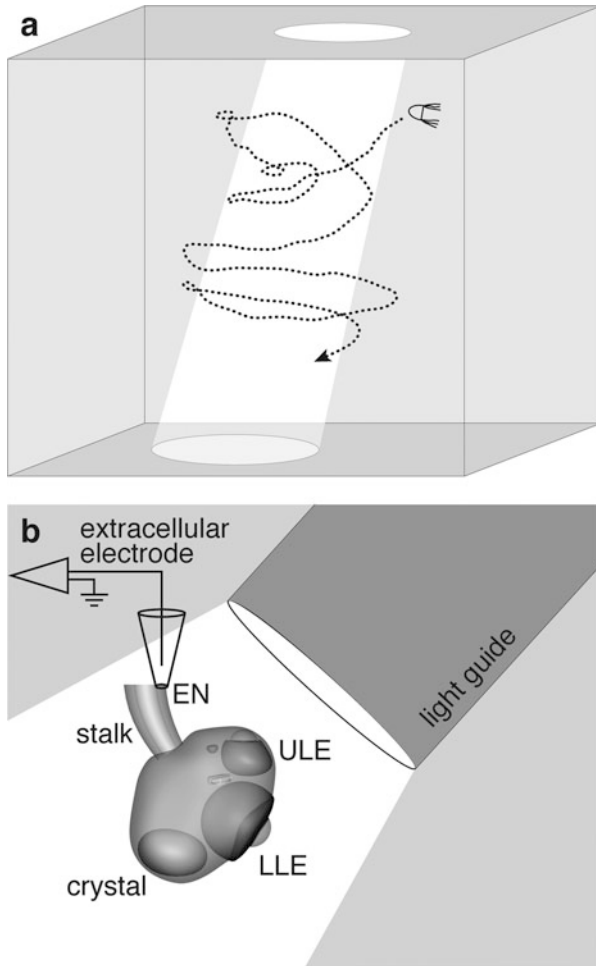
### 27.5.2 *Foraging: Light Shaft Detection*

Another robust visual behaviour in *T. cystophora* is related to foraging in their habitat between the prop roots of the *Rhizophora mangle* mangrove trees. During the day *T. cystophora* prey on the copepod *Dioithona oculata* by swimming through a swarm and catch them on outstretched tentacles (Buskey 2003). The copepods are positive phototactic and congregate in huge numbers within light shafts created by sunlight penetrating the foliage of the mangrove trees (Figs. 27.7 and 27.9). The foraging behaviour is mediated by the lower lens eye in *T. cystophora* and, since the spatial resolution is insufficient for direct prey location (Buskey 2003; Nilsson et al. 2005), entails detection of the light shafts (Fig. 27.9; Garm and Bielecki 2008; Garm and Mori 2009).

Light shaft detection does not necessarily involve image formation but rather a rapid increase in ambient light intensity corresponding to an animal swimming from a shadow area to an area with full sunlight. The flush of light causes the swim contractions to cease and the animal to slowly glide through the water. Cubomedusae are negatively buoyant and will sink through the water column, so within approximately 10–15 s, the swim contractions will resume (Buskey 2003). This behaviour was supported by electrophysiological data (Garm and Bielecki 2008), where a sudden increase in light intensity (light-on) presented to the entire rhopalium resulted in a decreased pacemaker frequency (Fig. 27.9b). This response resembles the modus operandi of the lower lens eye when stimulated individually, indicating that light shaft detection is controlled by the lower lens eye. In contrast the upper lens eye modulates an increased pacemaker frequency when presented to a light-on stimulus (Garm and Mori 2009). Should the animal inadvertently exit the light shaft, it will perform a number of fast bell contractions, turn 180°, and try to relocate the light shaft (Fig. 27.9a; Buskey 2003). This is also supported by the electrophysiological data, where a light-off stimulus results in an instantaneous increase in pacemaker signal frequency (Fig. 27.9b; Garm and Bielecki 2008; Garm and Mori 2009). This behavioural modulation ensures most possible time spent in light shafts and thereby access to prey items.

### 27.5.3 *Obstacle Avoidance*

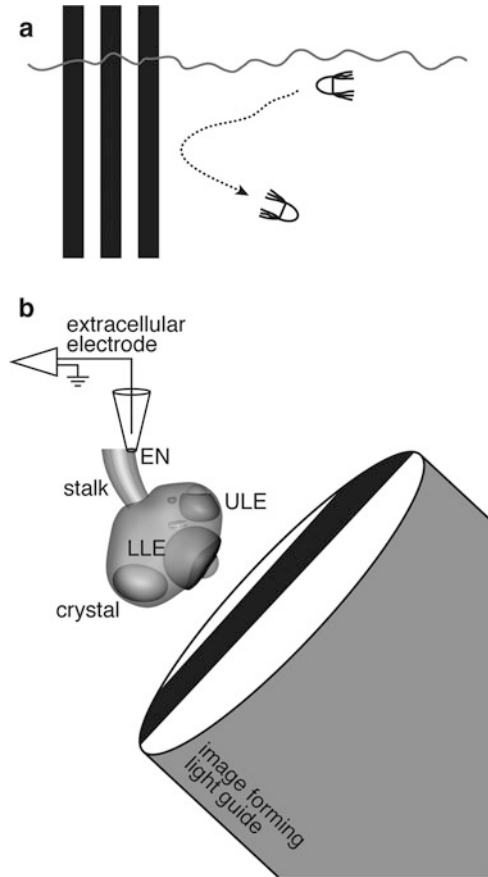
In the medusa stage, *T. cystophora* does not have natural enemies but face threats nonetheless. While searching for light shafts and prey, the animal must avoid colliding with the prop roots of their underwater habitat. The epidermis of their bell is merely one cell layer thick, and abrasion can cause infection and subsequently death. So when *T. cystophora* encounter an obstacle, the animal will perform 4–5 fast bell contractions, turn 120–180°, and swim away (Garm et al. 2007c, 2013; Fig. 27.10). Their obstacle avoidance behaviour is modulated by the lower lens eye and based on true spatial vision (Garm et al. 2013). In behavioural assays,



**Fig. 27.9** Light shaft detection. *Tripedalia cystophora* prey on positive phototactic copepods that congregate in light shafts created by sunlight penetrating the foliage of the mangrove trees (a). When the animal enters a light shaft, it experiences a sudden increase in the general light intensity, and the swim contractions cease for 10–20 s, and the animal drifts through the light shaft passively hunting copepods with outstretched tentacles. Once outside, the animal is faced with a sharp decrease in ambient light intensity and responds by increasing swim contraction frequency, circling back, and attempting to re-enter the light shaft (a). The visual stimuli that the animal is subjected to when interacting with light shafts were recreated in the lab (b). When the entire rhopalium was exposed to a light-on stimulus, a sudden decrease in pacemaker signal frequency could be recorded from the epidermal stalk nerve (EN) using extracellular techniques (extracellular electrode). Upper lens eye (ULE) and lower lens eye (LLE). (a) Inspired by Buskey (2003)

*T. cystophora* was exposed to vertical, horizontal, and oblique bars on the perimeter wall of a behavioural arena (Fig. 27.10a; Garm et al. 2013). Here, the animals displayed graded obstacle avoidance behaviour, depending on the direction of the

**Fig. 27.10** Obstacle avoidance. When *Tripedalia cystophora* is confronted with an obstacle, it will perform four to five fast bell contractions, turn 120–180°, and swim away (a). Electrophysiological experiments confirm the behavioural data (b). When simultaneously showing the lower lens eye (LLE) a moving bar and recording the pacemaker signals from the epidermal stalk nerve (EN), a sharp increase in pacemaker signal frequency was observed when the bar travelled across the middle of the visual field. This is consistent with the obstacle avoidance response observed in behavioural experiments



depicted bars. The avoidance response in *T. cystophora* was most prominent when exposed to vertical bars, then oblique, and the least response to horizontal bars (Garm et al. 2013). This response pattern corresponds to the directionality of the roots in the habitat, and it seems that the visual system of the animal is tuned to detect this specific pattern on the retina (cf. matched filters in Wehner (1987)). In addition to the directionality, *T. cystophora* shows a graded avoidance response to varying contrasts of the bars, with higher contrast yielding greater response. It has been suggested that this response is an inherent measure of distance under water. Contrast dissipates quickly through the water column, so objects with less contrast are further away than objects of more contrast (Garm et al. 2013).

Obstacle avoidance behaviour has been confirmed by electrophysiological experiments. Moving bars projected into the lower lens eye result in a sharp increase in swim pacemaker signal frequency when the bar travels across the middle of the visual field (Fig. 27.10b). This instantaneous increase matches the behavioural response to an obstacle. A uniform decrease in light intensity of the entire visual field, of the same magnitude as the decreased intensity when the bar was moving

across the visual field, did not elicit the same type of response from the behavioural modulators proving that the animals see the root as an object in an image (manuscript in prep).

## 27.6 Future Research Perspectives

We want to obtain a system-level understanding of the cubozoan visual system and follow the information pathway from the retinal photoreceptors through the rhopalial nervous system (RNS) to the motor neurons. Neural physiology and signal transmission strategies are highly conserved throughout metazoan evolution, so we can possibly gain a general understanding about nervous systems by studying cubozoan signal transduction mechanisms and network organisation. If we can understand the algorithm used in neural image analysis in a simple system such as *Tripedalia cystophora*, we have a high chance of discovering universal filtering and processing units used by all animal visual systems including humans. However, at present we are merely able to measure the retinal response to different light stimuli by performing ERGs on the retinas (Garm et al. 2007a; O'Connor et al. 2010; Bielecki et al. 2013a) and to determine the neural output by monitoring the activity in the epidermal stalk nerve (Garm and Bielecki 2008; Garm and Mori 2009; Bielecki et al. 2013b). Unfortunately, what happens between the visual input and the neural output (Fig. 27.2) is contained within the proverbial black box.

The advantages of using the small transparent connectome of *T. cystophora* seem obvious, but the RNS comprises small neurons and is protected by a defiant impenetrable epidermis, which discourages intracellular electrophysiological recordings and makes it impossible to identify cells until backfilling them post-recording. Other approaches are therefore warranted. Recently developed optogenetic reporters, genetically encoded calcium indicators (GECIs) and voltage indicators (GEVIs), have provided tools to monitor neuronal activity in real time. In contrast to conventional calcium- or voltage-sensitive dyes, optogenetic reporters can be expressed in specific cell types and will reliably report on the state of the cell (Akemann et al. 2010; Ahrens et al. 2012, 2013; Alford et al. 2013; Akerboom et al. 2013; St-Pierre et al. 2014; Lin and Schnitzer 2016; Yang et al. 2016).

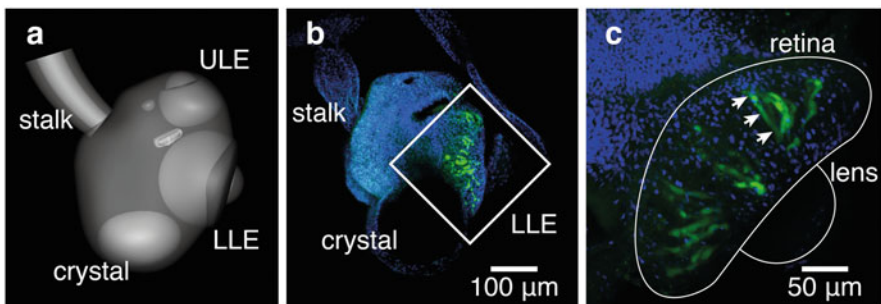
The central nervous system of *T. cystophora* is probably the poster child of live cell imaging: transparent, small, and separated from the motor effectors. Due to the proximity of the CNS and the eyes, we can monitor the neural response to a visual stimulus from the photoreceptors, across the processing circuits and integration in the pacemaker cells, to the motor neuron output signal, all in the same imaging frame and with single cell resolution.

For the imaging approach, it is imperative that the model animal is completely immobile. To resolve this issue, mice are head fixed, and zebrafish are embedded in agarose under the microscope. At best, this causes discomfort for the animal, but in worst-case scenario, it produces an unnatural response pattern. In *T. cystophora* the motor effectors can be separated from the motor signal, and the behaviour related to

a specific visual stimulus is accurately predicted by electrophysiological monitoring of the pacemakers inherent to the RNS. Of course, feedback information is lost from the other rhopalia and putative sensory systems on the bell, but since one driving rhopalium tends to override the output of the others (Petie et al. 2011; Stöckl et al. 2011), valuable information can still be extracted from one transected rhopalium.

Unfortunately, we have not yet succeeded in culturing mature females and therefore do not have access to recently fertilised embryos for microinjection. Granted, the obstacle to microinjection resides with the inabilities of the researchers, not the animals, but the issue has to be resolved nonetheless. A solution to the lack of embryos could be utilising a viral vector to express the optogenetic reporters in the RNS neurons, and one such option is the vesicular stomatitis virus (VSV). The virus' genome is antisense RNA coding for the necessary molecular machinery for viral gene transcription and RNA metabolism and thereby independent of interaction with the host cell nucleus and potentially applicable for use in a wide variety of organisms (Mundell et al. 2015). In most applications using VSV, a GFP is situated behind the ubiquitous T7 promoter and will infect and express in all cell types but spreads anterograde transsynaptically (with the native glycoprotein). This means that it is possible to follow interconnected neurons and the putative signal from point of injection to the processing units. Currently, the VSV is used as a neuro-neuronal tracing tool and has not yet been designed to carry optogenetic reporters. However, we succeeded in transducing GFP in the photoreceptors of the lower lens eye using the VSV vector (Fig. 27.11), but, while it is quite remarkable that a vector produced for mammalian transduction purposes works in the cnidarian system, unfortunately transsynaptic transfection still eludes us (Mundell et al. 2015).

Other viral vectors carry optogenetic reporters: adeno-associated virus (AAV), the preferred transduction workhorse for the mouse scientific community (Gao et al.



**Fig. 27.11** GFP expression in the lower lens eye in vivo. A challenge to using *Tripedalia cystophora* as a model for visual information processing is the limited access to genetic tools. Specifically, the lack of stable transgenic adult jellyfish. However, we have successfully expressed green fluorescent protein (GFP) in the photoreceptors of the lower lens eye (LLE) in vivo using a vesicular stomatitis virus (VSV) vector (b). Higher magnification (c) of the square area in (b) shows individual photoreceptors expressing GFP (arrowheads). The orientation of the rhopalium in (b) is depicted in the schematic (a). VSV(VSV-G)GFP (green), post-fixed (PFA), and DAPI stained (blue). (b) and (c) modified after Mundell et al. (2015)

2005), translocates into the host cell nucleus where it forms a replicative double-stranded DNA of the AAV genome. AAV infects all cell types, but the inserted gene will only be expressed if it is situated behind a cell type-specific promoter. We do not yet know if AAV will infect cells of the cnidarian system, but even if it did, the genome of *T. cystophora* has never been sequenced, and we therefore lack promoter sequences specific to the RNS neurons. It is possible that out of pure serendipitous luck that the commercially available AAV-Synapsin-GCaMP construct (Akerboom et al. 2013) will readily transduce *T. cystophora* rhopalial neurons, but this remains to be determined.

Only time will tell, whether it is possible to produce stable lines of transgenic box jellyfish expressing dynamic optogenetic reporters, but it could greatly enhance our understanding of visual information processing on the cellular level, as current model systems do not yet offer the imaging resolution, depth of field, and field of view necessary to contain the entire processing network within the same recording. This is the true advantage of the box jellyfish model system, and, even though the present account is far from exhaustive, we hope to have inspired box jellyfish awareness for future research.

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