Hideharu Numata · Barbara Helm
Editors

Annual, Lunar, and Tidal Clocks

Patterns and Mechanisms of Nature's Enigmatic Rhythms

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This book is dedicated to Eberhard Gwinner,
chronobiologist, ornithologist, and untiring
pursuer of circannual rhythms.
After obtaining his doctorate with Konrad
Lorenz on the behavior of ravens,
Ebo was drawn wholeheartedly to the topic
of avian chronobiology. He first conducted
research under the mentorship of Jürgen
Aschoff and later followed him in his
position as Director of the Max-Planck
Institute of Behavioural Physiology and
Ornithology in Erling-Andechs in Southern
Germany. Ebo made extraordinary
contributions to our understanding of
circannual rhythms in general, and of the
circadian rhythms, circannual rhythms, and
migrations of birds. The European
Biological Rhythms Society and
the Japanese Society for Chronobiology
commemorate Ebo Gwinner by a named
plenary lecture during each biennial
meeting. Beyond all else, however, Ebo
was a remarkably humane, kind, and
visionary colleague, mentor, and friend,
who certainly will not be forgotten.

Professor Eberhard Gwinner (1938–2004). This photograph was taken by one of the editors (HN) during the International Conference "Complex Clocks" held at the University of Edinburgh, Scotland, 20–24 March 2000.

Foreword

Rhythmic events, suggesting regulation by some sort of negative feedback, are common in biological systems and range in frequency from milliseconds to several years. In 1964, Jürgen Aschoff drew attention to four particular biological rhythms whose periodicities closely matched those environmental cycles caused by the effects of planetary movements. These rhythms, which he called "circarhythms," were tidal (circatidal), daily (circadian), lunar (circalunar), and annual (circannual). When isolated from their natural environments these rhythms express their endogenous ("free-running") periodicities or τ values close to the tidal cycle (about 12.5 h), the day (about 24 h), the lunar month (28–29 days), or the year (about 365 days). To these four circarhythms should be added a fifth, circasemilunar, matching the period between successive high or low spring tides (14–15 days).

These five circarhythms have a number of properties in common. Firstly, as outlined above, their endogenous periods have evolved to match relevant periodicities in their natural habitat. Secondly, under free-running conditions these periods are frequently temperature-compensated. Thirdly, the rhythms are entrained by environmental variables to provide an adaptive synchrony and phase relationship between the behavior or physiology of the organism and the regular changes in their environment. The appropriate environmental signals or *Zeitgeber* may include such factors as tidal stimulation, daily cycles of light or temperature, moonlight, or annual changes in photoperiod. In their entrained steady state these endogenous rhythms may then act as biological clocks enabling the organisms to emerge, become active, or to reproduce at the most appropriate time of their particular environmental cycle.

In recent years significant advances have been made in our understanding of the physiological and molecular events underpinning the autoregulatory feedback loops controlling circadian rhythms, together with some understanding of their entrainment. Such matters concerning the other circarhythms, however, remain conjectural, hence the "enigmatic" reference to these clocks in this book's subtitle. We know little about these enigmatic (non-circadian) clocks beyond their overt periodicity, relevant *Zeitgeber*, and, perhaps, their ecological importance and evolutionary significance. In particular, we know next to nothing about how these rhythms are generated at the physiological and molecular levels.

The book is organized in two parts. The first, consisting of ten chapters, deals with the shorter-period clocks—circatidal, circasemilunar, and lunar—in a variety of organisms. The part opens with a masterful account of tidal and semilunar rhythmicity in the intertidal midge *Clunio marinus* by Dietrich Neumann, now sadly deceased. This is followed by chapters on tidal and lunar rhythms in annelids, crickets, crustacea, corals, and fish, attesting to the widespread occurrence of such rhythms in a diverse array of organisms. In most cases, the endogeneity of the rhythms is known together with the manner of their entrainment to relevant environmental *Zeitgeber*. Some chapters speculate on possible "molecular machinery" behind such rhythms, and most conclude that it is likely to differ from the better known circadian case.

The second part of the book (six chapters) concerns circannual rhythmicity in mammals, birds, and the carpet beetle, *Anthrenus verbasci.* Once again, the basic properties of circannual rhythmicity in the various taxa are comparatively well known, but knowledge of the genetic and molecular events underpinning such periodicity is in its infancy compared to similar information concerning the circadian system. However, since most of these "enigmatic" clocks may be expressed in organisms as diverse as unicellular algae, plants, coelenterates, annelids, insects, and vertebrates, it is likely that they have evolved independently and on numerous occasions, so that the regulating mechanisms are likely to be equally diverse. As most of the contributions to this book suggest, unraveling the molecular complexities of these systems promises to provide a challenging future.

Edinburgh, UK David S. Saunders

Preface

This present collection of articles on enigmatic biological clocks had its beginning during the XII Congress of the European Biological Rhythms Society in association with the Japanese Society for Chronobiology, held at the University of Oxford, 20–26 August 2011. While the emphasis of this meeting was on the exciting progress in our understanding of circadian clocks, the organizers were mindful of the driving interest of chronobiology in unravelling nature's geophysical cycles across timescales. By including a session on annual, tidal, and lunar clocks, they brought together many of the book's authors and spurred excitement about this topic beyond the event, sparking the initiative for the present book. We are convinced that studying "non-circadian" clocks contributes significantly to a full understanding of biological rhythms in general, and in particular to understanding clocks "in the real world", where organisms are usually confronted with more than a single temporal scale of geophysical cycles. Insights on non-circadian clocks may in their own right enable future progress and inspiration for unforeseen applications, much as circadian rhythms have done in the past. The time has come to re-vitalize the interest in non-circadian rhythms: Tools are rapidly becoming available for investigations from molecular detail to environmental context, bringing the unravelling of mechanisms and functions of biological clocks within reach. Since the initiation of the idea to create this book, exciting new publications have indicated that the rapid development of research on non-circadian biological clocks is already taking place. We are pleased to think that the book is therefore very timely, and we hope that it will invigorate research on these fascinating, yet still largely unexplored, natural processes.

Creating a book of this kind is a difficult task that requires the help and patient support of many people and is ultimately subject to various walks of life. In the present case, most unfortunately, two of the authors did not live to see the completion of the book. Dietrich Neumann, who wrote the introductory overview of tidal and lunar rhythms, passed away before he could produce the final version of his chapter. We are immensely grateful to Tobias Kaiser, who, in close coordination with Dietrich's family, brought the chapter to completion. Dietrich Neumann was a giant in his area of research, and although the field is now deprived of one of its great pioneers, we are fortunate and grateful for his inspiring legacy. Dietrich's chapter presents a thoughtful summary of the research developments that he had witnessed and had contributed to over many decades. We also heard with sadness that Franziska Wollnik, a coauthor of Chap. 14, passed away as the final touches were being put on this chapter. Both researchers will be painfully missed by their colleagues and friends.

Other circumstances in people's lives also affected the course of this book, in particular the relocation of one editor (BH) to the United Kingdom, which led to unfortunate delays. We would like to thank all authors for their patience during these events. We gratefully acknowledge the inspiration of Russell Foster, who was the main organizer of the chronobiology meeting in Oxford. During the development of the book, Kaoru Hashimoto and Momoko Asawa, Springer, Japan, kindly gave us expert advice. Finally, we thank our families and friends who encouraged us not only during the period of editing the book, but also over the long periods of our research in this field. One editor (BH) expresses a special thank-you to Tim, Lotte, and Edda, for tolerating and supporting the many hours of dedicated work. This book, and the passion for biological rhythms expressed in it, are ultimately inspired by the vision of the pioneers of chronobiology, especially Ebo Gwinner, to whom we dedicate it.

Kyoto, Japan Hideharu Numata
Glasgow, UK Barbara Helm

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Part I
Tidal, Semilunar, and Lunar Rhythms

Chapter 1
Timing in Tidal, Semilunar, and Lunar Rhythms

Dietrich Neumann

Abstract Tidal, semilunar, and lunar rhythms are particularly pronounced in intertidal organisms, which are affected by strong fluctuations of environmental factors with the tides. For a number of species it has been shown that their tidal, semilunar, or lunar rhythms in behavior and reproduction are not a direct response to the tides, but reflect the action of endogenous biological clocks. This chapter reviews current knowledge on the properties and mechanisms of circatidal and circa(semi)lunar clocks as shown by laboratory experiments and highlights major open questions for future research.

Keywords Biological clock • Chronobiology • *Clunio* • Marine • Tides

1.1 Introduction

The interface between sea and land represents an extremely harsh environment as a result of the strong periodic impact of rising and falling tides (called 'flood' and 'ebb,' respectively), which superimpose the periodic alternations of day and night as well as that of the seasons. It is a fascinating world for any ecophysiologist who is interested in the complex timing adaptations that have evolved in the algae and animals of this intertidal zone.

In contrast to the multitude of experimental studies on daily and photoperiodic timing and on the involvement of circadian clocks in plants, animals, and in humans as well, the first carefully controlled laboratory experiments on tidal or lunar biorhythms did not start until the late 1950s. Since then, a considerable variety of

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Sadly, Dietrich Neumann passed away while he was still working on this chapter. Tobias Kaiser kindly has taken on the responsibility for the chapter and, thanks to his efforts, it has been brought to completion.
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intertidal animals has been examined, mainly with regard to locomotor behavior or reproductive timing. The gradual increase in knowledge in this field of chronobiology has been documented in books and reviews (Bünning 1973; Palmer 1973, 1995; Enright 1975; DeCoursey 1976; Neumann 1981; Naylor 2010). As a selection from the large body of literature, this introductory chapter presents those case studies that give the most detailed and comprehensive information and could be the basis for further research. Biological phenomena correlated with the synodic month of the moon in terrestrial or limnetic biotopes remain unconsidered in this overview.

1.2 General Definitions

1.2.1 Tidal Regimes

On the average, *tidal cycles* have a period of 12.4 h at most coastal locations and estuaries, reflecting the lunar day (24.8 h). It is important to notice that the intertidal range of these *semidiurnal tides* varies in parallel to the phases of the moon. *Spring tides*, with their maximum tidal range, occur every 14.77 days (or half a synodic month of 29.53 days) when the earth, sun, and moon are situated in an approximately straight line and the tide-generating forces of the moon and sun reinforce each other. They occur around days of full and new moons and alternate with *neap tides* of smaller amplitude. The latter occur around the first and the last quarter of the moon when the sun–earth axis and the moon–earth axis form a right angle, so that the tide-generating forces of the sun and moon counteract maximally and the resulting tidal amplitude is at a minimum. The levels of spring high water and spring low water define the extension of the *intertidal zone*.

The semidiurnal tides with their marked situations of high and low water shift from day to day by about 50 min as a result of the delayed meridian transit of the moon on each day. The daily delays of about 0.8 h add up to 12 h during half a synodic month of 14.77 days. As a consequence, any distinct tidal situation (e.g., ‘low water time on a spring tide day’) recurs at the same time of day after half a synodic month (14.77 days). The tides are further influenced by coastline geography, resulting in two important limitations to this general rule. First, this rule is true for each distinct coastal location, but the respective time point will differ between coastal locations; that is, ‘low water time on a spring tide day’ will differ between geographic locations but not at a given geographic location. Second, in places with regular semidiurnal tides this rule is more or less valid for any tidal situation, for example, also for ‘high water on a neap tide day.’ However, at some coasts, coastline geography produces so-called *mixed semidiurnal tides*, which may have interesting daily and seasonal irregularities in the successive levels of high and low waters. Such complex tidal inequalities are mentioned only in context with corresponding biorhythmic adaptations here. In any case, reliable tide tables are available today for nearly all coasts and for any time of the year, so that a chronobiologist who is interested in intertidal timing phenomena does not need to record anew the complex tidal regime of a distinct place; the tide tables allow looking up specific locations where a particular physiological timing adaptation of an intertidal organisms can be expected to be found with regard to part of year, time of synodic month, or time of day.

1.2.2 Intertidal Areas

Intertidal areas occur along the coastline of oceans and their subseas or along the shore of estuaries. They differ in their local maximum *tidal amplitude* (between a few decimeters and far more than 10 m, depending on various geophysical conditions of the sea basin and its shore). Correspondingly, the *spatial extension* of the resulting intertidal zone can be very different. At coasts with strong tidal amplitude, this zone will be relatively small at rocky locations with a steep shore, but considerably larger at, for example, intertidal mudflats, fringing reefs, or tropical mangrove forests. In the Mediterranean Sea, the intertidal area is a relatively narrow zone as a consequence of small tidal amplitudes, meaning that wind from land or sea can more significantly determine the exposure or flooding of this biotope. In any case, intertidal species living between sublittoral and supralittoral areas (for littoral zoning, see Mann 2000) are confronted with the impact of fluctuating physical forces, for example, with strong sea surf at unsheltered coastlines (especially during rising tide), with high solar irradiation during exposure of the intertidal area (especially in subtropical areas during summer), or even with strong water flow and changes of salinity in the range of estuaries.

1.2.3 Intertidal Organisms

Most species that have settled in this intertidal world are of marine origin, such as sessile periphytic algae, sessile and vagile zoobenthos, and littoral fish. Most are physiologically more active during inundation and tolerate exposure to terrestrial-like conditions twice a day, at least in the midlittoral zone. In contrast, some marine crustaceans (such as fiddler crabs), the greenish turbellarian *Symsagittifera roscoffensis* (originally named *Convoluta roscoffensis*), and some genera of terrestrial origin (adults of marine insects and other arthropods) have adapted their activities to intervals when the intertidal biotope is falling dry. In the case of mangroves, even trees have colonized the intertidal zone. Wherever these intertidal organisms may have originated, a reliable timing of some of their physiological features is of considerable selective advantage, may it be in metabolism, color change, locomotor behavior (including migrations), or in development and reproductive timing. At the beginning of experimental studies, the principal question arose as to whether the reliable synchronization of biological events to the very strict conditions of the intertidal habitat is forced by direct responses to distinct tidal or lunar factors or whether these physiological adaptations are actively controlled by endogenous physiological timing mechanisms and their specifically selected tidal or lunar time cues. The general answer is that physiological clocklike mechanisms are involved in the timing of behavior and reproduction time.

1.2.4 Selected Types of Biological Rhythms

Various biological rhythms resulting from evolutionary adaptations can be distinguished in intertidal organisms: (a) *tidal rhythms*, which are correlated with the semidiurnal cycle of tides and shift daily by about 50 min (for exceptions of *diurnal tides* and *mixed tides* at distinct coastlines, see Barnwell 1976), (b) *semilunar rhythms* (more precisely, *semimonthly-lunar* rhythms), which are correlated with the cycle of spring or neap tides (mean period, 14.77 days), and (c) *lunar rhythms* (more precisely, *monthly-lunar* rhythms), which are correlated with every second spring or neap tide event (mean period, 29.53 days).

As already outlined (Sect. 1.2.1), in the case of semidiurnal tides a certain tidal situation reoccurs at approximately the same time of day every 14–15 days (or, in more chronobiological terms, the same *phase relationship* of the daily cycle and the tidal cycle reoccurs every 14–15 days, i.e., in a semilunar cycle). Because of that, it may be advantageous for intertidal species to couple their independent semilunar or lunar timing processes to a daily timing process. An additional timing process comes into play during reproduction events, when the overt semilunar or lunar activity only occurs during a certain season.

1.3 Classic Examples

The nuptial dances of the Pacific palolos are a widely known example of a lunar rhythm in an intertidal invertebrate species. The palolos, which are the sexually mature, posterior part of the bisexual polychaete *Eunice viridis*, are released from the worms on the island of Samoa at about midnight only during neap tide high-water times on days of the waning moon in October or November, when the fringing reefs are flooded but the surf is mild. Their sexual maturation has already begun during the previous months. This predictable temporal occurrence has seasonal, lunar, and daily components (Hauenschild et al. 1968). This highly regular natural phenomenon has found a place in the cultural heritage of the indigenous people of these islands, as the considerable epitokous parts of the worms (full of sperm or eggs) present a festive dish once or sometimes twice a year.

Carefully conducted field observations by natural biologists in the nineteenth and early twentieth century revealed further clear examples of both lunar and tidal rhythms of intertidal species. One of the first may have been discovered by René Chevrel (1894), whose report ‘Sur un diptère marin’ was overlooked for many decades. He observed the tiny, short-lived midge *Clunio* at the coast of Normandy in France and named it *Clunio syzygialis* (later synonymized with *Clunio marinus*) because its reproduction in the lower intertidal zone only occurred between April and October at about the afternoon low-water time on days with spring tides correlated with the ‘syzygy’ (which is the astronomical term for full and new moon positions, when earth and moon have the same heliocentric ecliptic longitude). Today we know that the female *Clunio* prefer the felt-like substrates of red and brown algae and fine sand on rocks of the lower midlittoral (which is only exposed on these days and which is the best substrate for larval development) for egg deposition. Their development takes from at least 6 weeks to about 1 year (depending on latitude and season); each of the tiny adult midges (1.5–2.0 mm) is only capable of reproduction for about 1 h directly after eclosion (Neumann 1966, 1986).

A much more dramatic semilunar-periodic event is the run of the grunion fish (*Leuresthes tenuis*) on sandy beaches of California. These fish swim to the moist upper beach during nocturnal high tides about every spring tide (on 2–4 successive days) between February and August. The females bury their eggs in the sand and attract mature male fish, which fertilize the eggs. The fertilized eggs develop on the warm beach during the following days (which have a smaller tidal amplitude) until the larvae can hatch when the next spring tide washes the upper beach again (Walker 1949).

In general, tidal activities of intertidal species are more difficult to observe than semilunar or lunar activities, because most of them take place when the terrain is flooded. Examples of exceptions are the locomotor behaviors of fiddler crabs (*Uca* spp.) and the turbellarian *S. roscoffensis*. The latter harbor symbiotic phototrophic algae in their digestive tracts (which give them their characteristic green color) and expose their bodies to sunlight during the ebbing tide when the sandy habitat becomes exposed; they return into the substrate before the next rising tide arrives.

1.4 Tidal Rhythms

1.4.1 First Laboratory Experiments

During the past 50 years a variety of tidal biorhythms have been studied in selected intertidal species kept under controlled laboratory conditions, in absence of tidal influences and nocturnal moonlight. The first persistent free-running tidal biorhythms recorded were the locomotor activity rhythms of individual fiddler crabs, *Uca* (Bennet et al. 1957), and green shore crabs, *Carcinus* (Naylor 1958).

On the basis of these early results, two opposing interpretations were put forward to explain the free-running tidal rhythms: direct influence of weak geophysical influences of the moon (possibly modulated at least in the laboratory) or overt rhythms of an endogenous clockwork, which would (in the field) be controlled by environmental time cues analogous to circadian biorhythms. Using an actograph, Naylor (1960) recorded ‘dock crabs’ from nontidal docks and ‘shore crabs’ (*Carcinus maenas*) from the intertidal zone. The ‘dock crabs’ [kept under 24-h light–dark (LD) cycles] showed mainly nocturnal activity peaks, but in continuous bright or dim light the activity peaks drifted with a period of about 12–13 h for 5 to 6 days. In nontidal aquaria the initial strong tidal rhythm of the ‘shore crabs’ became less and less pronounced until after 4–8 weeks they behaved quite the same as the ‘dock crabs’ when put in continuous conditions. Naylor concluded that the similar daily drift of the rhythm in both ‘shore crabs’ and ‘dock crabs,’ irrespective of the original tidal or nontidal habitat and of the entrained behavior, reflects an endogenous tidal rhythmicity. In the discussion of a paper on tidal rhythmicity in marine organisms by Fingerman at the now-famous Cold Spring Harbor Symposium of 1960, Enright presented the clear-cut locomotor activity rhythm of groups of the sand-beach amphipod *Synchelidium* from a Californian beach where mixed semidiurnal tides occur and successive tides on any given day can differ considerably in amplitude and period (see Fingerman 1960, discussion). His activity recordings of freshly caught specimens, made at 15- or 30-min intervals under constant conditions (Fig. 1.1), reflected both amplitude and timing of the actual local tide pattern but with a significantly longer period than that of the tidal cycle in the field; the rhythm persisted for the following days until damping. In this context, Enright was presumably the first to use the term “circatidal” for such a free-running tidal pattern, ‘by analogy with circadian’ (and thus opposing speculations on a direct influence of any weak geophysical influences) and ‘in contrast with previous reports of long continued precision of tidal rhythms in other organisms’ (these reports not being confirmed in later studies).

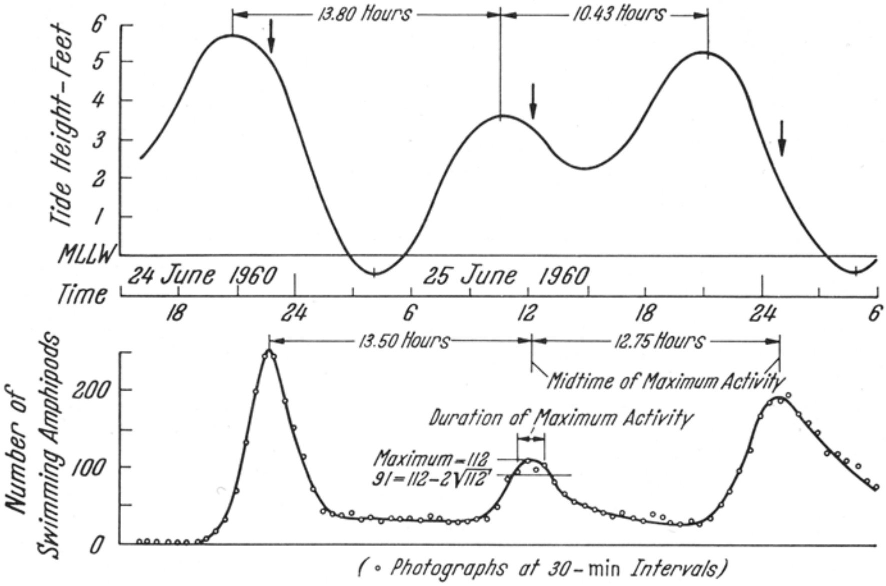

Fig. 1.1 Experiment under constant light and temperature conditions demonstrating the ‘circatidal’ rhythm of freshly collected amphipods (*Synchelidium* sp.) from a Californian sand beach characterized by mixed semidiurnal tides (*upper graph*). Counts of locomotory activity at 30-min intervals (*lower graph*). The height of the activity peaks mirrored the tidal pattern of that day, but by a longer period of 26.25 h, which was then persistent for some weeks. (Data from Enright 1963, Fig. 1; reproduced by permission of Springer-Verlag, Berlin)

1.4.2 Free-Running Rhythms and Entrainment Experiments

Various recording devices were invented for continuous registration of the tidal behavioral activities of groups or of single specimens of marine organisms (cf. Neumann 1981; Palmer 1995). These devices allowed recordings to be made either in 24-h LD cycles, in constant light (LL), or in time schedules of simulated tidal factors as mechanical disturbances of the seawater, changes of hydrostatic pressure or temperature, etc. A high individual variability of the actograms was frequently observed for single specimens: the trend of free-running locomotor rhythms of about 12.5–13 h was superimposed by shorter or longer activity bursts (see Fig. 1.2). Some of the captured specimens did not show any rhythmic regularity. Palmer (1995) presented an overview of all these tide-associated studies, including a compilation of methods for time-series analysis and results on phase setting and entrainment to physical factors. Two different interpretations on these free-running rhythms with regard to an underlying, clearly endogenous pacemaker were put forward: on the one hand, the existence of a *circatidal oscillator* of approximately 12.5-h period, and on the other hand a quasi *circabitidal* 25-h *oscillator*[1], which can

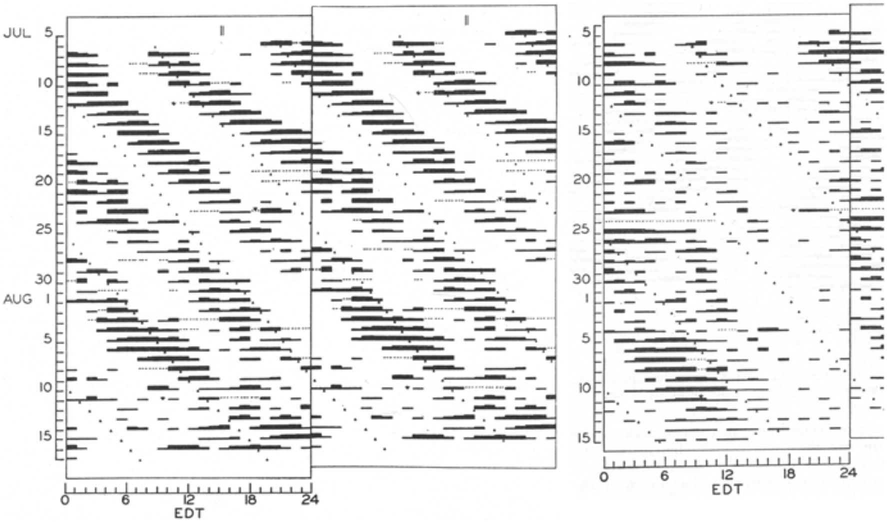

Fig. 1.2 Locomotory activity of two fiddler crabs (*Uca minax*; collected at the Chapoquoit marsh near Woods Hole, MA, USA) in continuous dim light (below 10 lx). The *small points* indicate the times of high tide at the beach. The actograph tracings (*left*: plotted twice) show the duration of activity for each hour, measured as 0, 25, 50, 75, or 100 % of that interval. (Data from Barnwell 1966, Figs. 4 and 5)

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[1] The synonymous term 'lunidian' is avoided in this overview. It correlates these rhythms with the lunar-day rhythm corresponding with the 24.8-h period between two consecutive culminations of the moon, which are the basic cause of the 12.4-h cycle of the tide-producing forces. However, all rhythms of intertidal organisms are primarily responses to the phases of the local tides with all their local modulations. The parallelism with the culmination period of the moon is of secondary importance.

occur in about 180° antiphase with a second one, as observed on coastlines with mixed semidiurnal tides (see Fig. 1.1).

The results of two additional experiments support the existence of an autonomous circatidal pacemaker as seen in green shore crabs (*C. maenas*). Williams and Naylor (1967) reared *Carcinus* larvae to the crab stage in a 24-h LD cycle with no experience of tides; when first subjected to continuous, dim red light they showed a free-running locomotor rhythm of 24–25 h with a peak during subjective night (circadian response), and after an additional chilling period (4 °C for 15 h), a free-running circatidal period of 12–13 h that indicated an inherited period. Reid and Naylor (1989) collected *Carcinus* crabs in winter when they exhibited no overt circatidal rhythmicity in the laboratory; by exposure to hypo-osmotic seawater (7 ppt) during dusk as well as dawn, circatidal rhythms were initiated.

However, two essential characteristics of circatidal actograms differ from those of circadian rhythms in many terrestrial animals: (1) the free-running rhythms often faded after a few days, and (2) additional short phases of both activity and rest occurred in an addition to the dominating circatidal activity pattern and were regarded as ‘noisy’ or ‘ultradian’ activity bursts. Concerning the first characteristic, Naylor hypothesized a multiple clock model, that is, a number of circatidal oscillators that drift out of phase relatively quickly—a hypothesis that cannot be tested without integrated neurophysiological studies. As to the second point, the quasi-stochastic activity bursts may also document inadequate actograph conditions that are too different from the natural habitat, or a specific readiness for direct responses to disturbances by irregularly occurring wind and wave action on the shore (see Sect. 1.4.4).

1.4.3 Further Challenging Studies on Circatidal Pacemakers

Oscillator-driven rhythms such as self-sustained circadian rhythms are characterized (a) by temperature independence of the free-running period, (b) by a restricted range of entrainment to periodic zeitgeber cycles, (c) by phase control with advances or delays in response to effective environmental time cues, and (d) by different effects of strong and weak external time cues (Hoffmann 1969). Some of these properties have already been analyzed in some of the circatidal studies (compare studies cited in Neumann 1981; Naylor 2010). However, the experimental simulation of tidal forces and their temporal patterns is far more difficult than the simulation of 24-h day–night conditions. In general, the previous actograph conditions for individuals or groups of intertidal organisms were often rather simplified and deviated strongly from the species-specific habitat conditions with regard to flooding and sites for feeding and shelter. A radioactive marker technique within small seawater basins with simulated tidal water change was successfully tested in fiddler crabs (Lehmann et al. 1974), so that animals could be studied in a close-to-natural environment without noticeable intervention by the experimenter or data-collecting devices. In any case, it is neither necessary to assume that all intertidal organisms evolved one and the same circatidal (or circabitidal) timing mechanism, nor do these timing mechanisms need to be directly derived from the circadian clock and its genetic inventory.

In conclusion, the free-running records reveal a wide range of unsolved problems. Solving these will require supplementary records of activity and rest within the natural habitat (Green 1970), differentiation between strong and weak times cues for entrainment (Hoffmann 1969, for circadian rhythms), localization of the pacemaker (together with molecular genetic tests), and last but not least, analysis of the perception of tidal time cues.

1.4.4 Noncircatidal Timing Principles

In a study on two fiddler crab species, clear-cut tidal activity rhythms could be evoked under natural-like laboratory conditions (see foregoing) and recorded using a radiomarker technique (Lehmann et al. 1974). These activity rhythms were not subjected to daily modulation when a 24-h LD cycle was added. But under constant conditions, the crabs showed non-circatidal bursts of activity (as already documented by Barnwell (1966) (Fig. 1.2, right panel) and mentioned by many other authors). Neither a preferred duration of activities nor of rest was observable in actograms; the frequency distribution of the durations of both phases could be fitted by negative exponential functions. No significant correlation between one burst and its following rest was found; in other words, the change from an activity burst to rest and from rest to activity was described by a time-independent probability resulting in a stochastic pattern. Under the simulated tidal conditions the frequency distribution of the activity bursts remained unchanged, but the resting periods were lengthened to periods of 7–11 h. The resulting activity ‘pattern’ was fairly well correlated with the simulated tides, but the correlation was only based on lengthening the resting periods.

An example of tidal hourglass timing was discovered in an artic intertidal population of the 1-h midge *C. marinus*, which reproduces twice a day during midsummer when the range of its sandy beach habitat is exposed and warms up by a few degrees. Although no free-running circatidal rhythms were observed in substrate samples from the beach in the laboratory, in newly bred cultures a small temperature increase resulted in a simultaneous sharp eclosion peak by those pupae that were already pharate (mature) for eclosion and immediate swarming. The peak was followed by a second one about 13 h later, before an irregular eclosion pattern reoccurred after about 18 h. Thus, the tidal rhythm in the field must result from the coincidence of the single 13-h programming (hourglass type) during the preceding ebb and a direct response triggered by the increasing temperature pulse of the actual ebb (Pflüger 1973).

1.5 Circasemilunar and Circalunar Rhythms

1.5.1 First Laboratory Breeding Experiments

Hauenschild (1960) was the first to succeed in demonstrating, in a small-scale experiment, a free-running lunar reproduction rhythm in a laboratory stock of the semelparous Mediterranean polychaete *Platynereis dumerilii*. By superimposing a regular 24-h LD cycle with 5–7 successive nights of simulated moonlight (or bright light) every 30 days, he induced an accumulation of reproducing specimens during certain periods of this artificial moonlight cycle. It was the first evidence of an endogenous, oscillating circalunar clock that controls the development of maturing individuals via neurosecretory cells of the brain. In the years following his experiment it was repeatedly discussed whether one moonlit night would be enough to induce an effect, and what happens in the field when the moonlight zeitgeber signal is disturbed by dense cloud cover. Franke (1985) experimented with the iteroparous Mediterranean polychaete *Typosyllis prolifera* in which the lunar timing can be analyzed not only in entrained groups as in *Platynereis dumerilii*, but in each laboratory reared individual because of the recurring lunar stolonization (up to 18 times). In this context, stolonization is the transformation of the hind part of the body into a specialized reproductive individual (*stolon*) that detaches from the rest of the body for reproduction. Franke showed that for the synchronization of stolonization one or two nights of artificial moonlight is not enough to be effective; additional stimulating nights are needed to induce entrained lunar rhythms (Fig. 1.3). The phase of

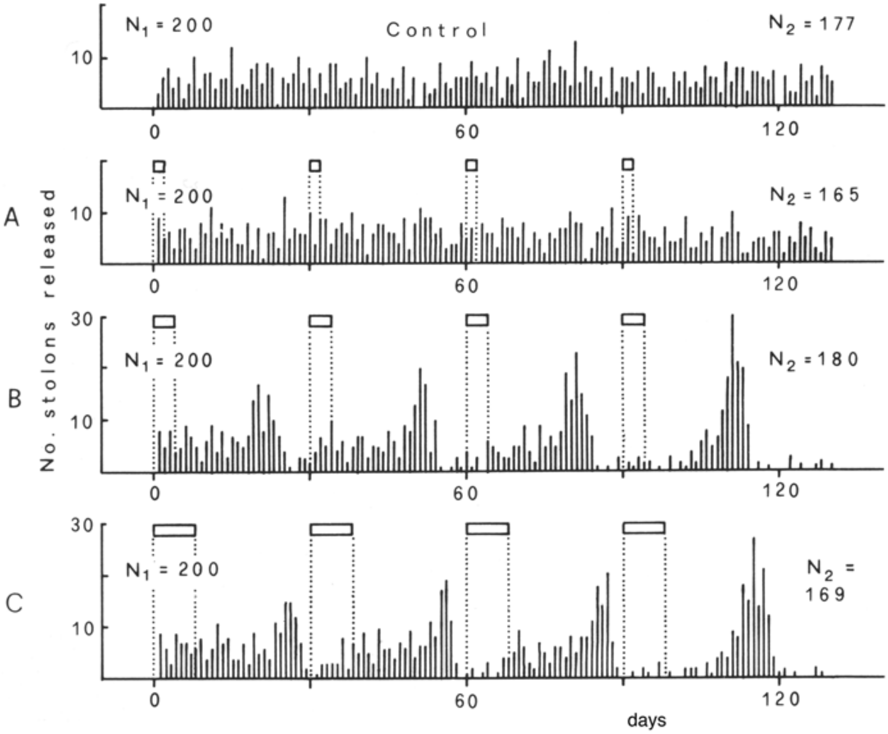

Fig. 1.3 Stolonization, that is, transformation of the hind part of the body into a specialized reproductive individual (*stolon*), which detaches from the rest of the body for reproduction, in laboratory cultures of the polychaete *Typosyllis prolifera* in a 30-day cyclic illumination program with 2–8 successive nights of 0.3–0.5 lx (*horizontal bars*) in initially asynchronous cultures (further conditions, LD 16:8 at 20 °C). (Data from Franke 1985, Fig. 3; reproduced by permission of Springer-Verlag, Berlin)

these rhythms was correlated with the last moonlight night of the laboratory 30-day cycle. Therefore, some cloudiness during the night should be unimportant, particularly if the nocturnal light sensitivity is very high.

1.5.2 Free-Running Lunar Rhythms

Further studies on the lunar timing of reproduction events were carried out on the brown algae *Dictyota* (Bünning 1973) and on the 1-h midge *Clunio* (Neumann 1966). The latter turned out to be particularly suited for further laboratory analyses of the properties of both semilunar and lunar rhythms. Strong swarming rhythms of this chironomid insect could be evoked in cultures (dishes of 20 cm diameter with up to about 400 larvae of mixed age structure) by artificial moonlight zeitgeber cycles (mostly four successive nights with about 0.3 lx every 30 days in an otherwise 24-h LD cycle, and this independently of the natural synodic month of the moon; compare Fig. 1.4, left). When cultures synchronized in this way were transferred into continuous conditions of 24-h LD cycles, free-running rhythms were observed for at least 6–8 weeks. In the case of the Normandy stock, the period was 13–14 days, and for a stock from the Basque Coast at the Biscaya Bay it was 26–27 days, corresponding to the semilunar, respective lunar rhythms of their populations in the field (Neumann 1966). In other words, the period of this long-term rhythm is a population-specific property. In the field, it is correlated with the days of spring tides, in the case of

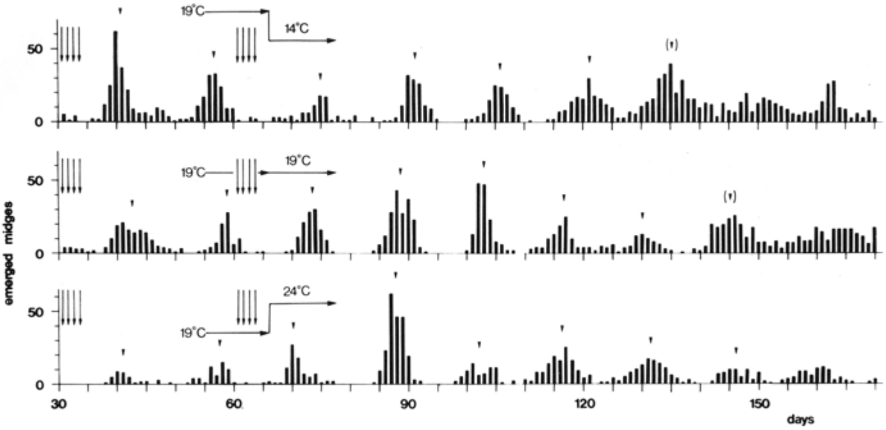

Fig. 1.4 Free-running semilunar emergence rhythms of males in laboratory cultures of the 1-h midge *Clunio tsushimensis* at three different temperatures during light–dark (LD) 12:12 conditions. The semilunar synchronization of the three groups occurred by 30-day illumination cycles of LD 12:12 at 19 °C with artificial moonlight (0.3 lx, *downward arrows*) during nights 1–4 (emergence not plotted until day 31) and nights 31–34 and 61–64. On day 66, groups 1 (*top*) and 3 (*bottom*) were transferred to different temperatures (14 and 24 °C, respectively). *Arrowheads* represent medians of the individual emergence peaks. (Data from Neumann 1988, Fig. 1; reproduced by permission of Springer-Verlag, Berlin)

Normandy with each spring tide during summer around the times of full and new moons (Chevrel 1894), and at the Basque Coast only with new moon spring tides (Neumann 1966). In unsynchronized *Clunio* cultures these free-running lunar rhythms could be also induced by a single treatment with three or four successive nights of 0.3 lx, that is, with no information on any semimonthly or monthly lunar periods, confirming the autonomous physiological property of the period length.

The true nature of the period component was tested in various ways. Three possibilities were considered, as discussed by Bünning (1973): the free-running period could (a) really correspond with a long-term oscillator, or (b) be related to a circadian clock combined with a physiological counter mechanism for about 15 or 30 day–night cycles, or (c) depend on the coincidence of a circadian clock and a circatidal (or circabitidal) clock every 15 (or 30) days ('beat hypothesis'). Corresponding breeding experiments were performed on *Clunio* stocks. Neither in continuous darkness (Neumann 1988) nor in any other sophisticatedly combined LD cycles (Neumann 1976) were the periods of the *Clunio* rhythms disturbed, so that one can conclude that at least the circasemilunar (or circalunar) reproduction rhythm of *Clunio* is characterized by a real long-term cycle as a self-sustained pacemaker with some properties analogous to those of the circadian clock.

1.5.3 Temperature Compensation of the Free-Running Period

Temperature compensation of a reliable timing system is a decisive property of the circadian oscillator and its entrained, overt physiological rhythms. In the case of semilunar rhythms it was tested over an ecophysiologically reasonable temperature range of 14–24 °C in a series of experiments with the subtropical species *Clunio tsushimensis*, which displays a rather clear-cut semilunar rhythm of eclosion in the laboratory (Fig. 1.4). The temperature independence of the free-running period was not significantly different from a Q_{10} of 1.0 (range, 1.03–1.18). In other words, the period of the endogenous circasemilunar clock of *Clunio* that finally triggers the days of reproduction is also temperature compensated. Corresponding results were noticed in the lunar rhythm of *Typosyllis* ($Q_{10} = 1.04$) (Franke 1985).

One has to consider that developmental processes are involved in free-running circasemilunar and circalunar rhythms. Therefore the question arises as to what stage of development represents a physiological 'waiting phase' that is triggered by the endogenous pacemaker, that is, a stage just before the reproduction event or one that perhaps occurs up to a few weeks before. In *Typosyllis* this waiting phase seems to occur directly before the stolonization (Franke 1985), but in *Clunio* this phase already happens about 18 days before as a 'switching point' (Neumann and Spindler 1991). In synchronized *Clunio* cohorts, this physiological 'waiting stage' for the slowly growing larvae occurs at the beginning of the last larval instar, LIV (*Clunio* is characterized by four larval instars, as is true for any other chironomid insect; the developmental time of the first three instars can vary between about 2 and 4 months, or even considerably more when a winter diapause occurs). The imaginal disc development of the final metamorphosis to an adult midge starts with the molting to instar LIV and is easily observable through the transparent larval cuticle (see Neumann and Spindler 1991; Fig. 1.1). In synchronized cultures both growth and further differentiation stop at an early stage in LIV (stages two and three of a total of nine stages until pupation), so that larvae of similar physiological stage accumulate in number. From that point, growth and metamorphosis start again in synchronized cohorts every 15 or 30 days (depending on the physiological race of the respective *Clunio* stock), which is correlated with a short increase in ecdyson titer (Neumann and Spindler 1991). In *C. marinus* (Helgoland stock), this final development before the mature (pharate) pupae is largely independent of temperature (tested here at 11 and 19 °C). Thus, the temperature independence of the free-running rhythm not only reflects the period of the circasemilunar clock but simultaneously demonstrates the temperature-compensated developmental process of final metamorphosis before eclosion (Neumann and Spindler 1991). The final period of metamorphosis until pupation required about 14 days in circasemilunar stocks (Neumann and Spindler 1991; Fig. 1.5) and about 20 days in circalunar stocks (stock Vigo/Spain, Neumann, unpublished data). Thus, the lunar periodically synchronized metamorphosis of *Clunio* larvae draws attention to a previously overlooked phenomenon that may occur in ectothermic animals. How such temperature independence of developmental processes could be endocrinologically controlled is one of the open questions in the field of semilunar and lunar rhythms.

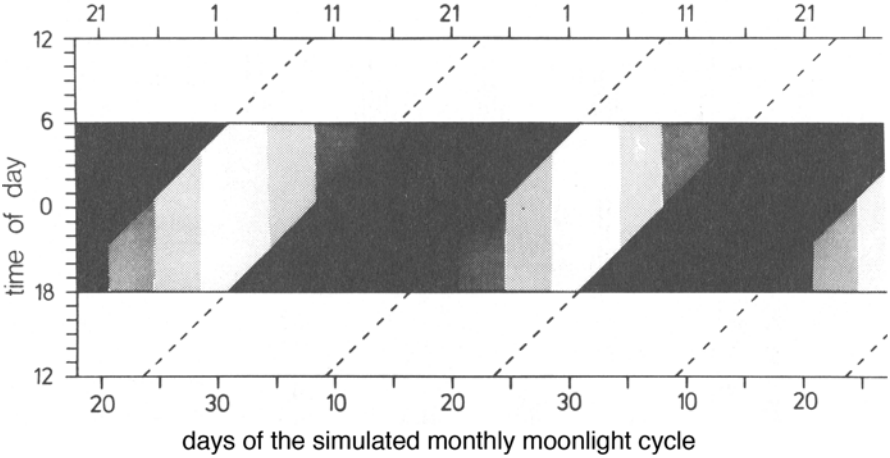

Fig. 1.5 An artificial moonlight regime simulating the 24.8-h period of the lunar day as well as the changing light intensity. The lunar-day period was triggered by a modified synchronous motor-driven 24-h clock; the intensity of small fluorescent tubes was damped stepwise by different *grey* filters to values ranging from 0.3 lx to about 0.07 and about 0.007 lx. (Schedule from Neumann 1985, Fig. 5; reproduced by permission of Springer-Verlag, Berlin)

1.5.4 The Perception of Nocturnal Light as Zeitgeber

The experimental verification that roughly simulated moonlight cycles can entrain these long-term rhythms calls attention to two open issues. (1) In nature, effective moonlight may occur not only during days of full moon but even on many days between the quarters of the crescent and waning moon; furthermore, the effective exposure to moonlight depends on the phases of the tides, with a strong reduction during flooding. (2) The perception of nocturnal light requires a photoreceptor mechanism that should not be disturbed by daylight.

The first problem was examined in a stock of *C. tsushimensis* with the aid of an artificial moonlight program that roughly imitates natural moonlight (Fig. 1.5; Neumann 1985). It simulates the daily 0.8-h shift of moonrise and moonset as well the increasing and decreasing nocturnal light intensity between the quarters of the moon. To further simulate the effect of the tides, the moonlight-like program can be modified by reducing the moonlit time to the hours when the midlittoral would effectively be exposed during low tide, resulting in a very narrow gate of moonlight exposure. Depending on the phase relationship of the local semidiurnal tides to the lunar cycle, the gate may be an early one (in this case, the nocturnal light program was switched on for 3 h just after 'moonrise'), or a late one (3 h per day just before 'moonset'), or it was set in the middle between moonrise and moonset. The results are documented in Fig. 1.6. When comparing the effect of 'four nights of 0.3 lx' with 'simulated moonlight', one can state that (1) the semilunar synchronization was hardly more concentrated in the simulated nocturnal light schedule, and (2) the light stimuli of both series produced the same phase relationship of the entrained rhythm. The other three programs with a reduced gate of simulated moonlight showed no better synchronization of the adult emergence rhythm; obviously the light stimuli were less optimal here. However, there was a clear influence on the phase relationship of the entrained rhythms: a significant advance in the 'early gate' program and a delay in the 'late gate' program, meaning that the end of these tidally modulated dim light programs determines the phase of the entrained rhythm, as in the case of *Typosyllis*.

Two conclusions could be drawn for the evaluation of entrained semilunar rhythms in the laboratory and in intertidal zones: (a) the daily shift of natural moonlight seems to be of no relevance, and (b) the time schedule of the local tides modulates the effectiveness of moonlight and must have influenced the selected phase relationship between biorhythm and time cue conditions. Comparing different *Clunio* locations along the coastlines where the semidiurnal tides are phase shifted (e.g., Atlantic coast of France and the coast of the English Channel), one has to expect a correcting parameter in the timing mechanism which compensates the phasing effect in the semilunar (or lunar) timing mechanism, so that swarming can occur at each location around the days of the spring tides (Neumann 1985; Kaiser et al. 2011). These evolutionary modifications are treated in detail in Chap. 7.

The second open issue concerns the photoreceptor dilemma: a moonlight receptor system must not be disturbed by sunlight. In experiments on *C. tsushimensis* in

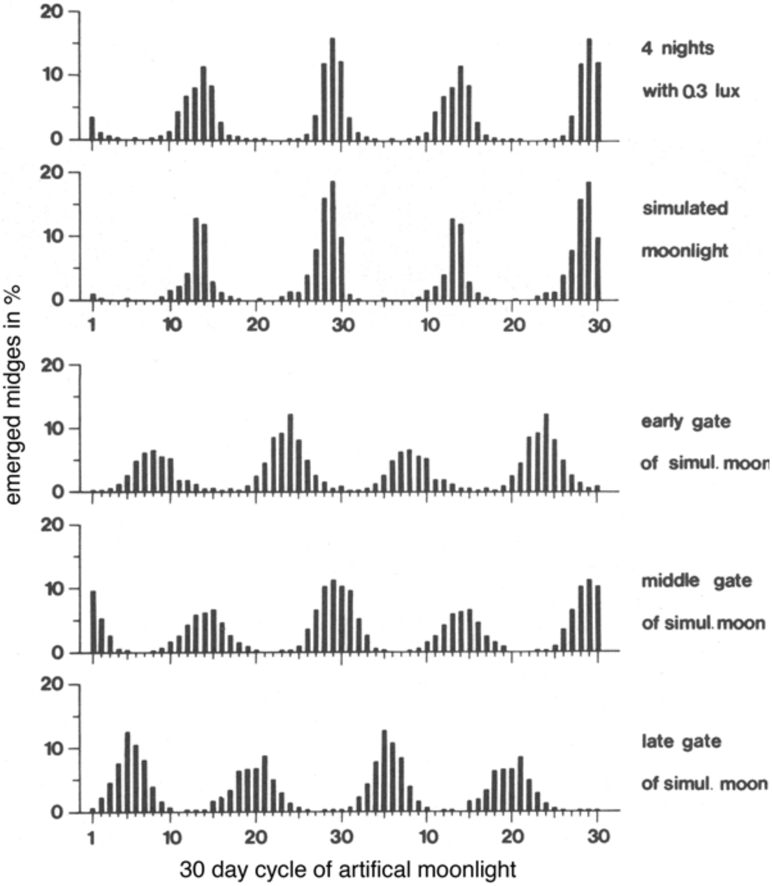

Fig. 1.6 Entrainment experiments with laboratory cultures of *Clunio tsushimensis* in different artificial moonlight programs. *First panel*: Standard laboratory protocol with four nights of artificial moonlight (constant intensity of 0.3 lx). *Second panel*: Simulated natural moonlight regime as shown in Fig. 1.5. *Panels 3–5*: Simulated natural moonlight regime plus additional superposition effect with an semidiurnal tidal regime restricting the changing moonlight intensity to a smaller gate of moonlight exposure, as it occurs in a midlittoral habitat; for details see text. The percentages of emerged midges are summation results of five cultures of three 30-day cycles; they were plotted twice for better visual comparison. The sample size in each experiment was some thousands. (Data from Neumann 1985, Fig. 6; reproduced by permission of Springer-Verlag, Berlin)

which the cultures were exposed to a full-light fluorescent tube for four consecutive nights every 30 days (1,000 lx instead of the 0.3-lx artificial moonlight), the lunar entrainment was even improved. Thus, a "weak-light" receptor for nightly moonlight need not be discussed. As moonlight is simply reflected sunlight and is thus only slighted modified in the range of visible light, it is not possible to distinguish between sunlight and moonlight by wavelength, so that moonlight perception as time cue requires particular physiological properties.

In an experimental series with various *Clunio* stocks that were exposed to different LD cycles (LD 10:10, LD 11:11, LD 12:12, and LD 15:15), the entrainment by the standard 30-day moonlight program turned out well in LD cycles of 22 and 24 h only; in those with shorter or longer LD periods, no semilunar (or lunar) synchronization occurred (Neumann 1989). This finding demonstrates that LD cycles of close to 24 h act as a sort of second time cue condition; in other words, the perception of nocturnal light seems to be embedded in a circadian organization. Outside the range of entrainment of the circadian clock, no synchronization can be caused by moonlight.

In a next experimental series, artificial moonlight was supplied at different times of the day every 30 days over a 4-day period of total darkness (Fig. 1.7). Only ‘moonlight’ presented during the hours of ‘subjective midnight’ was effective. Thus, a circadian clock function must be involved for the perception of the entraining moonlight stimulus. This experiment gives information on the properties of a putative moonlight receptor and thus offers a starting point for an analysis of the photosensory physiology of moonlight perception.

In *Clunio*, studies on nonvisual photoreceptors and other specializations in the larval ocelli have already begun. On the one hand, extraretinal putative photoreceptors cells have been found using both immunohistological as well as ultrastructural methods (with antibodies against ciliary opsins in the larval instars LIII and LIV, which stain cells in the optic lobes, in the intestinal wall, in abdominal ganglia, and below the intersegmental hypodermis; Fleissner et al. 2008a). On the other hand, the larval ocelli show a lunar rhythmic change of shielding pigment transparency so that these photoreceptor cells may function as very sensitive photometers (Fleissner et al. 2008b; Falkenberg et al. 2013). However, no circadian changes were observed in either the extraretinal cells or in the larval ocelli cells, so that a central neural filtering (Nilsson 2009) may be involved in the circadian-modulated perception of moonlight.

1.5.5 Geographic Adaptation: A Tidal Factor as Zeitgeber

Moonlight cannot be a reliable and dependable time cue during summer months in northern areas of the temperate zone because the duration of the night is relatively short and the northern horizon remains relatively bright. Despite this fact, *Clunio* populations display a clear lunar periodicity even in such geographic regions (e.g., Helgoland/North Sea, latitude 54°N; Bergen/Norway, 60°N). In agreement with this environmental situation, *Clunio marinus* stocks from Helgoland (54°N) and Bergen (60°N) could not be entrained by the artificial moonlight cycles. However, a clear-cut semilunar synchronization could be achieved in the Helgoland stock when an artificial 12.4-h tidal cycle was used in combination with the 24-h LD cycle (Fig. 1.8). In the case of the Helgoland stock, the effective tidal stimulus was a mechanical disturbance that lasted continuously for 6 h and which simulated both turbulence and underwater vibrations caused by the surf of the rising flood, with a frequency spectrum of 100–200 Hz and 20–30 dB over the background noise level (Neumann 1978). In this tidal schedule the *Clunio* larvae were exposed to the same

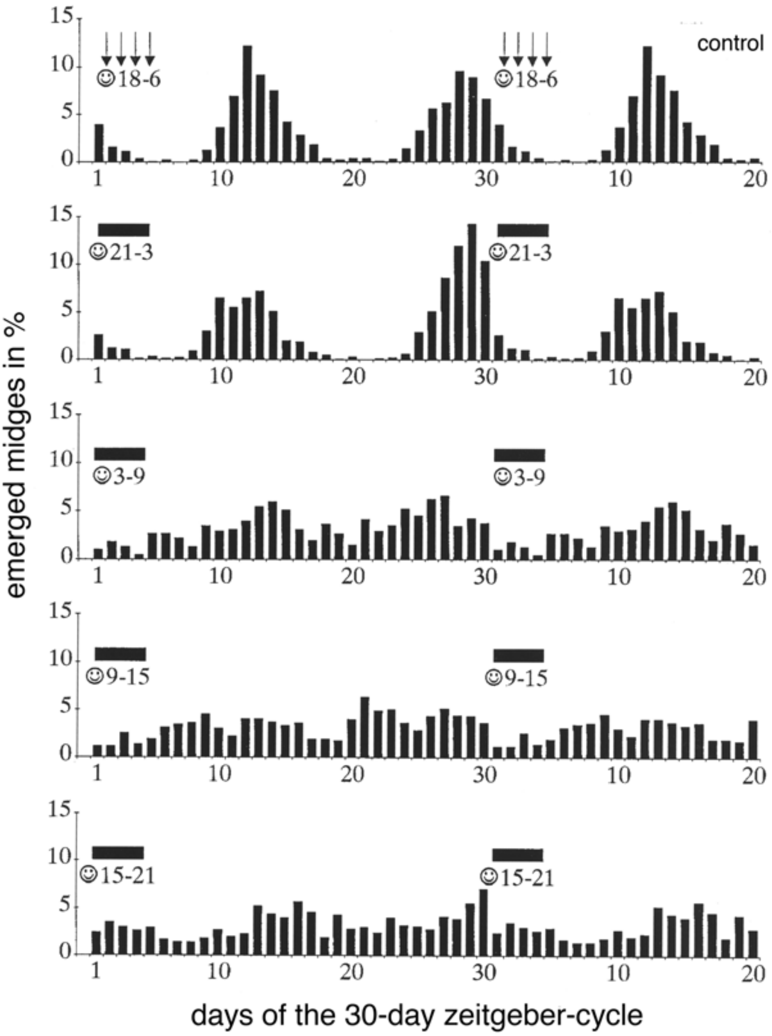

Fig. 1.7 Test on limited nocturnal light sensitivity of *Clunio* larvae (*tsushimensis* stock), as observed in the eclosion of the adults. *Above*: Control in standard program in 24-h LD cycles (*light* 6–18, *darkness* 18–6) and artificial moonlight (see *arrows*). *Below*: Four experiments with 4 days of darkness every 30 days (see *black bar*), but artificial moonlight at different daytimes during the second to fourth 'subjective night' only. Pretreatment in each series: 3 months with the identical program. *Result*: Semilunar entrainment in the second series with moonlight during 21–3 only. (Data from Neumann 1995, Fig. 6; reproduced by permission of Springer-Verlag, Berlin)

daylength and to the same number of hours of mechanical stimulation by tidal turbulence cycles each day, but both conditions had the same phase relationship only every 15 days. The range of 1 of these 15 phase relationships (recurring every 15 days) offers the effective time cue condition of the circasemilunar clock. The Bergen population is localized in sheltered fjords with relatively small tidal

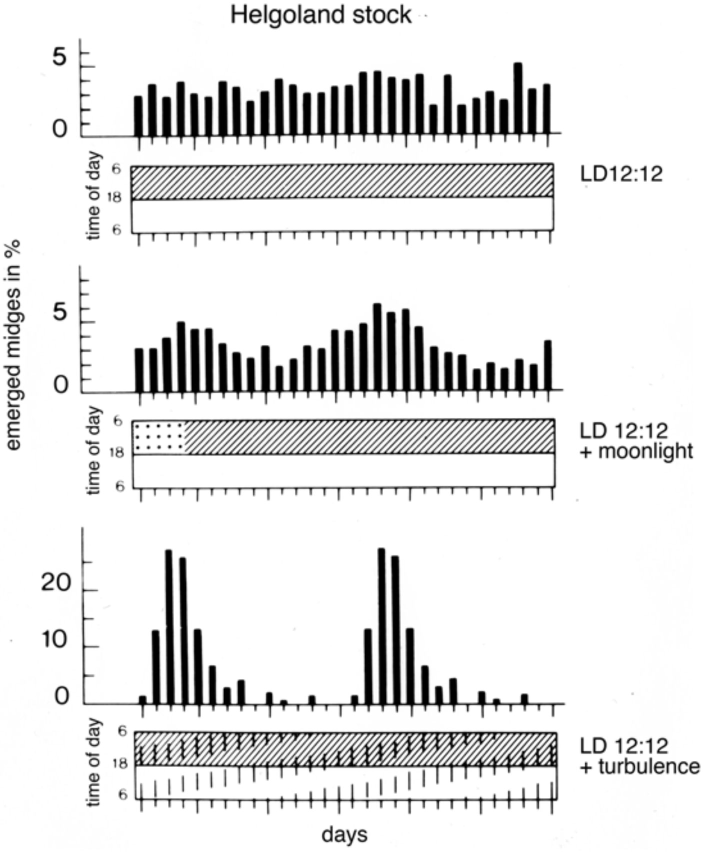

Fig. 1.8 Semilunar entrainment experiments with a northern stock of *Clunio marinus* (Helgoland/North Sea) by artificial moonlight and a simulated cycle of 12.4 h of water disturbance in LD 12:12 (see time schedule below). In the *bottom panel*, *vertical lines* mark the time of "turbulence" in the simulated tidal cycle of water disturbances and its phase relationship with the 24-h LD cycle. (From Neumann 1985, Fig. 3; reproduced by permission of Springer-Verlag, Berlin)

amplitudes and a weak surf; its stock could be entrained by 12.4-h tidal temperature cycles combined with 24-h LD cycles (the amplitude of the temperature cycle was 3–5 °C, which simulated tidal temperature changes of as much as 2 °C during calm weather in the field (Neumann and Heimbach 1984). The effectiveness of these tidal stimuli is also limited by a narrow range of circadian entrainment to LD cycles between 22 and 26 h, so that also here the time cue perception is based on a circadian organization of the *Clunio* larvae (Neumann and Heimbach 1985). Thus, while southern populations of *Clunio* rely on moonlight for entrainment of their circasemilunar timing mechanisms, northern populations (in the absence of reliable moonlight cues in summer) use other time cues for synchronization. This observation demonstrates a perfect adaptation of the circasemilunar timing mechanism and its endogenous semilunar clockwork to reliable time cue conditions.

1.5.6 Daily Timing of Lunar Rhythms

In general, the reproduction time in semilunar and lunar rhythms is not only correlated with the days of spring or neap tides, but also with the time of day when the optimal conditions for swarming or egg deposition occur, particularly at locations with large tidal amplitudes (e.g., at locations where palolo, grunion, and *Cluni*o are found). As outlined in the beginning (Sect. 1.2.1), the time of day of a particular tidal phase on a particular day of the lunar cycle (e.g., the ‘time of low tide on a spring tide day’) can vary along the coastline in response to the influence of coastline geography on the tidal currents. Thus, the optimal ecological situations for semilunar (or lunar) reproduction times occur at different times of day at different locations. Correspondingly different physiological races can be expected to exist and were detected along the coasts of France and southern England. In *C. marinus* populations, the daily timing of eclosion could be identified as a gene-controlled circadian timing mechanism (Neumann 1967) (see Fig. 1.9). The ongoing analysis of daily and lunar phases of the timing mechanisms in various *Clunio* populations is discussed in Chap. 7.

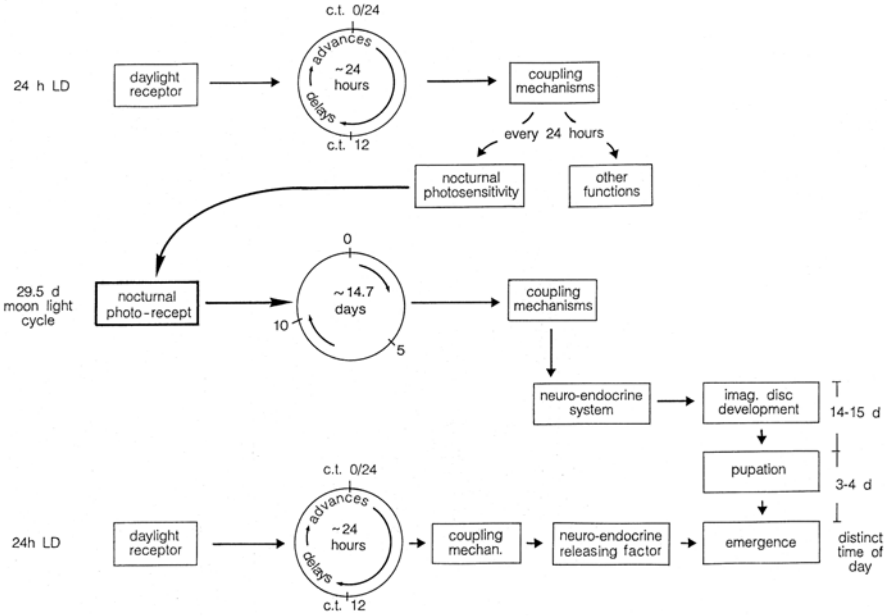

Fig. 1.9 Schematic representation of the physiological components of the semilunar timing mechanism of the intertidal 1-h midge *Clunio* synchronized by 24-h LD cycles and 29.5-day moonlight cycles. *Above*: Circadian clock for the control of nocturnal photosensitivity for perception of moonlight (time cue: 24-h LD). *Middle*: Circasemilunar clock (time cue: nocturnal light of some hours on few days every 30 days) for control of the switching point of the imaginal disc development in early larval stage IV Circadian clock for control of time of day of eclosion in pharate images (*c.t.* times of the circadian oscillator for phases of ‘advances’ and ‘delays’). (Scheme from Neumann 1995, Fig. 7, English version; reproduced by permission of Springer-Verlag, Berlin)

A particular case of daily timing combined with a lunar rhythm occurs in the species *Clunio mediterraneus*. The tidal amplitudes of the European Mediterranean Sea are relatively low, so that spring low-water conditions can be reinforced by continuous offshore wind, and in contrast strongly reduced by onshore wind. In *C. mediterraneus* (Rovinj population), the time of eclosion and swarming correlates with the spring low water in the early morning. This is the time when the tidal amplitude is reinforced by offshore wind in the summer; only then is the upper sublittoral range (which is the most suitable larval habitat) exposed, permitting egg deposition.

1.5.7 Circalunar Clock as Central Component of a Complex Timing Mechanism

The main physiological components of a semilunar periodic timing mechanism as established in the 1-h midge *Clunio* are summarized in Fig. 1.9. The results of the experiments indicate that the circasemilunar (or circalunar) timing during a developmental switching point is coupled to the circadian organization of the organism in two ways. First, nocturnal photosensitivity to moonlight (and potentially sensitivity to any other time cue of the semidiurnal tidal cycle) is under circadian control. Second, daily eclosion time is tuned to the ecologically proper tidal situation, which reoccurs every 15 or 30 days. It may be expected that a corresponding scheme may be also valid for other semilunar or lunar rhythms of species of the intertidal zone.

Particularly important in any such timing mechanism are the components responsible for the ecologically suitable phase relationship of the overt rhythm with the environmental cycles. The general circadian organization seems to be extremely important: (1) in case of the perception of the complex zeitgeber conditions (day–night cycle, moonlight, or tidal factors such as tidally fluctuating turbulence or temperature), (2) regarding the gene-controlled and evolutionarily selected phase relationship between the physical cycles and the controlled physiological process, and (3) in tuning to different zeitgeber conditions (e.g., from photic to mechanical stimuli). The different geographic races arise from genetically different versions of the timing mechanism and offer possibilities of determining timing parameters by genetic analysis (see Chap. 7 by Kaiser, this volume).

Concluding Remarks

Experimental studies on the physiological components of tidal and lunar timing mechanisms are still underway. During the past few decades, important physiological components and properties of endogenous ‘clockworks’ have been revealed through long-term registration of locomotor behavior (in the case of circatidal timing) and by breeding experiments with polychaetes and an intertidal insect and its local physiological races (in the case of lunar rhythms). Many new questions have arisen, such as to zeitgeber effectiveness, zeitgeber perception (especially nocturnal moonlight in contrast to daylight), zeitgeber transfer to the respective internal clock, as well as to gene-controlled components of circadian and circalunar phase relationships. It is still difficult to understand those components of the circadian system that are on the one hand tuned to produce a time-invariant, constant output but on the other hand can be subject to selection by environmental cycles (i.e., must have a certain degree of variation). This apparent antagonism not only applies in the context of semilunar and lunar rhythms but represents an unresolved problem in the study of circadian rhythms in general. The perception of stimulus duration in time cue perception and the identity of the pathways connecting different photic time cue receptors are still open issues in receptor physiology. The population genetics of ecophysiological differentiation along various coastlines offer ideal topics of study in the field of evolutionary biology. Today these open questions on endogenous timing processes can be rendered more precisely, which may eventually allow solving the remaining enigmas in biological timing.

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Chapter 2
Circatidal Rhythms and Their Entrainment to the Tidal Cycle in Insects

Aya Satoh and Hideharu Numata

Abstract Some intertidal insects show endogenous activity rhythms that synchronize with the tidal cycle, that is, circatidal rhythms. In this chapter, we collected studies of the circatidal activity rhythms of intertidal insects and the entrainment of the circatidal rhythm to the tidal cycle. Until now, circatidal activity rhythms have been reported in three taxonomic groups: springtails (Collembola), beetles (Coleoptera), and crickets (Orthoptera). The mangrove cricket *Apteronemobius asahinai* is known as the only insect species that shows clear and persistent circatidal activity rhythms. When the inundation cycle with a period of 12.4 h was provided by artificial means, the circatidal rhythm of *A. asahinai* responded to the given periodic inundations in a phase-dependent manner. Furthermore, the molecular mechanism of the circatidal clock of *A. asahinai* is likely to be different from the circadian clock. Advances in studies in *A. asahinai* will lead to better understanding of the circatidal rhythm in insects.

Keywords *Apteronemobius asahinai* • Intertidal insects • Mangrove cricket

2.1 Introduction

The tides flood and ebb twice each day in most coastal regions. This tidal cycle shows a period of approximately 12.4 h. The tidal cycle is an important factor for animals living in the intertidal and sublittoral zones to determine the timing of activity. For many aquatic animals, high tides are the most suitable time of activity. The activity of terrestrial animals in intertidal zones should, however, be strictly limited to the time of low tides, when their living space emerges from the sea.

Then, how do animals determine the "correct" timing of activity? There are two solutions to this problem: by using external environmental cues related to the tidal cycle or by using endogenous biological clocks. For example, fiddler crabs, *Uca uruguayensis*, open their burrows during the periods of simultaneous light and low

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tide and close them at the times of high tide or darkness (De la Iglesia et al. 1994). De la Iglesia et al. (1994) showed that the rhythm of burrow plugging did not show clear endogenous tidal components under experimental conditions, but that crabs plugged their burrows in response to the entrance of water into them. Direct responses to environmental stimuli, however, may lead the organism to be active at the "wrong" time by responding to nontidal events such as rainfall or storm surge. Alternatively, many marine organisms show tidal activity rhythms that persist under constant conditions, that is, circatidal rhythms (Gibson 1965; Barnwell 1966; Enright 1972; Akiyama 1995). Thus, these organisms determine suitable timing for activity using one or more endogenous biological clock(s).

Biological clocks usually show a periodicity that is not exactly the same as the periodicity of the environmental cycle. In the field, the endogenous rhythm is entrained to the environmental cycle; as a result, the period of the rhythm equals the period of the environmental cycle. An environmental stimulus that can act to entrain biological clocks is called a "zeitgeber" (Johnson et al. 2003). Many environmental cues related to tidal cycles have been reported as the zeitgeber for the entrainment of circatidal activity rhythms, such as periodic changes in water turbulence (Enright 1965; Klapow 1972; Ehlinger and Tankersley 2006), hydrostatic pressure (Reid and Naylor 1990; Morgan 1965; Northcott et al. 1991; Akiyama 2004), salinity (Taylor and Naylor 1977), and water temperature (Naylor 1963; Holmström and Morgan 1983).

In this chapter, we review studies of the circatidal activity rhythms of intertidal insects and the entrainment of the circatidal rhythm to the tidal cycle.

2.2 Life of Insects in Marine Environment

Not many but some insects spend at least part of their life cycle in the marine environment, which includes many habitats from the upper intertidal to the open ocean (Cheng 2003). Usually people do not even think that they can meet insects in marine environments. However, actually we can find many taxonomic groups of insects at the seaside, such as springtails (Collembola), crickets (Orthoptera), true bugs (Heteroptera), beetles (Coleoptera), moths (Lepidoptera), flies (Diptera), and ants (Hymenoptera) (Cheng 2003).

Here we introduce some unique lifestyles of marine insects. Larvae of moths are generally herbivorous, but some moth species at the seaside use unique feeding habitats. For example, larvae of *Tatobotys aurantialis* (Crambidae) can be found at the high-water line of spring tide on rocky shores and eat the intertidal red alga *Bostrychia tenella* at night (A. Satoh, personal observation). Another example is the mangrove moth *Hymenoptychis sordida* (Crambidae), the larvae of which feed indiscriminately on decaying wood, leaves, and green algae on mangrove forest floors (Murphy 1990).

Some marine insects live in burrows on tidally inundated ground. These insects have to solve the important problem of how to prevent water from intruding into the burrow and to maintain airspace within the burrow during submergence. The mangrove ant *Polyrhachis sokolova* (Formicidae) nests in the muddy soil of the mangrove forest. For this species, when the tide reaches the nest entrance, loose soil particles at the nest entrances collapse and function as a stopper, which prevents water from intruding into the nest (Nielsen 1997). When the water withdraws, the ants reopen the entrances as soon as they are above the water level, and resume foraging before the soil dries (Nielsen 1997). Similarly, the subsocial beetle *Bledius spectabilis* (Staphylinidae) also has a burrow in the mud in the intertidal zone, which it leaves to eat algae on the surface of mud during low tides. For this beetle, a critical minimum diameter of the burrow entrance (2–3 mm) prevents sudden flooding by exploiting a surface entrance effect, giving beetles time to block the entrance hole with mud (Wyatt 1986). Females of this species lay eggs around the burrow from inside of it, and take care of their young until approximately 1 week old, which is why this beetle is categorized as a 'subsocial' insect. The importance of maternal care is indicated by the fact that orphaned first instars did not block or reopen the burrow in accordance with the state of the tide (Wyatt 1986).

Although all insects in the marine environment, except those in a pelagic habitat, must be influenced by the tidal cycle to some greater or lesser degree, only a few studies have demonstrated circatidal activity rhythms and their entrainment to the tidal cycle for insects. Circatidal activity rhythms have been reported only in the following three taxonomic groups: springtails (Collembola), beetles (Coleoptera), and crickets (Orthoptera).

2.3 Circatidal Rhythms

2.3.1 Springtails

Many species of springtails can be found in the soil of the forest, but they are also found in the marine environment. The salt-marsh collembolan *Anurida maritima* (Neanuridae) is distributed in various types of intertidal habitats, such as tidal marshes and rocky shores, throughout the Holarctic. Foster and Moreton (1981) found that this species becomes active or inactive in accordance with the tidal cycle: a large proportion of the individuals emerge from their underground refuges shortly after the retreat of the tide, forage there for a few hours, and retire underground at least 1 h before the return of the tide. This tidal activity rhythm persisted in the laboratory under constant light for at least 7 days (McMeechan et al. 2000).

2.3.2 Beetles

Beetles are observed rather often in marine environments. The nocturnal beetle *Thalassotrechus barbarae* (Carabidae) inhabits the rocky intertidal zone on the western coastline of the United States (USA) and Mexico (Evans 1976). Adults of this species show circadian rhythms in their locomotor activity under constant conditions; however, they are more active during the period corresponding to low tide than at other times of night. It appears that the circatidal rhythm modifies the predominant circadian rhythm by inhibiting activities during high tides (Evans 1976).

Larvae of the intertidal tiger beetle *Callytron inspecularis* (Cicindelidae) live in well-formed ground burrows at the high-water line of spring tide on muddy estuary riverbanks in East Asia. Usually, larvae wait patiently at their burrow opening for prey to approach within striking distance throughout the day and night. In spring tides of the lunar cycle, however, larval activity is interrupted during high tides because the larval burrows become submerged. In this period, they show a unique rhythmic behavior: They plug their burrow opening with soil before submergence at high tide and open it again soon after the water recedes. Satoh et al. (2006) observed that a larva plugged its burrow periodically under constant conditions in the laboratory for 3 days after being subjected to the artificial tidal cycle (Fig. 2.1). The mean interval between consecutive burrow plugging was 12.45 h (Satoh et al. 2006), suggesting that this burrow-plugging behavior is governed by an endogenous circatidal rhythm. A similar rhythmic plugging behavior was also observed in larvae of the tiger beetle *Callytron yuasai okinawense* on the mangrove forest floor (Satoh and Hayaishi 2007), although it was not confirmed whether this behavior was endogenous. On the other hand, the activity rhythm in adults of these tiger beetle species, which walk over the intertidal flats for foraging and mating, has not yet been studied.

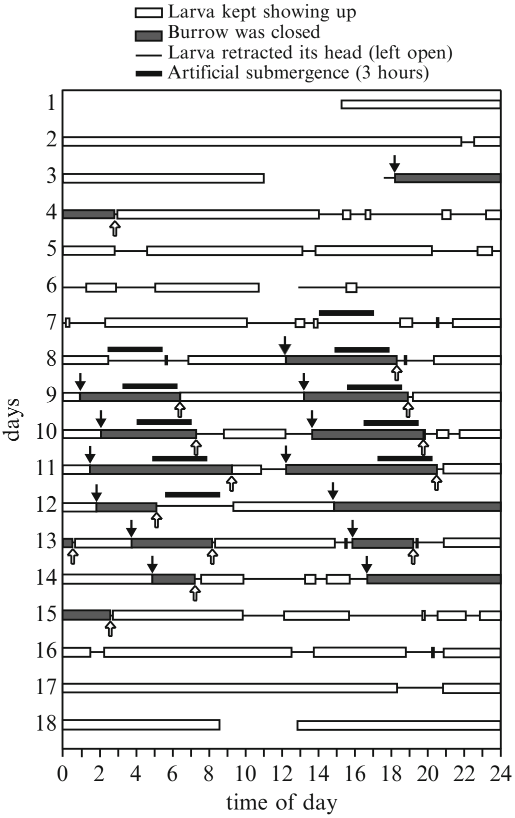

Fig. 2.1 Example of a larval activity record of the tiger beetle *Callytron inspecularis* under constant light at 25 °C. Larval behavior was recorded using time-lapse photography for 18 days. An artificial submergence (3 h) was provided ten times, at intervals of 12.4 h from day 7. *Black arrows* indicate times of burrow plugging; *white arrows* indicate times of burrow opening. (From Satoh et al. 2006)

2.3.3 Crickets

The mangrove cricket *Apteronemobius asahinai* (Trigonidiidae) is known as the only insect species that shows a clear circatidal rhythm persisting for a long period under constant conditions (Satoh et al. 2008). *A. asahinai* is distributed only on the floors of mangrove forests in East Asia. In the field, this species is found to be active during low tide and to rest on mangrove stems or mounds built by the mud lobster *Thalassina anomala* during high tide, regardless of whether it was day or night.

Under constant conditions, crickets showed clear circatidal activity rhythms that persisted for at least 24 days (Satoh et al. 2008). The activity rhythm consisted of active phases of about 10 h alternating with inactive phases of about 2 h (Fig. 2.2). The mean free-running period was 12.56 h. After the light condition switched from constant darkness to a light–dark cycle, the amount of activity was suppressed in the photophase, but the endogenous rhythmicity itself was not influenced by the light–dark cycle (Fig. 2.3a).

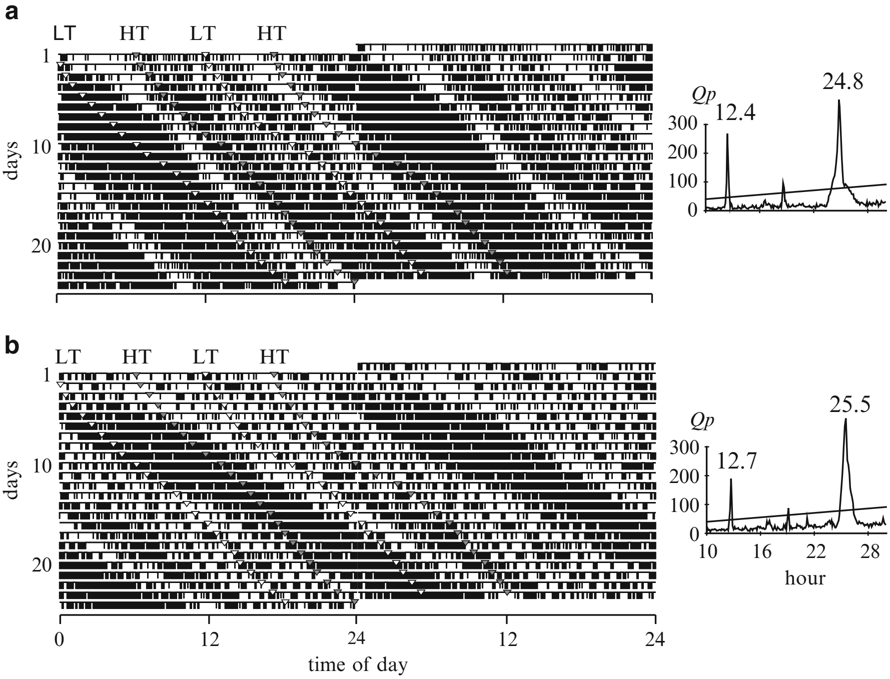

Fig. 2.2 Two examples (**a**, **b**) of the locomotor activity of *Apteronemobius asahinai* under constant darkness at 25 °C. The locomotor activity rhythm is represented by a *double plotted actogram* over a 48-h time period. *Triangles* indicate times of expected low (LT; *white*) and high (HT; *gray*) tides in the field. Rhythmicity was determined by chi-square periodogram analysis (*line graphs at the right side*). An oblique line in the periodogram indicates significance level of $P = 0.05$; a peak value above the line is designated as significant. *Qp* is statistic value (ratio of variances) of chi-square periodogram (Sokolove and Bushell 1978). (From Satoh et al. 2008)

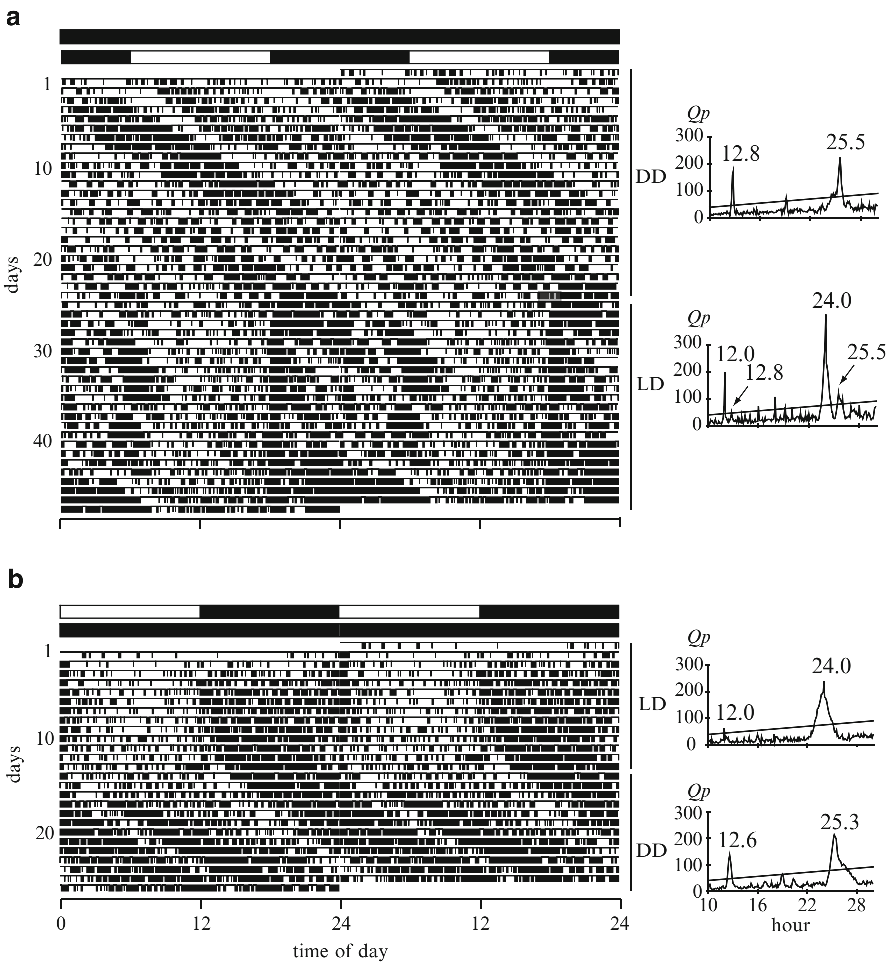

Fig. 2.3 Two examples (**a**, **b**) of the locomotor activity of *Apteronemobius asahinai* under constant darkness (*DD*) and a light–dark cycle (*LD*) at 25 °C. In (**a**), the constant darkness was interrupted by turning on a fluorescent lamp for about 2 h on the 25th day (within the period shown by *shaded region* on the last day of DD) for mechanical maintenance. *Black* and *white bars above the actograms* indicate light and dark phases, respectively, during LD. Displays of actograms and rhythmic analysis are same as in Fig. 2.2. *Qp* is statistic value (ratio of variances) of chi-square periodogram (Sokolove and Bushell 1978). (From Satoh et al. 2008)

On the other hand, intense activity alternating with weak activity was observed in some crickets under constant darkness (Fig. 2.2b). The intense activity phase was observed during expected nighttime during the first few days in captivity. In addition, when the locomotor activity was first recorded under a light–dark cycle, and then under constant darkness, the endogenous rhythm under constant darkness was more intense in the active phase continuing from the dark phase than from the light phase of the preceding light–dark cycle (Fig. 2.3b). These tendencies demonstrated the presence of circadian modulation, that is, the circadian rhythm superimposed on the circatidal rhythm (Satoh et al. 2008).

2.4 Entrainment of Circatidal Rhythms

The zeitgebers for circatidal rhythms in insects have rarely been identified. Satoh et al. (2006) demonstrated that cyclic submergence acted as the zeitgeber for the circatidal burrow-plugging rhythm of larvae of *C. inspecularis*. However, the conclusion of this study was based on observations of only one specimen (see Fig. 2.1). Exceptionally, the entrainment of the circatidal activity rhythm was well demonstrated quantitatively for *A. asahinai*. The inundation acted as the zeitgeber for the circatidal rhythm of this species (Satoh et al. 2009). When an inundation pulse (30 min) was provided four times at intervals of 12.4 h (Fig. 2.4), the circatidal rhythm of *A. asahinai* responded differently to the zeitgeber at different phases of its cycle (Fig. 2.5). When periodic inundations were initiated during the first half of the active phase, the activity onset was considerably delayed (Fig. 2.5a), whereas when periodic inundations were initiated during the latter half of the active phase, the activity onset was considerably advanced (Fig. 2.5b). After treatment, the specimen became inactive around the times when inundation was expected (Fig. 2.5a, b). On the other hand, when periodic inundations were initiated during the middle of the active phase or during the inactive phase, the treatment had little effect (Fig. 2.5c, d).

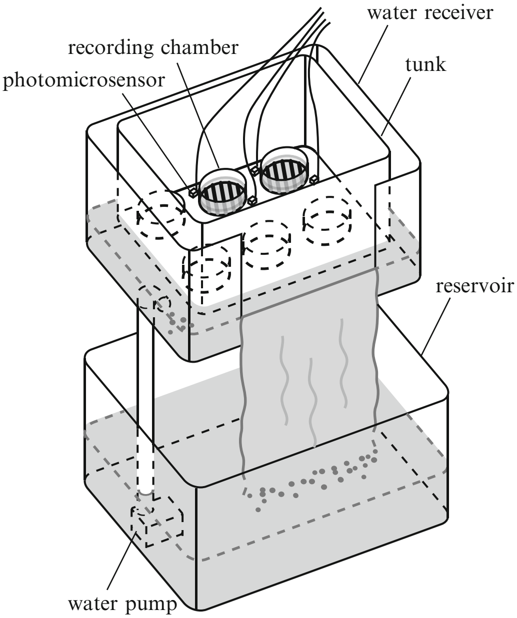

Fig. 2.4 Simplified diagram of the recording apparatus that provides an inundation stimulus to *Apteronemobius asahinai*. Crickets were housed individually in recording chambers with several slits in the bottom. When the receiver was filled by pumping tap water from a reservoir, the water flowed into the chambers through the bottom slits, thereby providing an inundation stimulus. Crickets could escape from drowning by climbing the chamber wall. When the water pump was stopped, the water immediately drained back into the reservoir. A photomicrosensor was used to record locomotor activity. (Modified from Satoh et al. 2009)

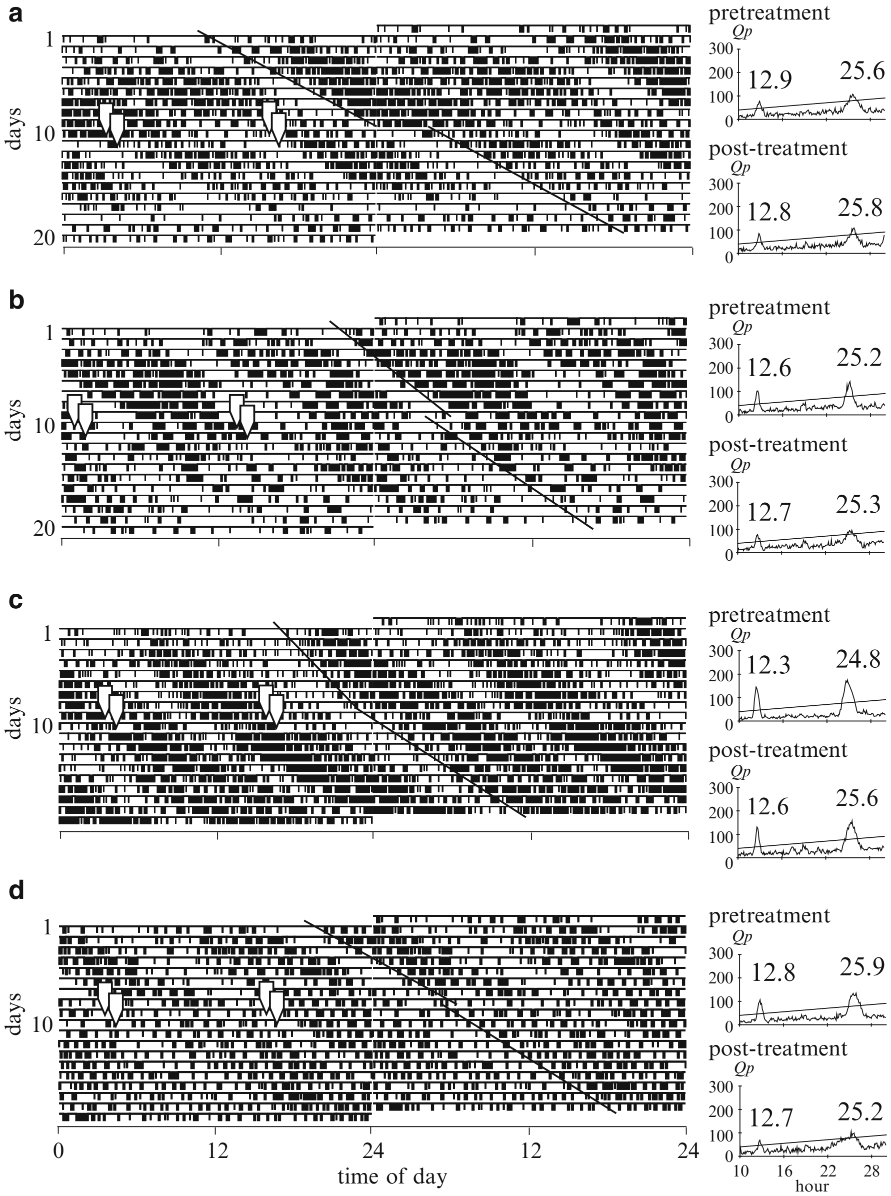

Fig. 2.5 Four typical examples (**a**–**d**) of the phase responsiveness of the circatidal rhythm in *Apteronemobius asahinai* to periodic inundations under constant darkness at 25 °C. Periodic inundations (*arrows*) were initiated during the first half of the active phase (i.e., subjective low tide) (**a**), during the second half of the active phase (**b**), during the middle of the active phase (**c**), and during the inactive phase (i.e., subjective high tide) (**d**). The phase shifts are visualized by *regression lines*, separated for the time before versus time after inundations. The regression lines were fitted by eye through the sequential points of activity onset. Displays of actograms and rhythmic analysis are the same as in Fig. 2.2. *Qp* is statistic value (ratio of variances) of chi-square periodogram (Sokolove and Bushell 1978). (From Satoh et al. 2009)

A phase-response curve (PRC) is the description of the phase-dependent response of the endogenous rhythm to the zeitgeber. The circadian PRCs are generally constructed on the bases of phase responsiveness to a single light pulse under constant darkness (Johnson 1999). In our study, on the basis of the phase-response data of *A. asahinai*, a PRC was constructed by plotting the phase shift of a circatidal rhythm as a function of the phase at which the first inundation pulse was initiated (Fig. 2.6). The comparison of the shape of this circatidal PRC to those of circadian PRCs gives useful information about the nature of the underlying oscillator of the circatidal rhythm. Periodic inundations started during the first half of the subjective low tide caused a phase delay of the circatidal rhythm of *A. asahinai*, whereas those during the second half of the subjective low tide caused a phase advance (Fig. 2.6). This response is similar to the effective action of light pulses against the circadian rhythm; light pulses given during the first half of the subjective night caused a phase delay, whereas those during the second half of the subjective night caused a phase advance. In addition, periodic inundations had less phase-resetting efficacy during the subjective high tide, which is similar to light pulses during the subjective day. The crossover point between the delays and advances existed near the middle of the subjective low tide in the PRC of *A. asahinai*. This characteristic is common with type 1 PRC of circadian rhythms, which show a continuous transition between delays and advances in the middle of the subjective night. The similarity between the PRC on a

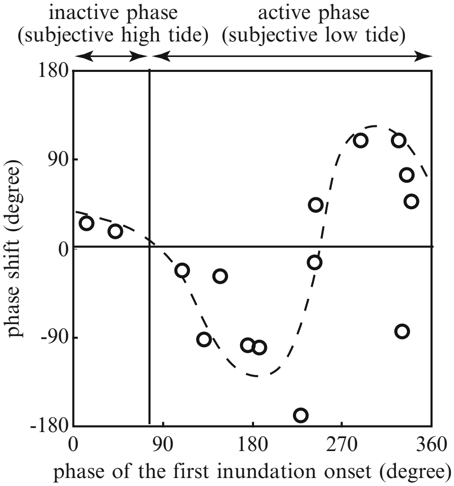

Fig. 2.6 Phase-response curve (PRC) of the circatidal rhythm of *Apteronemobius asahinai* treated with periodic inundations. The period under constant conditions before the treatment is shown in terms of phase angle (0–360°). The phase shift of activity (*vertical axis*) and the phase of first inundation onset (*horizontal axis*) are expressed as angular proportions of the free-running period of each individual. The phase shift of activity was determined from each actogram (see Fig 2.5) by measuring the distance between the two regression lines extrapolated to the day the first inundation was provided. *Dotted line* indicates the form of the PRC as determined by eye. *Arrows* indicate periods of subjective high and low tides, respectively. (From Satoh et al. 2009)

tidal (12.4 h) time base and circadian PRCs indicates that an underlying oscillator of the circatidal rhythm of *A. asahinai* is similar to a circadian oscillator but that its period is approximately 12.4 h, that is, a circatidal clock.

2.5 Molecular and Physiological Basis of Circatidal Rhythms in Insects

The molecular and physiological basis of circatidal rhythms had not been examined in insects until Takekata et al. (2012) studied the circatidal rhythm in *A. asahinai*. As mentioned earlier, this species shows two kinds of rhythms simultaneously in its locomotor activity: a circatidal rhythm producing active and inactive phases and a circadian rhythm modifying the activity intensity of circatidal active phases. Takekata et al. (2012) examined the role of the circadian clock gene *period* (*per*) in these two rhythms by RNA interference (RNAi) of the gene. RNAi is a method to suppress expression of a target gene product by introducing double-stranded RNA that is complementary in sequence to the target gene and is effective in many insect species (Mito et al. 2011). The *per* gene is one of the key components of the circadian clock in insects (Tomioka and Matsumoto 2010), and *per* RNAi completely disrupts the circadian rhythm in the two-spotted cricket *Gryllus bimaculatus* (Gryllidae) (Moriyama et al. 2008).

Adult male crickets collected from the field were used for the RNAi experiment. Their locomotor activities were recorded under a light–dark cycle, and they were anaesthetized (control), injected with double-stranded RNA for the control gene (*β-lactamase*), or injected with double-stranded RNA for *per* on the 6th day. Then, to analyze the effect of RNAi on the circatidal rhythm and circadian modulation, the locomotor activity was recorded under constant darkness from the 11th day (Fig. 2.7). The circatidal rhythm and circadian modulation under constant darkness were detected by the following method. The circatidal rhythm was detected by the presence of a single peak between 10 and 15 h in the periodogram above the 0.05 confidence level. Because the peak of the circadian rhythm was not clear in the periodogram, the presence of the circadian modulation was explored by the following method: The activity level was counted for every circatidal period, and the differences between the activity level of even- and odd-number cycles was examined by analysis of covariance (ANCOVA; Fig. 2.8). Activity was judged to be influenced by the circadian rhythm when the slope or the elevation was significantly different between even- and odd-number cycles ($p \leq 0.05$).

Most of the control crickets, that is, intact and injected with double-stranded RNA for *β-lactamase*, still showed a circatidal rhythm and a circadian modulation of activity under constant darkness after the treatment (Fig. 2.7a, b). After *per* RNAi, however, most crickets did not show circadian modulation of activity, but the circatidal rhythm persisted without a significant difference in the period from controls (Fig. 2.7c). There were significant differences in the proportion of crickets with a circadian rhythm between the *per* RNAi group and the control groups (Takekata et al. 2012), which means that *per* is involved in the circadian rhythm but has no role or a less important role in the circatidal rhythm.

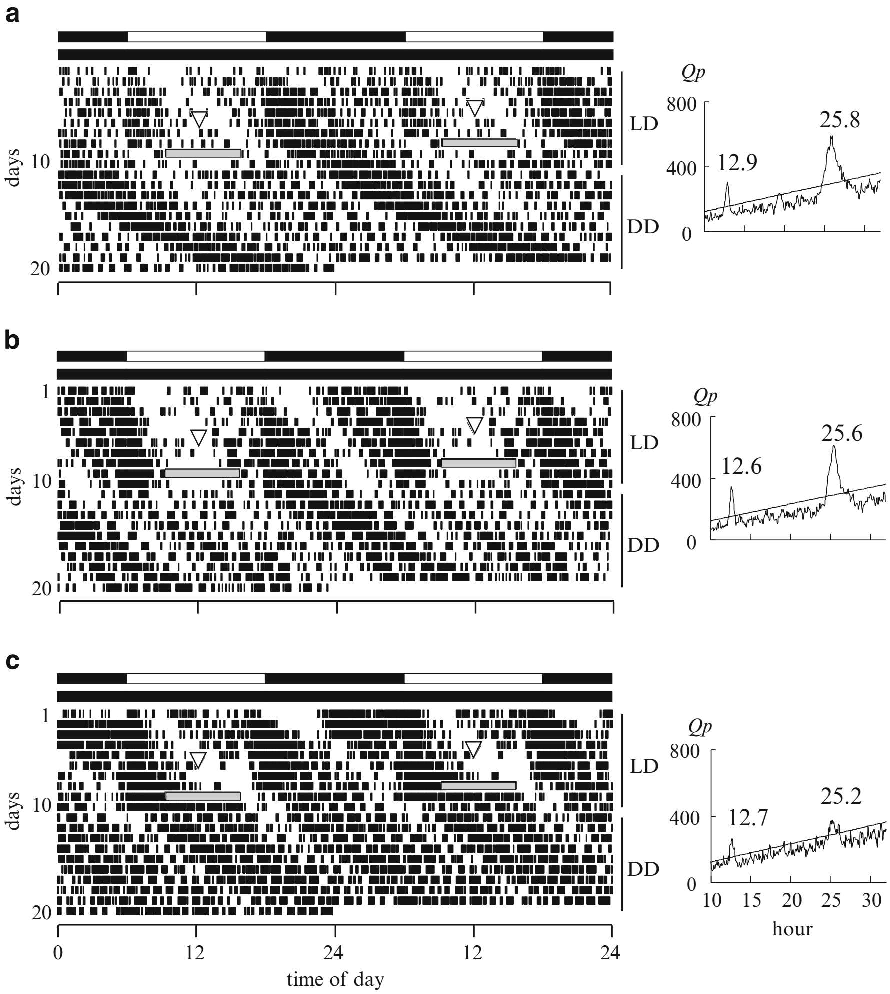

Fig. 2.7 Examples of the locomotor activity of *Apteronemobius asahinai* anaesthetized (control) (**a**), injected with double-stranded RNA for the control gene (*ß-lactamase*) (**b**), and injected with double-stranded RNA for *per* (**c**). Injection was performed on the 6th day (*triangles*). Rhythmicity under DD was determined by chi-square periodogram analysis (*line graphs at the right side*). *Black* and *white bars above the actograms* indicate light and dark phases, respectively, during LD. *Gray boxes in actograms* indicate no data. Circadian modulation under DD was significantly detected for the free-running rhythm of (**a**) and (**b**) but not for (**c**) by using the method described in Fig. 2.8. *Qp* is statistic value (ratio of variances) of chi-square periodogram (Sokolove and Bushell 1978). (From Takekata et al. 2012)

Furthermore, Takekata et al. (2014) examined the role of the optic lobe in the circatidal rhythm. The optic lobe in the brain is the circadian clock locus in crickets (Tomioka and Abdelsalam 2004). The surgical removal of the optic lobes of *A. asahinai* disrupted the circadian modulation but did not affect the circatidal rhythm,

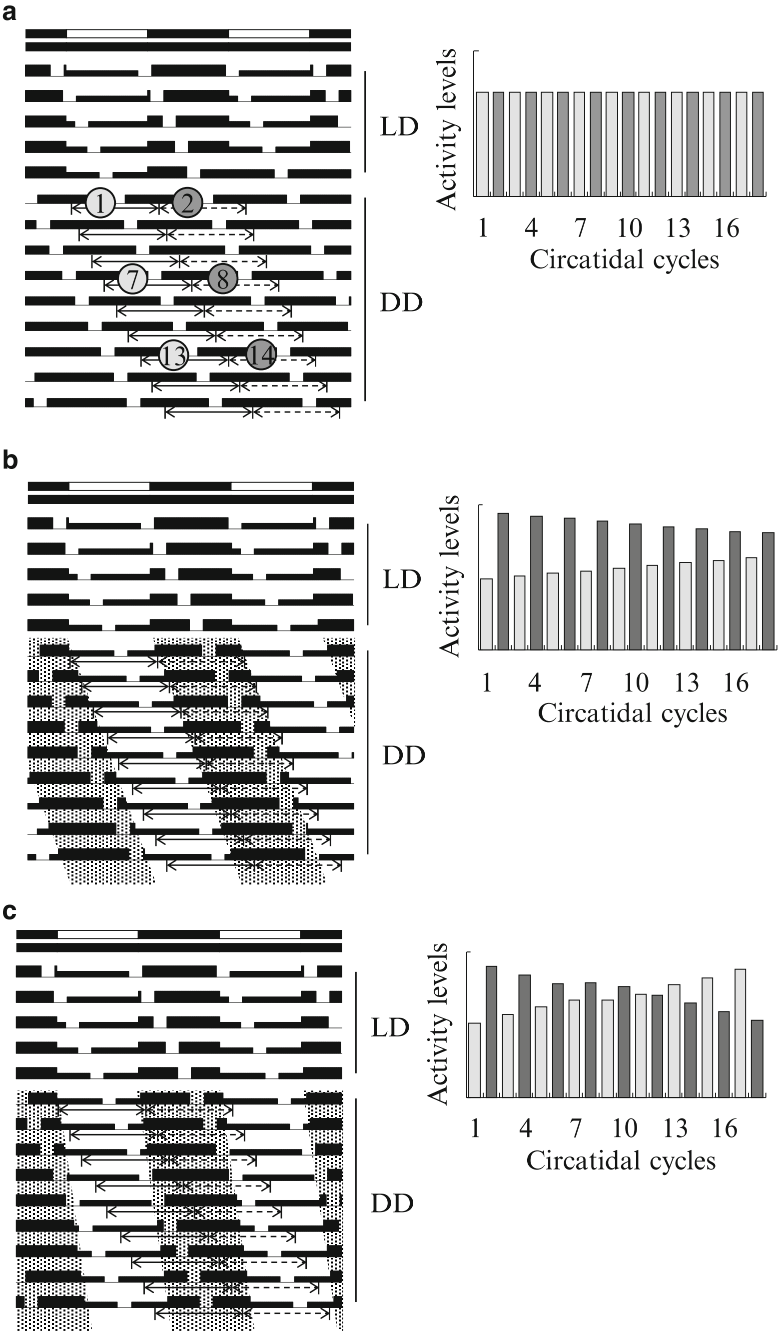

Fig. 2.8 Method for detecting circadian modulation of activity in *Apteronemobius asahinai* that show a circatidal rhythm. It is assumed that crickets under light–dark cycles (LD) were transferred to constant darkness (DD) just after the injection of double-stranded RNA. Double-plotted actograms (*left*) and histograms of activity levels counted for each circatidal period in DD (*right*) are shown.

which was maintained with no remarkable changes in its free-running period (Takekata et al. 2014). Although the circatidal rhythm in *A. asahinai* is controlled by a circatidal clock of which the phase responses are similar to those of the circadian one (Satoh et al. 2009), the molecular and neural bases of the circatidal clock appear to be different from the circadian clock.

2.6 Conclusions

There are not enough studies to discuss the general characteristics of circatidal rhythm in insects. Collecting outputs of endogenous activity rhythms for many species is primarily needed. Many intriguing insects in the marine environment are yet to be studied. By analyzing many outputs of endogenous activity rhythms, we can also know whether the circatidal rhythm is widely observed among intertidal insects. The zeitgeber and the physiological and molecular basis also need to be studied as the next step.

Finally, we refer briefly to the origin of the circatidal rhythm in insects. Intertidal insects are essentially terrestrial and have been derived from the inland ancestor by penetrating secondarily to marine environment. It is, therefore, easy to accept that the circatidal rhythm in intertidal insects is controlled by a clock originating from the circadian clock widely distributed in insects. At present, one negative answer to this implication is, however, obtained by the study of *A. asahinai*. As already mentioned, Takekata et al. (2012, 2014) indicated that the circatidal rhythm in *A. asahinai* is controlled by a circatidal clock of which the molecular and neural mechanism is different from that of the circadian clock. This observation might suggest that the tidal clock has evolved from the genetic background unrelated to the circadian clock. Nevertheless, circadian clock genes other than *per* may possibly be involved in the clock. Only after we determine the circatidal clock genes and the genes related to those could we answer clearly the question about the origin of the circatidal clock. Advances in studies of *A. asahinai* as a model organism will lead to better understanding about the circatidal rhythm in insects and, possibly, its evolutionary basis.

Fig. 2.8 (continued) *Black and white bars above the actograms* indicate light and dark phases, respectively, under LD. The *shaded areas* show subjective nights under DD in crickets with the circadian rhythm. The difference in the activity levels counted for each circatidal period of odd (*light gray*) and even (*dark gray*) number cycles was examined by analysis of covariance (ANCOVA) on the assumption that the activity levels regress linearly. In a cricket without the circadian rhythm, the activity levels are the same between adjacent circatidal rhythm, and there is no difference in the elevations and the slope (**a**). In a cricket with the circadian rhythm of which the free-running period almost coincides with twice of the circatidal rhythm, the elevations of activity levels are different between circatidal even- and odd-number cycles because the subjective day corresponds to odd or even circatidal cycles throughout the experimental period (**b**). When the circadian free-running period slightly deviates from twice of the circatidal rhythm, the slopes of the activity levels are different because the subjective day moves from odd to even circatidal cycles or vice versa during the experimental period (**c**). Thus, the activity was judged to be influenced by a circadian rhythm when the slope or the elevation was significantly different between odd and even number cycles ($p \leq 0.05$)

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Chapter 3
Daily and Tidal Rhythms in Intertidal Marine Invertebrates

Christopher C. Chabot and Winsor H. Watson III

Abstract Intertidal animals are faced with strong rhythmic environmental signals on both daily and tidal frequencies, and their behavioral output is often complex because these two competing influences affect the endogenous clocks that drive their behaviors. This chapter reviews the nature of the clocks that govern the behaviors in intertidal organisms as well as the inputs and outputs from these clocks. The relative importance of the environmental factors that synchronize these rhythms is discussed, as well as the different types of physiological and behavioral outputs that have been observed. The molecular underpinnings of the circadian clocks are presented as well as the hypothesis that the clocks that drive circatidal rhythms are composed of the same molecular "machinery" used by circadian clocks.

Keywords Circadian • Circalunidian • Circatidal • Clocks • Horseshoe crab

3.1 Overview

There are many reasons one might refer to tidal rhythms as mysterious and enigmatic. First, although the presence of an endogenous timing system controlling tidal rhythms has been known for more than 50 years (Bennett et al. 1957), virtually nothing is known about the underlying physiological/molecular mechanisms that produce this approximately 12.4-h rhythm. Second, this approximately 12.4-h rhythm can vary somewhat in different locations because, although the tides in some areas occur twice daily (semidiurnal), in other areas there are once-daily tides, unequal semidiurnal tides, or even a lack of tides! It is not at all unusual for different populations of the same species to occur in areas that are exposed to all these types of tidal cycles. Finally, perhaps as a result of the underlying timing mechanisms combined with the variety of environmental cues that synchronize the endogenous tidal clocks, circatidal

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rhythms (with periods of about 12.4 h) tend to be less robust than circadian rhythms (periods ~24 h) and thus are more difficult to analyze in the laboratory or field. When all these issues are considered, it is no wonder that Palmer (1995a) warned: "Until one reaches tenure, it would be best not to enter this branch of the field"!

Nevertheless, during the past few decades, significant strides have been made in the understanding of the formal properties of these rhythms, similar to the state of the field of circadian rhythms biology in the early 1970s before the discovery of the *period* gene by Konopka and Benzer (1971). Since that time, the endogenous circadian clock "black box" has been opened and great strides have been made in the understanding of the molecular underpinnings of the circadian clock. Although only hints about the molecular basis of the circatidal clock have emerged thus far, the advent of clear, robust outputs from the clock and the availability of deep sequencing RNA and DNA technologies, makes these discoveries all but certain in the near future.

The aim of this chapter is to provide a summary of what is known about the timing systems, and environmental cues, that control the physiology and behavior of selected marine invertebrates. Because there are several excellent previously published reviews of tidal rhythms, we have chosen to focus our attention on a subset of marine invertebrates that express both daily and tidal rhythms, with an emphasis on the tidal rhythms, because we find this ability particularly enigmatic and thus appropriate for this volume. We have organized the chapter into three sections. First, we present a discussion of the types of behavioral and physiological outputs we use to monitor biological rhythms. Next, we outline the inputs or entraining agents that help to synchronize circatidal clocks to tidal cycles. Last, the formal properties that govern the clocks that control these circatidal rhythms are presented.

3.2 Outputs

Although coastal indigenous peoples have long been aware of the influence of the tides on intertidal organisms, endogenously generated circatidal rhythms were first reported in fiddler crabs by Bennett et al. (1957). As did many circadian biologists of the time, the early researchers used some type of overt behavior as their "window into the clock," because it is relatively easy to measure. Many of these clock pioneers used tilt-cage "actographs" as a way to monitor the activity of these intertidal species.

3.2.1 Laboratory Studies of Tidal Rhythms

Most of the early work on tidal rhythms focused on the European shore crab (known elsewhere around the world as the invasive green crab or green shore crab; Williams and Naylor 1969) and several species of fiddler crabs (*Uca* spp.; Bennett et al. 1957; Palmer 1963). Although these studies paved the way for many future experiments, they also shed light on many of the challenges inherent in studying tidal rhythms. Most importantly, it was clear that the tidal rhythms expressed by these animals were not as robust and persistent in constant conditions as circadian rhythms.

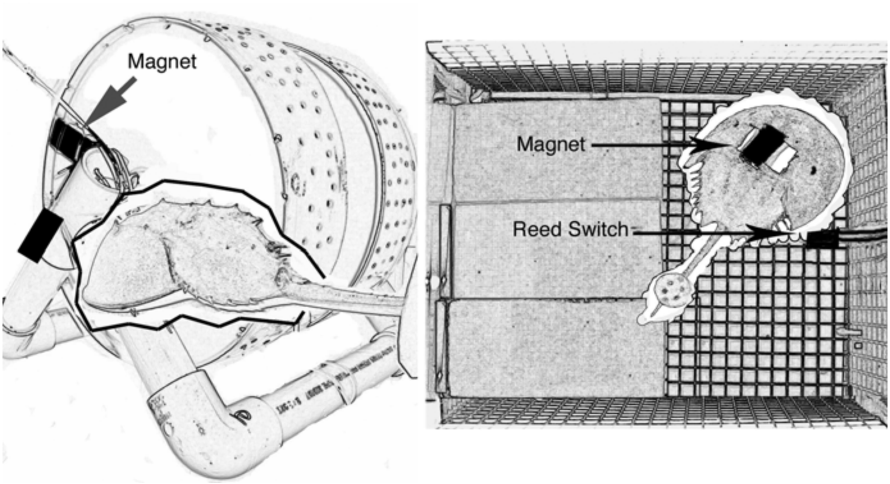

Fig. 3.1 Computer-rendered photos of a *Limulus* “running wheel” (*left panel*) and activity “square” (*right panel*). *Left:* Part of the running wheel has been “cut away” to reveal the position of the animal. Note the circular “tail limiter,” magnetic reed switch (*black rectangle* attached to the running-wheel leg), and magnet (*black rectangle*). *Right:* The ceiling of the square as well as some of the brick “roof” (*left*) has been cut away to reveal the animal. Note the magnet affixed to the dorsal carapace and the magnetic reed switch (*black rectangle at right* of diagram). (Reproduced from Chabot et al. 2007 with permission from Elsevier)

Another challenge with the study of tidal rhythms is developing appropriate methodology for recording the activity of aquatic species for many days at a time. Much of the early work was carried out using the aforementioned tilt cages, but recent technological advances have allowed scientists to use infrared beams, magnetic reed switches, and digital video analysis systems to monitor activity. Each of these techniques has its own set of advantages and disadvantages, and we have used all of them to obtain long-term recordings from both lobsters (Jury et al. 2005) and horseshoe crabs (Fig. 3.1; Chabot et al. 2007).

3.2.1.1 Examples of Tidal Rhythms Expressed by Selected Marine Invertebrates

Circatidal rhythms have been demonstrated in many intertidal invertebrates, including the shore crab *Carcinus maenas* (Naylor 1958), the isopod *Excirolana chiltoni* (Klapow 1972; Enright 1976), and the shrimp *Crangon crangon* (Aladhub and Naylor 1975). Because these investigations were conducted in the laboratory, in constant conditions with respect to tidal cues, the evidence is clear that there are endogenous oscillators that control the circatidal rhythms of locomotion in these species. The presence of such an oscillator(s) would allow these species in their natural habitat to anticipate changes in water depth and currents and adjust their behavior accordingly. Recently, clear endogenous circatidal rhythms have also been documented in

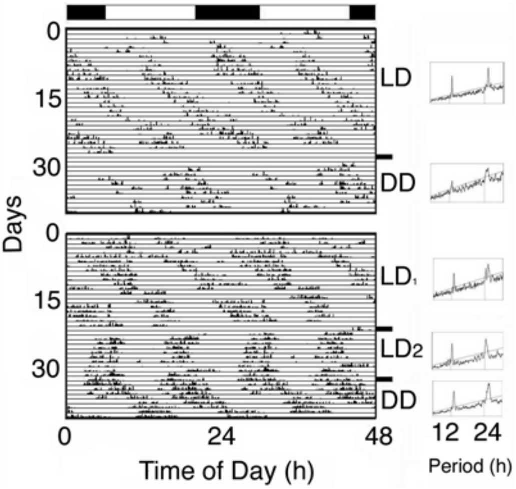

Fig. 3.2 Free-running locomotor rhythms expressed by two individual *Limulus polyphemus* exposed to a 14:10 light–dark (LD) cycle, clearly illustrating tidal patterns of activity (*left panels*). Data are double-plotted to facilitate visual inspection. Chi-square periodogram analyses of sections of the actograms (separated by *horizontal bar*) are presented in the *right panels*. LD_1 and LD_2 portions of the bottom actogram were analyzed separately to illustrate the different periods exhibited by this animals under this LD cycle [free-running (*Tau* = 12.45 h; LD_1) or entrained (*Tau* = 12.0 h, LD_2)]. The figure also demonstrates the robust persistence of free-running patterns under constant tidal conditions. (Reproduced from Chabot et al. 2007 with permission from Elsevier)

Limulus polyphemus, the American horseshoe crab (Fig. 3.2; Chabot et al. 2007). In this species, circatidal activity is seen in approximately two-thirds of the animals tested in the laboratory, at virtually all times of the year: summer (Chabot et al. 2004), later summer and fall (Chabot et al. 2007), and winter (Watson et al. 2009), so long as the water temperature is above 11 °C.

Circatidal rhythms of other physiological phenomena have also been observed in intertidal animals and, in general, these parameters are often related to overall activity. For example, a circatidal rhythm of oxygen consumption has been demonstrated in isopods (Marsh and Branch 1979), as well as circatidal rhythms of molting (Abelló et al. 1997), and heart rate (Styrishave et al. 1999) in the shore crab. Locomotion, heart rate, and ventilation activity are all closely correlated in horseshoe crabs (Fig. 3.3) and in sand crabs, *Portunus pelagicus* (Gribble and Broom 1996). The long-term synchrony between rhythms of heart rate and respiration is also supported by electrophysiological studies showing the close coordination of heart rate and gill ventilation in *L. polyphemus* (Watson and Wyse 1978) that occurs during daily activities (e.g., walking, gill cleaning). Interestingly, in the absence of activity, circatidal rhythms of respiration persist in *C. maenas* (Arudpragasam and Naylor 1964; unpublished data), demonstrating that locomotor activity expression is not necessary for the expression of respiratory rhythms. These results suggest that respiration and heart rate rhythms are not simply direct outcomes of locomotor activity rhythms and may indicate control by a common timing mechanism.

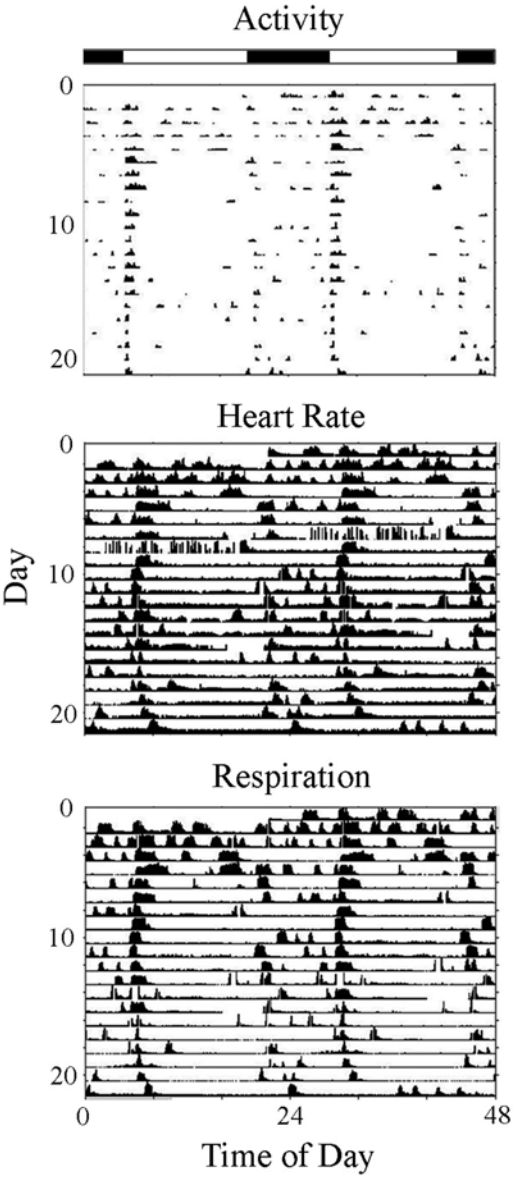

Fig. 3.3 Coordination of rhythms of activity, heart rate, and respiration in an individual *L. polyphemus* maintained for 21 days in a running wheel. The LD cycle (15:9) is indicated by *light/dark bars at the top*. Actograms are double-plotted, with size and position of *black marks* indicating the intensity and timing of activity (*top*), beats per minutes (*middle*), and respiration frequency (*bottom*). Heart rate and respiration were recorded after Chabot and Webb (2008)

3.2.1.2 Daily and Circadian Rhythms in Selected Marine Invertebrates

Time of day also influences the patterns of activity and associated physiological processes expressed by some marine invertebrates. In some cases, such as the color changes in fiddler crabs (Darnell 2012) and transcription and translation in mussels (Connor and Gracey 2011), there is good evidence that these daily rhythms are

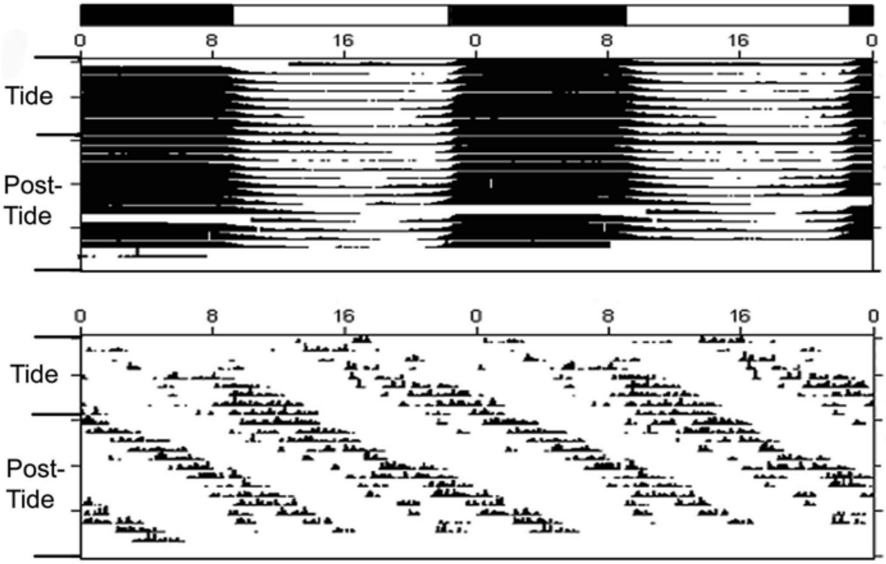

Fig. 3.4 Locomotor activity rhythms can be synchronized to artificial tides and are independent of daily rhythms of visual sensitivity. This animal was first exposed to changing cycles of water depth with a period of 12.4 h, for 8 days; then the imposed tides were turned off and the water level was kept at a constant intermediate depth for an additional 13 days. Although locomotion became entrained to the imposed tides (*bottom panel*), the electroretinogram rhythm remained entrained to the LD cycle (*top panel*). Units for the horizontal axes are hours. The LD cycle is indicated by the *light and dark bars*. (Reproduced from Watson et al. 2008 with permission from The Marine Biological Laboratory)

under the control of an endogenous circadian clock. Similarly, in horseshoe crabs, the sensitivity of their lateral eyes to light is controlled by a circadian clock that is located in the brain (Barlow et al. 1977; Barlow 1983). This change in sensitivity, controlled by input from a circadian clock, is the result of well over a dozen modifications of vision-related physiological parameters, such as dark adaptation (Kass and Berent 1988) and membrane shedding (Chamberlain and Barlow 1979). One might assume that these increases in visual sensitivity at night might serve to enhance the vision of a species that is primarily nocturnal and that the same circadian clock controlling vision would modulate locomotor activity. However, because locomotor activity tends to be expressed in circatidal frequencies (~12.4-h cycles), while eye sensitivity (as measured by electroretinograms) fluctuates with a circadian frequency (~24-h cycles), it is likely that they are controlled by different timing systems (Fig. 3.4; Watson et al. 2008). Thus, it appears that some marine invertebrates contain two different endogenous clocks.

Other life history stages of *L. polyphemus* also express both daily and tidal rhythms. After hatching, the larvae spend approximately 6 days in the water column where they are positively phototactic and nocturnally active (Rudloe 1979). Larvae collected in the field and brought into the laboratory will express a tidal rhythm of activity in constant conditions, becoming active near the start of the expected ebb tide, and can be entrained to 12.4-h cycles of agitation (Ehlinger and Tankersley 2006). Juveniles from both North Carolina (Casterlin and Reynolds 1979) and Massachusetts (Borst and Barlow 2002) are reported to be nocturnal in light–dark (LD) cycles. However, a close examination of the figure from the Borst and Barlow (2002) abstract shows two bouts of activity per 24 h, indicating the presence of circatidal rhythms at this stage as well. In a recent larger study of juvenile horseshoe crab biological rhythms (Dubosky et al. 2013), 63 % of animals exposed to a LD cycle expressed daily rhythms of activity whereas 25 % expressed circatidal rhythms. Of those that displayed a daily rhythm of activity, 47 % preferred to be most active in the day (diurnal activity) and 20 % preferred to be most active at night (nocturnal activity). Interestingly, when the LD cycles were terminated so that animals were in constant darkness (DD), more animals (54 %) began to express a circatidal rhythm, suggesting that exposure to the LD cycle may have "masked" expression of an underlying circatidal clock. Thus, in horseshoe crabs there is an interesting and complex interplay between LD cycles, daily rhythms, and circatidal rhythms, which is addressed in more depth in Sect. 3.3 below.

3.2.2 Biological Rhythms of Marine Invertebrates in Their Natural Habitat

Despite dozens of studies that have addressed activity rhythms of intertidal species, our understanding of the movement patterns displayed by free-living individuals is rather limited, primarily because of the challenges inherent in studying aquatic species. A main limitation is in our ability to obtain continuous data for many days in a row. Nevertheless, there are several examples in which tidal or daily rhythms of activity have been demonstrated. For example, New Zealand mud crabs exhibit circatidal rhythms in situ that are modulated by the solar day (Williams et al. 1985). In green crabs, *C. maenas*, it has been demonstrated that the depth at which animals are held influences the impact of LD cycles on their activity: LD has little or no influence on subtidal individuals (Lynch and Rochette 2007), although it does influence the behavior of individuals that inhabit the intertidal zone (Naylor 1963; Reid and Naylor 1989; Styrishave et al. 2003).

3.2.2.1 Daily and Tidal Rhythms Expressed by Horseshoe Crabs in Their Natural Habitat

It is well known that horseshoe crabs approach mating beaches to spawn during the high tides in the spring and early summer. However, just because groups of animals consistently appear at these beaches at the high tides, it does not mean they are inactive during other portions of the tidal cycle. Using ultrasonic telemetry we have shown that horseshoe crabs express a tidal pattern of behavior during the mating

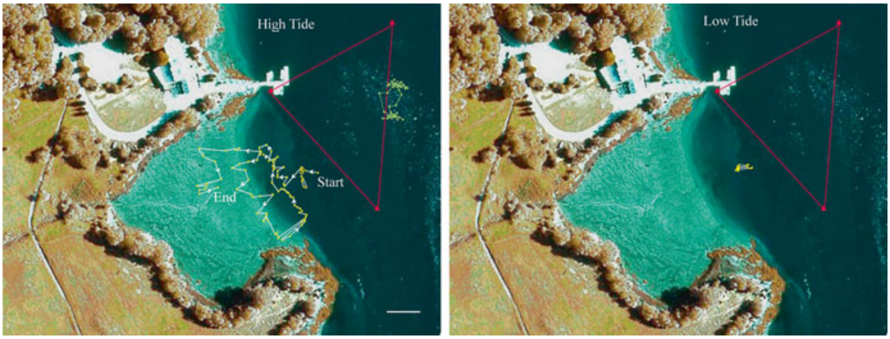

Fig. 3.5 Movements of a male horseshoe crab during a 5-h period when the tide was either high (*left*: peak high at 11:50 a.m.; data shown start at 12:30 p.m. and end at 5:30 p.m.) or low (*right*: peak low was at 7:30 a.m.; data shown start at 7:00 a.m. and end at noon). Each point represents one positional fix obtained from a VEMCO radio-linked acoustic positioning (*VRAP*) tracking system. The points have been superimposed on an infrared image of the cove that was colorized to look more "natural." The *red lines* connect each of the three VRAP buoys used to track the horseshoe crabs. In the *left panel the light green dots and lines* represent the position of the reference transmitter during the same time period (not shown in *right panel*). Note how it appears as if it moved, even though it was stationary, because buoy movements caused by the tides and waves generate small movement artifacts. *White scale bar on left panel* represents 20 m. (Reproduced from Watson and Chabot 2010, with permission from the Editorial Office, *Current Zoology*)

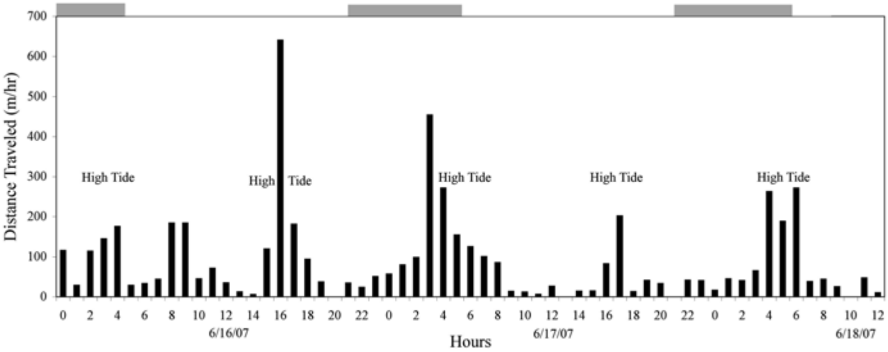

Fig. 3.6 Activity of a male horseshoe crab in June 2007, illustrating consistent increases of activity during high tides. Night hours are depicted by the *gray bars on the top* of the graph and peak high tides are also noted. (Reproduced from Watson and Chabot 2010, with permission from the Editorial Office, *Current Zoology*)

season (Figs. 3.5 and 3.6), and this tendency continued throughout most of the year, from May to October. Observations of these animals using SCUBA (self-contained underwater breathing apparatus) indicated that this tidal rhythm was most likely related to excursions onto intertidal mudflats to feed. Recent studies in the Great Bay estuary have revealed that there is intensive foraging activity by horseshoe crabs in these habitats (Lee 2010). Behavior in the field is also affected by LD cycles: telemetry studies of animals that expressed a tidal rhythm show a preference for activity during the daytime high tide (Watson and Chabot 2010). Similar results have also been seen in animals that are held in recording devices in the same estuary where the telemetry studies were conducted (Chabot and Watson 2010).

3.3 Inputs: Natural Stimuli That Entrain or Synchronize Biological Rhythms

Although light is clearly the most important zeitgeber, or cue, for entraining and synchronizing circadian clocks to natural LD cycles, there are many potential cues that may serve this purpose for tidal rhythms, including temperature, salinity, turbulence, current, turbidity, and depth. The influence of water-level changes on circatidal rhythms has been most thoroughly investigated, and this cue can entrain the activity of the amphipod (*Corophium volutator*; Harris and Morgan 1984), the portunid crab (*Liocarcinus holsatus*; Abelló et al. 1991), and the fiddler crab, *Uca crenulata* (Honeggar 1973), as well as many other species. Agitation or turbulence is an effective entraining agent for the mole crab *Emerita talpoida* (Forward et al. 2007), the amphipod *Synchelidium* spp. (Enright 1963), and the isopod *Eurydice pulchra* (Hastings 1981). The effects of tidal cues have been especially well-studied in *C. maenas*. This species can be entrained to cycles of temperature, inundation (Williams and Naylor 1969), or hydrostatic pressure (Reid and Naylor 1990). *C. maenas* is also one of the very few species in which the relative importance of tidal cues has also been assessed in situ (Styrishave et al. 1999, 2003).

3.3.1 Entrainment by Water Depth Changes and Inundation in Limulus

3.3.1.1 Laboratory Studies

In the laboratory, juvenile and adult horseshoe crabs exhibit endogenous circatidal rhythms that can be entrained to both water-level changes and inundation (Chabot et al. 2008; Dubosky et al. 2013), during both breeding and nonbreeding times of year. Large water-level changes (Fig. 3.7) or inundation cycles are effective entraining signals in this species; small water pressure changes are much less effective. For example, we found clear evidence of entrainment to water-level changes of 0.5 m, whereas depth changes of 0.2 m led to synchronization of activity to the artificial tides, but not clear entrainment (the rhythm did not persist after the artificial tides were stopped; Chabot et al. 2008). Although the “threshold” between 0.2 and 0.5 m is somewhat greater than those reported for *C. maenas* (Naylor and Atkinson 1972) and some planktonic species (0.1 m; Knight-Jones and Morgan 1966), our study appeared to use a more stringent definition of entrainment than the

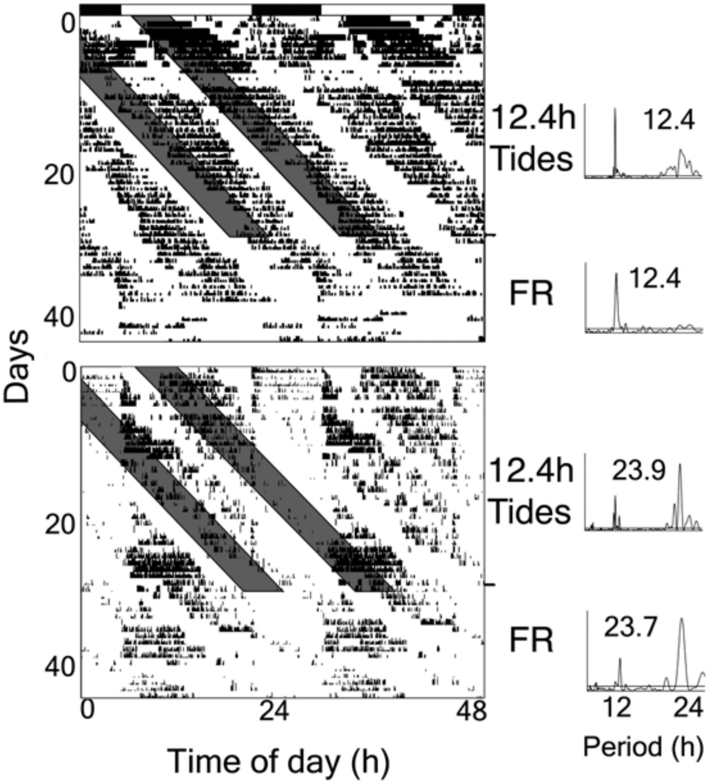

Fig. 3.7 Entrainment of tidal rhythms by artificial tides. These two animals were first exposed to 12.4-h artificial tides (dark parallelograms - high tides), and then the water depth was held constant and the entrained tidal rhythm of locomotion was allowed to free-run (*FR*). The light–dark cycle remained the same throughout the experiment (14:10). (Reproduced from Watson et al. 2008 with permission from The Marine Biological Laboratory)

previous studies. Some in situ results in horseshoe crabs also support the finding that stronger depth change signals increase the percentage of animals that entrain: 40 % of animals exhibited tidal rhythms in deeper water that experienced a 60 % change in water levels during each tide cycle, whereas nearly 80 % of those in running wheels anchored to the bottom in shallower water, and experiencing 300 % water pressure changes, showed tidal rhythms (Chabot and Watson 2010).

3.3.1.2 Rhythms Expressed by *Limulus* in Their Natural Habitat

Given the strong influence of artificial tides on the activity rhythms expressed by horseshoe crabs in the laboratory, one would expect most freely behaving horseshoe crabs to express tidal rhythms in the field. We tested this hypothesis in two different experiments. In the first, we found that most of the animals confined to running

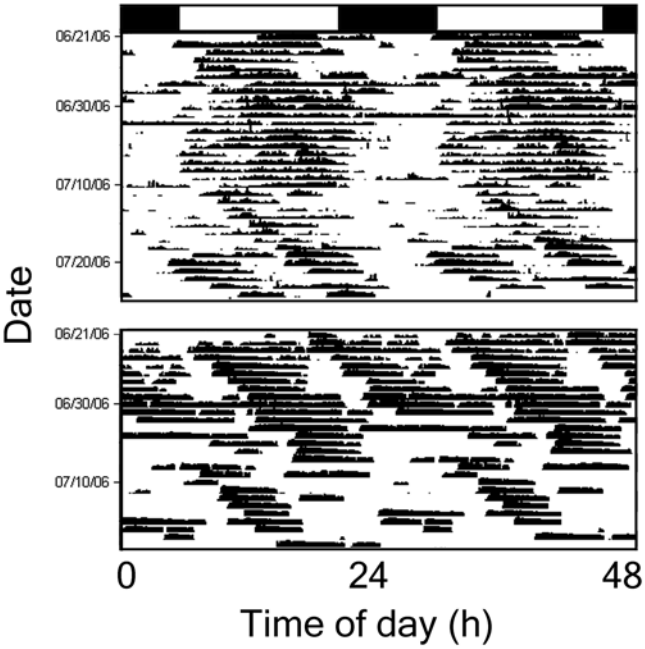

Fig. 3.8 Behavioral activity patterns expressed by two *Limulus* in running wheels that were either attached to a floating raft (*top*) or fixed to the bottom of the estuary (*bottom*). Approximate photoperiod is indicated by *LD bars at top*. Note that the animal attached to the floating raft was primarily diurnal (although with some evidence of tidal rhythmicity during the past 2 weeks) whereas the animal located at the bottom of the estuary was primarily tidal. (Reproduced from Chabot and Watson 2010, with permission from the Editorial Office, *Current Zoology*)

wheels in their natural habitat, where they were exposed to natural tide cycles, synchronized their activity to the tides (Watson et al. 2009; Chabot and Watson 2010; Fig. 3.8). In addition, we found that animals that were unable to sense the changes in water depth, because they were secured under a floating dock that rose and fell with the tides, failed to express a tidal rhythm of activity (Watson et al. 2009; Chabot et al. 2011) and exhibited clear daily rhythms instead. This observation that although factors such as current changes (0–0.2 m/s), temperature changes (Δ5 °C), salinity changes (Δ5 psu), and other physicochemical parameters may influence the behavior of horseshoe crabs, they are generally not sufficient to synchronize tidal rhythms. In the only other comparable field study of which we are aware, water pressure was also the primary tidal cue used by amphipods to synchronize their activity (Enright 1963): animals exposed to water pressure rhythms in situ entrained to the tidal cycles whereas those tethered to a buoy, like our animals tethered to a floating dock, did not. Overall, our data support the hypothesis that water-level changes are the dominant timing cue used by horseshoe crabs to synchronize their activity to the tides (Watson et al. 2009). However, in their natural habitat, when exposed to both tides and natural LD cycles, their rhythms are more complex than we anticipated.

We fitted animals with accelerometers that transmitted activity data to receivers located throughout the estuary so that we could gather activity data from animals that were freely moving in their natural habitat and exposed to all the normal cues. Interestingly, although many of these animals consistently expressed a tidal rhythm of activity, several were more active in the day or the night. Therefore, in their natural habitat, many other factors must be influencing the likelihood that they will express a tidal pattern of locomotion, or a pattern of activity that is the result of a variety of different cues.

The influence of water levels and depth changes on horseshoe crab activity can also be seen in *Limulus* breeding rhythms in the field. Populations that are exposed to tides of at least 0.5 m or so synchronize to the tides (Rudloe 1979, 1980, 1985; Cohen and Brockmann 1983; Barlow et al. 1986), whereas those that experience only microtides (of a few centimeters) do not (Ehlinger et al. 2003). Further, wind action leading to increased or decreased tidal heights significantly affects the number of animals breeding (Brockmann 2003). In addition, several studies have also shown a correlation between the height of the tides and the number of animals breeding, so that, for example, more animals are found on breeding beaches around the new and full moons in Massachusetts (Barlow et al. 1986) and Florida (Rudloe 1980). However, this relationship does not appear to be true at all breeding locations, most likely because different breeding beaches have different slopes, sizes, and sediment types.

3.3.2 The Influence of Temperature, Turbulence, and Salinity on Tidal Rhythms

Other tidal cues can also be effective in entraining or synchronizing tidal rhythms, depending on the species. Salinity changes appear to be effective synchronizing agents for the crabs *Rhithropanopeus harrissii* (Forward et al. 1986) and *C. maenas* (Reid and Naylor 1990), although periodic agitation is sufficient to entrain tidal rhythms in two species of isopods (Klapow 1972; Hastings 1981) and juvenile horseshoe crabs (Ehlinger and Tankersley 2006). Finally, in animals such as mole crabs (Forward et al. 2007), amphipods (Enright 1963), and isopods (Hastings 1981) that inhabit the swash zone, mechanical stimulation appears to be a very important stimulus for entraining rhythms.

In horseshoe crabs, temperature cycles do not appear to be as effective as water-level changes and turbulence cues appear to be less effective than temperature (Chabot et al. 2008). Interestingly, the magnitude of the cue seems to matter, suggesting a threshold effect for temperature as well as for water depth changes. Similar results were found in *Carcinus*: 11 °C cycles are effective entraining agents whereas 4 °C temperature cycles are not (Williams and Naylor 1969).

Artificial currents appear to be even weaker synchronizing agents. Whether in the lab or in running wheels attached to a floating raft in the estuary, horseshoe crabs do not entrain even if exposed to relatively strong currents of 0.2 m/s (Chabot et al. 2008). Interestingly, there is good evidence that larval *Limulus* entrain to cycles of mechanical agitation simulating the turbulence created by the incoming tide (Ehlinger and Tankersley 2006). However, other than during the mating season, juvenile and adult horseshoe crabs rarely inhabit areas where they would encounter significant wave- or wind-induced turbulence, or other types of agitation. Therefore, not surprisingly, the types of cues that are most effective in synchronizing and entraining the tidal rhythms of many marine invertebrates are closely related to their normal habitats and life history strategies. Even within a single species, such as the horseshoe crab, the best entrainment cues depend on both the life history stage and the location where they live.

3.3.3 The Influence of Light on the Expression of Rhythms

Although "The majority of tide-associated rhythms that have been described in different laboratories and different species, have not identified a solar-day influence" (Palmer 1995a), some intertidal animals do appear to be affected by LD cycles. Although many physical factors that fluctuate with the changing tides are likely to entrain or influence endogenous tidal rhythms, LD cycles have an entirely different period. Thus, one would predict that the tidal rhythms of most tidal species would not be affected by the LD cycle (DeCoursey 1983; Palmer 1995a). However, tidal-associated activity in *Sesarma pictum* (Saigusa 1992) and *U. crenulata* (Honeggar 1973) can be phase shifted by LD cycles. In addition, LD cycles appear to cause some transient synchronization of circatidal rhythms in other crustacean species (*Sesarma*, Saigusa 1988; *Hemigrapsus*, Saigusa and Kawagoye 1997).

In horseshoe crabs, we have shown in several studies that LD cycles influence the types of rhythms that horseshoe crabs express and that tidal rhythms can be influenced by LD cycles. Approximately one-third of animals studied in the laboratory exhibit daily rhythms, preferring to be most active during either the night or the day (Fig. 3.9). In several cases the circatidal rhythms expressed by some animals have been entrained by LD cycles and, when released into DD, these rhythms appeared to free run from the point of entrainment (Chabot et al. 2007). We have also observed a transient synchronization in many animals (Fig. 3.10), otherwise known as relative coordination (Johnson et al. 2004), which is indicative of a relatively weak entraining agent influencing an oscillator. Interestingly, beach isopod behavior is also affected by LD cycles, but only if well fed, when they avoid swimming during the day (Reid 1988). It is worth noting that our animals were not fed during the experiments cited here, so hunger could have influenced their propensity to express tidal rhythms.

There appear to be additional effects of LD cycles on circatidal rhythms. In two previous studies, LD cycles seem to allow for more robust circatidal rhythms in the semiterrestrial crab (Saigusa 1992) and the Pacific fiddler crab *Uca princeps* (Stillman and Barnwell 2004). Similarly, we have seen clearer tidal rhythms in LD than DD in horseshoe crabs (Chabot and Watson 2010). Although one would not expect photoperiod to have any effect on circatidal rhythms, because photoperiod is obviously not a strong predictor of tidal phase, light may indirectly act on the underlying oscillators

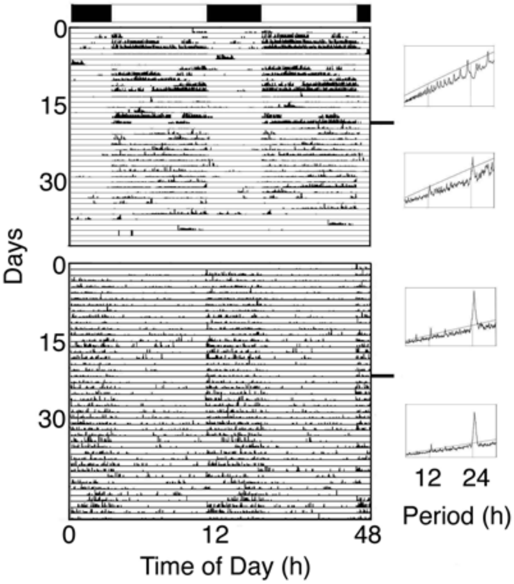

Fig. 3.9 Running-wheel activity of two horseshoe crabs exposed to a 14:10 LD cycle that exhibited a preference ($P < 0.05$) for daytime activity (*top*) or nighttime activity (*bottom*). (Reproduced from Chabot et al. 2007 with permission from Elsevier)

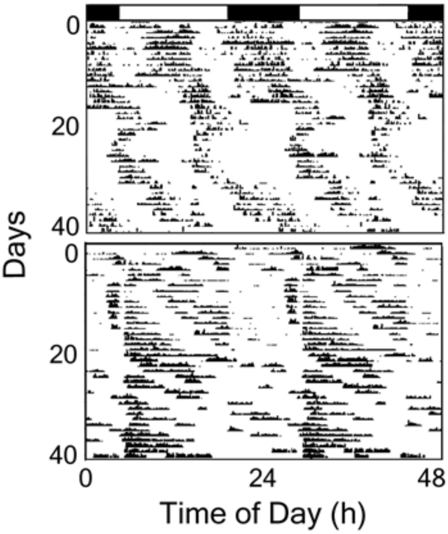

Fig. 3.10 Effects of LD cycles on locomotor activity in two *Limulus*. *Top*: Note the apparent transient synchronization to the LD cycles for the first 20–30 days and the subsequent free-run at approximately day 26. *Bottom*: Note the bouts of tidal activity that free-run through the first few weeks during L but not D. Note also the instant increase in activity upon lights on and the instant cessation of activity at lights off. (Reproduced from Chabot and Watson 2010, with permission from the Editorial Office, *Current Zoology*)

controlling locomotor activity. In particular, the lights-on and lights-off transitions appear to cause a large percentage of animals to begin to move (Fig. 3.10). In mammals, an initiation of activity can induce phase shifts in some species (Wickland and Turek 1991; Gannon and Rea 1995; Buxton et al. 2003). A similar feedback into the clocks that mediate circatidal rhythms may help to explain how light may affect circatidal rhythms. Another working hypothesis is that in bays and estuaries, characterized by turbid water that limits the penetration of light, horseshoe crabs are exposed to tidally fluctuating light levels during the day. In Great Bay, for example, light levels drop by about 1 order of magnitude for each 1 m of depth. Therefore, when horseshoe crabs are in shallow water they are exposed to changes in light levels with each tide cycle, and when they move into very shallow water to mate, the change is even greater. Thus, the clock controlling circatidal rhythms might be sensitive to changes in illumination to provide a redundant tidal zeitgeber.

Overall, it appears that for many species multiple, possibly redundant, cues enable intertidal organisms to synchronize to tidal cycles. Water-level changes seem to be the strongest entraining agent for horseshoe crab activity, but turbulence and temperature appear to contribute to entrainment as well. In a number of marine animals one cue is not sufficient to entrain their behavior, but when presented with a key zeitgeber the entrainment is further enhanced. One species, the amphipod *Corophium volunator*, entrains to cycles of 10 °C but shows greater entrainment when subjected to temperature cycles in conjunction with cycles of immersion (Holmstrom and Morgan 1983). Water pressure and immersion cycles appear to also be important synchronizers in *C. maenas*, and temperature may also serve to enhance entrainment to these cues (Naylor et al. 1971). Similar findings have been observed in other crustacean species as well (Williams and Naylor 1969; Naylor and Williams 1984).

3.3.4 Seasonal Changes in Activity: The Effects of Temperature and Photoperiod

There are few documented seasonal changes in circatidal activity patterns in intertidal invertebrates, and nearly all of these appear to be rhythms of larval release, not locomotion (Thurman 2004). However, the phasing of circatidal rhythms appears to be seasonally adjusted in the amphipod *Corophium volutator* (Harris and Morgan 1984), which the authors attributed to interactions between environmental temperature and endogenous rhythms.

Horseshoe crabs have very clear seasonal changes in behavior that appear to be modulated by temperature. In the spring, in New Hampshire, they become active when the water temperature reaches 8–10 °C or higher (Schaller et al. 2010) and breed at the water's edge during high tides for about a 1-month period in the late spring (Watson et al. 2008). The timing of the breeding season varies somewhat along the East Coast of the United States, with animals mating a little earlier in the year in more southern regions (Barlow et al. 1986; Rudloe 1980; Brockmann and Smith 2009). They remain very active during the remainder of the summer and into the fall (Schaller et al. 2010) and then become more and more sedentary and quiescent when the water temperature drops below about 8 °C (Watson et al. 2009; Watson and Chabot 2010).

Virtually the same pattern of activity occurs in horseshoe crabs exposed to different temperatures in the laboratory, regardless of the LD cycles to which they are exposed. When horseshoe crabs in running wheels were exposed to 17 °C, the majority of them expressed tidal rhythms of activity (Fig. 3.11) (Watson et al. 2009; Chabot and Watson 2010). In contrast, if the water temperature was reduced to

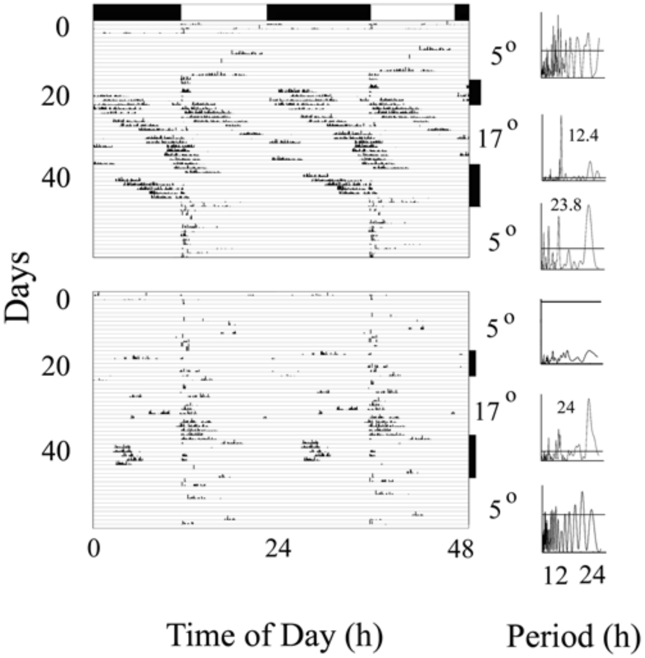

Fig. 3.11 Effects of water temperature on the expression of endogenous circatidal rhythms in two *Limulus* exposed to LD. Periods of increasing or decreasing temperatures are indicated by *black boxes* to the immediate right of the actograms. *Right panels*: Lomb–Scargle periodograms of portions of the activity records as delineated by temperature. *Horizontal line*: level of significance ($P < 0.01$). *Values above line*: highest peak period within the circatidal (10.4–14.4 h) or circadian range (22–26 h). (Reproduced from Chabot and Watson 2010, with permission from the Editorial Office, *Current Zoology*)

11 °C or below, they did not express a tidal rhythm of locomotion, even if they were exposed to an LD cycle of 14:10, typical of the spring and summer. At 4 °C, activity rhythms were completely suppressed and activity levels were greatly attenuated.

3.4 Endogenous Clocks Controlling Circatidal Activity

Although circatidal activity patterns have been documented in many intertidal species, the nature of the clock system that controls these rhythms is somewhat controversial (Palmer 1995b, 1997; Naylor 1996, 1997). An outstanding question in the field of biological rhythms is whether the locomotor activity of intertidal species is driven by two circalunidian clocks (clocks with a free-running periodicity similar to the periodicity of the lunar day, i.e., 24.8 h) or by one circatidal clock. Naylor and colleagues (Reid and Naylor 1989) have hypothesized that intertidal animals have one circatidal clock, with a period of about 12.4 h, that drives behavioral rhythms and one circadian clock which modifies those rhythms (Naylor 1958). Alternatively, the

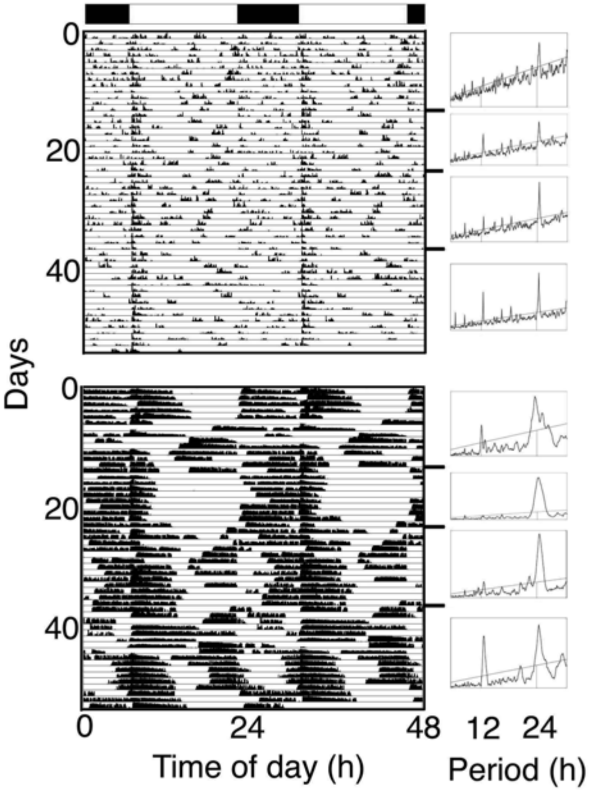

Fig. 3.12 Running-wheel activity of two horseshoe crabs exposed to14:10 LD and exhibiting "masking" (direct effects of lights-on or lights-off) as well as spontaneous changes in *tau* (*bottom panel*). *Top panel:* lights on, increased activity; *bottom panel:* lights off, increased activity. (Reproduced from Chabot et al. 2007 with permission from Elsevier)

circalunidian hypothesis, first proposed by Palmer and Williams (1986), proposes that circatidal rhythms can be best explained by the presence of two circalunidian clocks, each with a period of about 24.8 h (lunidian) and 180° out of phase with one another. Behaviorally, this can lead to major activity bouts every 12.4 h, just like a circatidal clock would. Currently, our data (Figs. 3.12, 3.13) seem to fit the three criteria put forth by Palmer (1997) as part of the circalunidian hypothesis: (1) evidence of two components of rhythms that scan the day at different periodicities (Fig. 3.12, bottom panel); (2) evidence of "skipping," sudden alternations of unimodal and bimodal patterns (Fig. 3.13, top panel, circled; and (3) "splitting," the separation of one component into two components (Fig. 3.12, bottom panel). All three of these criteria were most clearly met by the activity patterns illustrated in these figures (Figs. 3.12 and 3.13). Similar splitting of two "circalunidian components" has also been seen in the locomotor activity of the crab *Helice crassa* (Palmer and Williams 1986). However, the relative rarity of these phenomena in our data suggests that if there are two oscillators governing the timing of locomotion in *Limulus* they are strongly coupled. Thus, although we have some evidence to support the circalunidian hypothesis, convincing evidence supporting a coupled, two-oscillator system (such as is found in trout; Erikson 1973, cited in Pittendrigh 1981; hamsters Pittendrigh and Daan 1976; and *Drosophila* Stoleru et al. 2004) has yet to be obtained.

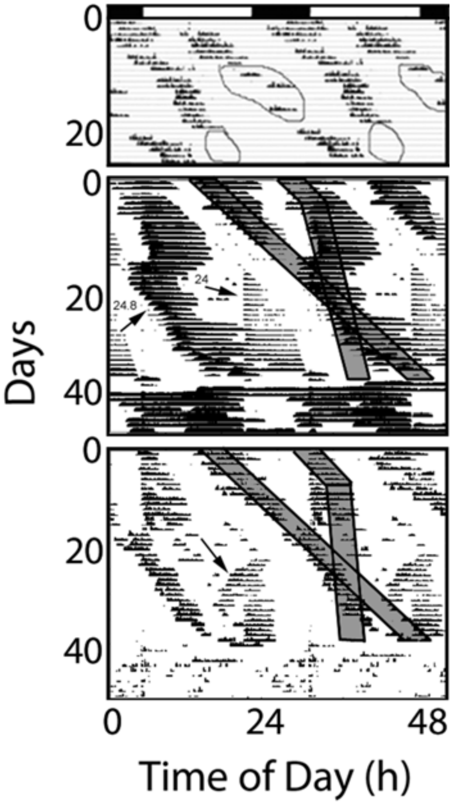

Fig. 3.13 Evidence for the presence of two circalunidian oscillators in *Limulus polyphemus* exposed to LD. *Top panels*: Free-running circatidal activity rhythm shows "skipping" of one of two components (*circled*). *Bottom two panels*: Effects of 12.4 and 12.1 cycles of water pressure on locomotor activity patterns in *Limulus*. Rising water levels indicated by *shaded boxes*: first 8 days, 2 cycles/day delivered each at 12.4 h; next 30 days, 2 cycles/day (one at 12.4 h; other at 12.1 h); remaining 15 days, constant water levels. Note the different periods of each component in the *middle panel* (*two arrows,* values = associated tau). Note also the apparent drift of at least one of the components (*arrow,* tau = 11.6 h) in the *bottom panel* when the 12.1-h tides were initiated. (Reproduced from Chabot and Watson2010, with permission from the Editorial Office, *Current Zoology*)

3.5 Molecular Mechanisms

Much is known about the molecular basis of circadian clocks, but virtually nothing is known about the molecular basis of circatidal clocks. The animal circadian clock has a core molecular mechanism based on the products of four or five genes that undergo a transcriptional and translational rhythm of approximately 24 h (Bell-Pedersen et al. 2005; Hardin 2009). Importantly, these core proteins appear to be conserved in marine invertebrates such as *Aplysia* and *Bulla* (Siwicki et al. 1989), and the lobster (Grabek and Chabot 2012). As it is thought that "evolution is a tinkerer" (Jacob 1977), it is tempting to speculate that the molecular underpinnings of the circadian clock may have been co-opted and modified to create clocks that help intertidal animals to synchronize to tidal cycles. As detailed earlier, one model for how circatidal rhythms might be controlled calls for two circalunidian clocks that keep time in the circadian range (periods of 24.8 h are still well within the circadian range) (Palmer 1995a), but are 180° out of phase, so that each clock could influence the probability that an animal would be active during one of the two high (or low) tides in a day. The presence of two clocks (that beat in this range) has been established in many organisms, including hamsters (Pittendrigh and Daan 1976) and fruit flies (Stoleru et al. 2004). In *Drosophila*, these two clocks couple the animal's activity cycle to dusk and dawn independently, and they are composed of two separate populations of cells in the fly's brain (Stoleru et al. 2004). Therefore, it is not too much of a stretch to imagine that circatidal rhythms might be controlled in the same way, by two circalunidian clocks of about 24.8 h. Efforts are currently underway in several laboratories to investigate this possibility.

3.6 Summary

The rhythms of marine intertidal invertebrates are truly enigmatic. Although many of the animals that inhabit the tidal zone exhibit tidal rhythms, these rhythms often disappear and reappear spontaneously. Although the disappearance of these rhythms in the field can be explained by strong winds, foraging, satiety, large salinity events, etc., the spontaneous disappearance of the rhythms in the lab is certainly enigmatic. The fact that tidal rhythms are not as robust as circadian rhythms may also be explained by comparing the high fidelity of LD cycles to the much lower fidelity of environmental tidal cycles. However, perhaps the observation that "muddies the water the most" (pun intended) is that behavioral tidal rhythms are often modulated, and even synchronized, by LD cycles. Although there are plausible explanations for this synchronization, none of them is completely compelling and a clear explanation of this phenomenon must await genetic and molecular analyses. What is clear is that a number of animal models have emerged to enable researchers to begin to open the "black box" and to identify the molecular gears that keep time in accordance with tidal and solar cycles.

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Chapter 4
Circatidal and Circadian Rhythms in Crustacean Swimming Behavior

Tadashi Akiyama

Abstract Marine animals occasionally exhibit complicated temporal activity patterns, which are affected by both tidal and solar day cycles. The cumacean *Dimorphostylis asiatica* (Crustacea, Peracarida) swims up in the water column around nocturnal high tides. In the laboratory, the animals show a circatidal swimming rhythm, entrained by hydrostatic pressure stimulus. During recording, the daily bimodal circatidal rhythmicity spontaneously changes to a circadian daily unimodal light-entrained rhythm.

Keywords Circadian • Circatidal • Entrainment • Splitting • Swimming activity rhythm

4.1 Introduction

Outputs of endogenous biological rhythms entrained by environmental tidal cycles, that is, circatidal rhythms, have been reported in many marine and semiterrestrial organisms (Neumann 1981; Morgan 1991; Palmer 1995; Naylor 2010). Occasionally, circatidal rhythms exhibit a rather complicated temporal pattern corresponding to environmental tidal and solar day periodicities.

The swimming activity rhythm of two sand beach peracarid crustaceans, the amphipod *Synchelidium* sp. and the isopod *Excirolana chiltoni*, has a bimodal activity pattern, and the two activities are conjugated (i.e., free-run together). The amplitudes of the conjugated activities are unequal; that is, one of two activity bouts on a single day is more intensive or longer lasting than the other. The rhythm occasionally changes between daily bimodal and daily unimodal patterns spontaneously, which was explained as environmental adaptation of the rhythmicity to the mixed semidiurnal tide that gradually changes according to the lunar phase (Enright 1962, 1972, 1976; Klapow 1972). Similar daily bimodal circatidal rhythms with unequal amplitudes have been reported in a few marine animals (Holmström and Morgan 1983a; Palmer and Williams 1986a; Northcott 1991; Sato et al. 2008).

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Marine animals are exposed to solar day cycles as well as to tidal cycles. As a result, their activity rhythms occasionally exhibit complicated temporal patterns including tidally synchronized and solar day-synchronized rhythmic components, other than the only tide-synchronized complicated rhythmic patterns already mentioned (Barnwell 1966; Stillman and Barnwell 2004; Zann 1973; Barlow et al. 1986). In the terrestrial crab *Sesarma*, timing of larval release, synchronized with the night high tide, is entrained by solar day cycles as well as moonlight cycles (Saigusa 1986, 1988). In the shore crab *Carcinus* and some fishes kept under non-tidal conditions, their daily bimodal circatidal locomotor rhythm changes to daily unimodal circadian rhythm (Naylor 1958; Gibson 1971, 1973). In the daily bimodal circatidal locomotor rhythm of the mangrove cricket *Apteronemobius asahinai*, the uneven amplitude of the conjugated activity is thought to be generated by a circadian component of the underlying pacemaker (Takekata et al. 2012).

Studies of the complicated rhythmicity as just mentioned, may provide some new insights into the evolutionary trait of circatidal clocks as well as their current divergence. In this chapter, I describe the spontaneous change of swimming activity rhythm between circatidal and circadian in the cumacean *Dimorphostylis asiatica* (Crustacea, Peracarida) from the Seto Inland Sea of Japan.

4.2 Temporal Emergence Pattern in the Field and Its Seasonal Variation

Planktonic animals show diurnal vertical migration, based on swimming upward in the water column (Pearcy 1970; Enright 1977). Tidal planktonic animals may perform similar movements. Cumaceans, consisting of about 1,500 species, live in the surface of sandy or muddy sea bottoms, from the intertidal to the hadal zone. The genus *Dimorphostylis*, consisting of 29 species, lives in shallow water in the Western Pacific and Indian Ocean, currently with only one deep-sea species (Băcescu 1988; Gamô 1968; Akiyama 2011). Field studies on shore and shallow-water cumacean species have also shown diurnal vertical migration, based on their nocturnal upward swimming (Corey 1970; Yoda and Aoki 2002). The temporal emergence pattern of *D. asiatica* from the Seto Inland Sea, at 1–3 m depth, where a mixed semidiurnal tide about 1.5–3.5 m in amplitude recurs, was investigated using an electric torch lamp attracting male specimens (Akiyama and Yoshida 1990). The emergence pattern in the field shows rather complicated rhythmicity, which varies with seasons (Fig. 4.1). In winter and spring, the animals emerge around the time of the night high tide every day, showing a daily unimodal tidal rhythm with a period of about 24.8 h (Fig. 4.1a). A similar emergence pattern in shallow-water habitats has been reported in other tiny crustaceans such as a cladoceran, a copepod, an amphipod, and a few crab larvae (Saigusa and Oishi 2000; Saigusa 2001).

In contrast to the emergence pattern in winter, which shows close synchrony with night high tides, in autumn the emergence mainly occurred within 1 h just after sunset (Fig. 4.1b), showing solar day synchronized rhythmicity. Small portions of the

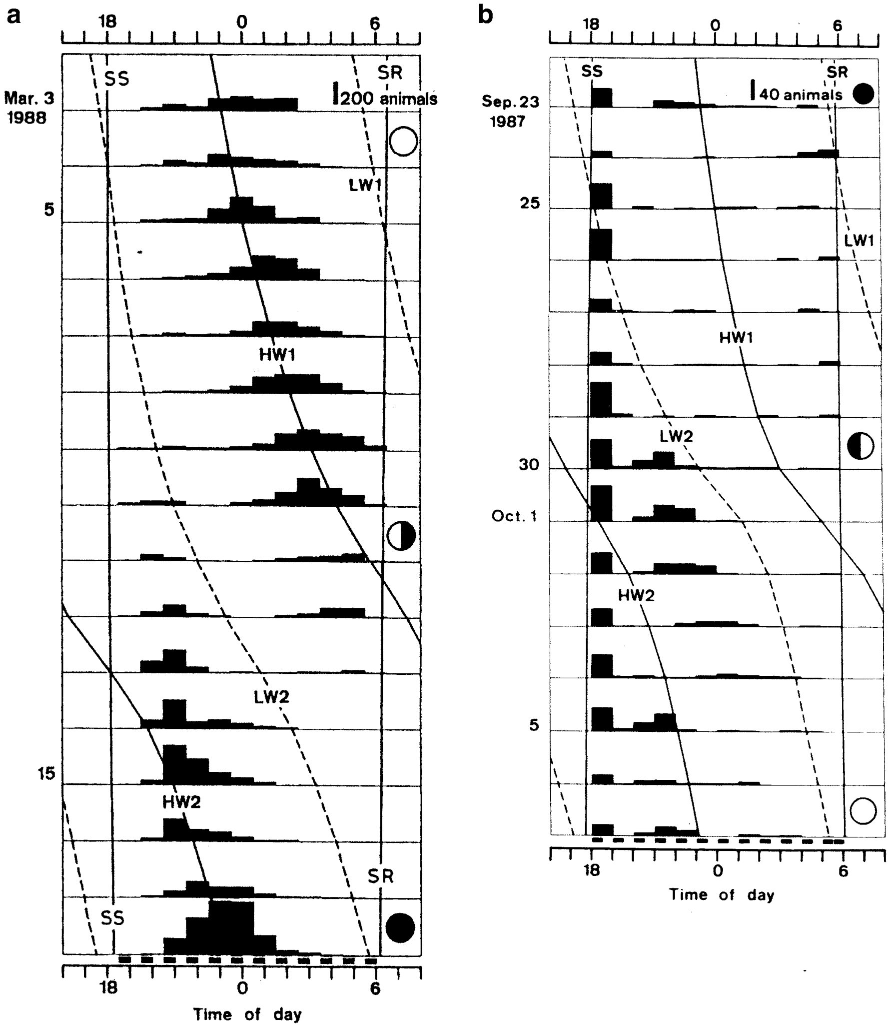

Fig. 4.1 Temporal emergence pattern of *Dimorphostylis asiatica*. Emergence during each time interval was indicated as number of adult male animals collected with an electric torch lamp for 30 min. (**a**) Emergence pattern in winter. *HW* high water, *LW* low water, *SS* sunset, *SR* sunrise; *open circle* full moon, *closed circle* new moon (Akiyama 1995). (**b**) Emergence pattern in autumn. (From Akiyama and Yoshida 1990; reproduced by courtesy of The Marine Biological Laboratory, Woods Hole, Massachusetts)

animals emerge during ebb (between 30 September and 4 October in Fig. 4.1b) or flow tides (5–7 October in Fig. 4.1b). Seasonal variation of tide-synchronized emergence rhythm between day and night in the field was also reported in the emergence rhythm of the marine midge *Clunio tsushimensis* (Oka and Hashimoto 1959; Saigusa and Akiyama 1995). In this case, the seasonal change of the temporal emergence pattern was related to the seasonal change of temporal tidal pattern. The height of the daytime low tide is lower than the height of the night low tide in late spring and summer, whereas in winter the opposite tidal pattern occurs at the sampling site.

4.3 Free-Running Activity Rhythm and Temperature Compensation

Diurnal vertical migration of many marine organisms based on their swimming activity shows circadian rhythmicity in the laboratory (Enright and Hamner 1967). Male specimens of *D. asiatica* show a clear swimming activity rhythm in constant darkness, indicating that this behavior is also governed by endogenous clocks (Fig. 4.2). In this species, the rhythmicity is regarded as circatidal but not circadian, which is based on the observations that in the presence of tidal periodicity (1) the daily bimodal activity pattern has a period length close to the environmental tidal period (12.4 h) and (2) the onset of activities coincides with the expected high tide on the first day of the recording.

The calculated free-running period of the daily bimodal rhythm was approximately 11.5 h on average, 1 h shorter than the environmental tidal cycle (Fig. 4.2a). Coincidence of the endogenous swimming activity with the expected high tide was rapidly lost during the first 1 or 2 days of the recording. Previous studies on the circatidal rhythms have focused on close similarity of the free-running periods to environmental tidal periods (Palmer 1995; Naylor 2010). As an extreme case, the sandy beach isopod *Excirolana* shows a temporal free-running activity pattern that is very similar to the complicated environmental tidal pattern (Enright 1972). The rather short free-running period of *D. asiatica* in its daily bimodal rhythm shown in my study may be a generic characteristic. An estuarine *Dimorphostylis* sp. from the Seto Inland Sea of Japan, kept in seawater at 15 ‰ salinity in the laboratory, exhibited a daily bimodal free-running rhythm with a period shorter than 24 h, which was similar to that of *D. asiatica* (T. Akiyama, unpublished data).

The specimens collected in all seasons exhibit distinct daily bimodal activity rhythms, at least during the early days of recording, and onsets of the first batch of activity on the first day of recording occur about 0.5 h before the expected high tide throughout a year (Akiyama 2004), which suggests that the circatidal clock of the animals operates in the field in all seasons. Therefore, the seasonal variation of emergence pattern in the field (shown in Fig. 4.1) should be caused by the environmental factors that directly affect their swimming behavior, not via endogenous clocks of the animals. In another peracarid crustacean, *Corophium voltator*, seasonal variation of circatidal activity rhythm was also observed in the laboratory (Holmström and Morgan 1983a).

Although the environmental tide in the Pacific Coast of southern Japan is a mixed semidiurnal tide, the bimodal circatidal swimming rhythm of most specimens is characterized by equal amplitude of two conjugated activities (Fig. 4.2a). A small portion of the specimens exhibit daily bimodal rhythms with unequal amplitude of conjugated activities. However, this phenomenon would not necessarily

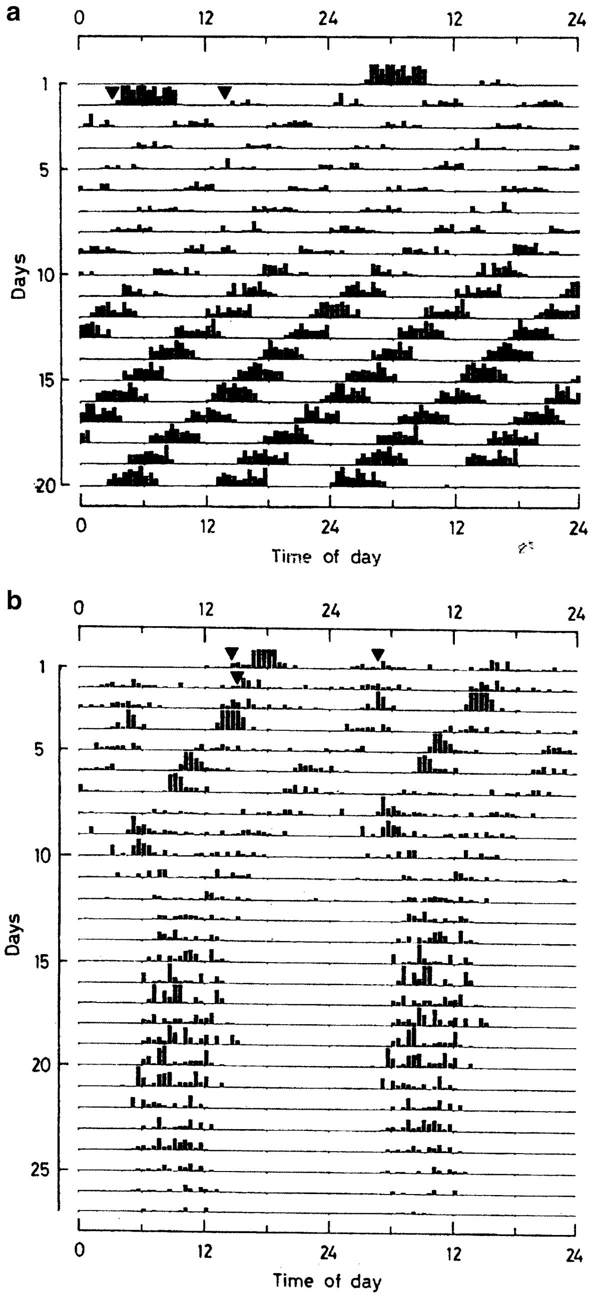

Fig. 4.2 Free-running swimming activity rhythm of two specimens of *Dimorphostylis asiatica* under constant darkness (Akiyama 1995). (**a**) A specimen that retained daily bimodal activity pattern throughout 20 days of recording. (**b**) A specimen whose activity pattern changed from daily bimodal to unimodal on days 10–12. *Arrowheads* indicate times of high water at the sampling site

be related to the environmental tidal pattern. In the mangrove cricket, a daily bimodal rhythm with unequal amplitude is explained as modification of the circatidal rhythm by a circadian pacemaker (Takekata et al. 2012).

In *D. asiatica*, the daily bimodal activity rhythm in about 90 % of the specimens examined spontaneously changed to the daily unimodal rhythm, within 10 days (it took 11–12 days in the specimen shown in Fig. 4.2b). In contrast to that just described for *Excirolana*, the change of the rhythmic pattern does not seem to be related to the environmental tidal pattern because the bimodal rhythms with equal amplitude of conjugated activity occur in most specimens in spite of the mixed semidiurnal tide at the collection site.

As in circadian rhythms, the free-running period of circatidal rhythms shows relatively small change according to change of ambient temperature (Naylor 2010). The free-running period of the daily bimodal rhythms in *D. asiatica* is 23.1 ± 1.6 and 22.9 ± 1.1 h at 10 and 15 °C, respectively. In the daily unimodal rhythm, the period is 25.5 ± 1.8 and 23.8 ± 0.6 h at 10 and 15 °C, respectively (Akiyama 1995). Temperature compensation was shown in the free-running periods of the daily bimodal and unimodal rhythms; Q_{10} values were 1.02 and 1.15, respectively, even though the latter value is slightly larger than values for circadian and circatidal rhythms of other animals. The difference in the compensation between the daily bimodal and unimodal free-running rhythms suggests that these rhythms are governed by pacemakers with different molecular oscillation systems. Otherwise, if identical pacemaker(s) govern the different forms of rhythmicity, neural components other than the molecular oscillation system may generate different periodicity. Unfortunately, previous studies on circadian and circatidal rhythms have not focused on comparison of temperature compensation accompanied by change of rhythmicity between daily unimodal and daily bimodal in a single species.

4.4 Administration of Hydrostatic Pressure Stimulus

Although circadian rhythms are dominantly entrained by sunlight (Binkley 1990), circatidal rhythms are phased by various kinds of time cues, such as mechanical agitation caused by water flow and wave action (Enright 1965, 1976; Klapow 1972; Hastings 1981; Morgan and Cordiner 1994), change of ambient temperature (Holmström and Morgan 1983b, c; Bolt et al. 1989), salinity change (Taylor and Naylor 1977; Bolt and Naylor 1985; Bolt et al. 1989), and periodic inundation of intertidal habitat (Holmström and Morgan 1983c; Yoshioka 1989; Chabot et al. 2008; Sato et al. 2009). Tide-synchronized larval release of a few species of terrestrial and semiterrestrial crabs is additionally phased by moonlight cycles or solar day sunlight cycles (Saigusa 1986, 1988, 1992).

For marine animals living in shallow, calm habitats, including *D. asiatica* from the Seto Inland Sea of Japan, periodic change of hydrostatic pressure stimulus caused by gradual fluctuation of tidal height would be a reliable tidal time cue for their circatidal clocks. The swimming behavior of tiny crustaceans responds to sudden change of hydrostatic pressure (Enright 1961; Digby 1972; Morgan 1984). Successful entrainment by a 12.5-h cycle of sinusoidal change of hydrostatic pressure was reported for the swimming behavior of an estuarine amphipod, *Corophium*

(Morgan 1965). Thereafter, entraining effects by hydrostatic pressure cycles on circatidal rhythms have been reported in the shore crab *Cacinus* (Naylor and Atkinson 1972), the horseshoe crab *Limulus* (Chabot and Watson 2010), and a few fish species (Gibson 1971, 1984; Northcott 1991; Northcott et al. 1991a, b).

A 12.5-h cycle of sinusoidal change of hydrostatic pressure of 0.3 atm, equivalent to 3-m amplitude of tidal height, entrains the circatidal swimming rhythm of *D. asiatica* (Akiyama 2004). Most specimens (*N*=41) examined displayed daily bimodal activity with periods of 24.9±0.6 h, as shown on days 1–10 in Fig. 4.3a, b,

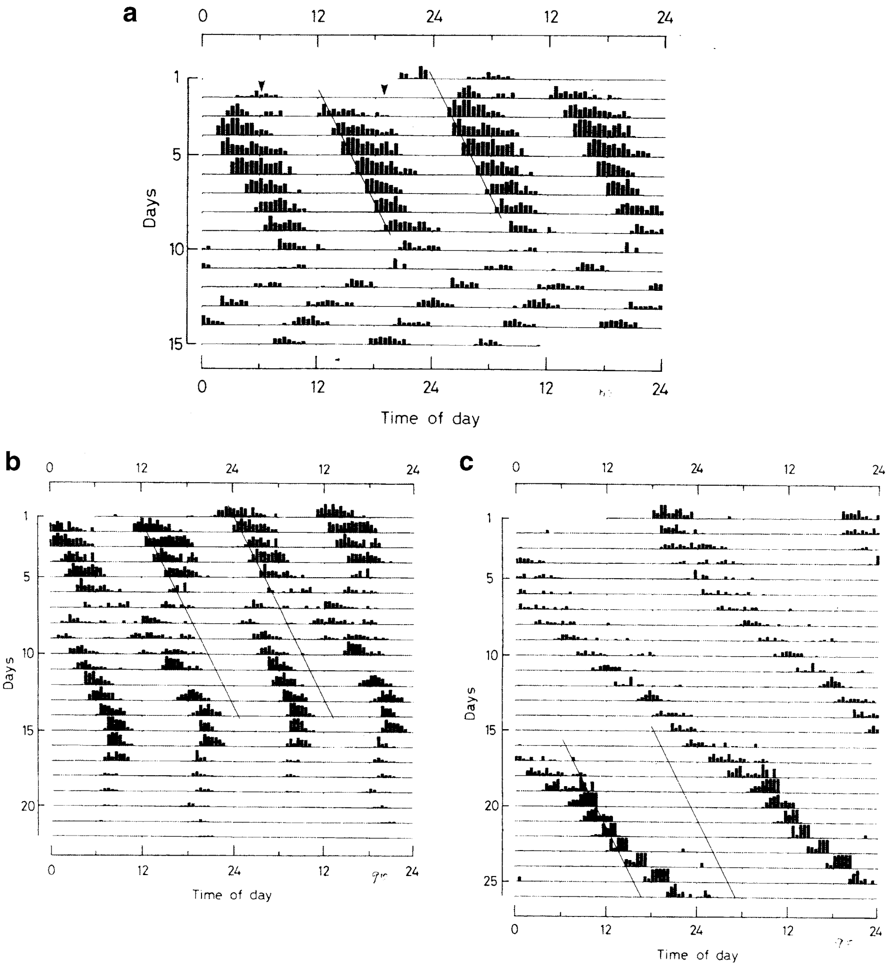

Fig. 4.3 Three *Dimorphostylis asiatica* specimens exposed to a 12.5-h cycle of sinusoidal change of hydrostatic pressure with 0.3-atm amplitude, under constant darkness. *Oblique lines* indicate times of maximum hydrostatic pressure (Akiyama 2004). (**a**, **b**) Specimens exhibiting daily bimodal rhythmicity. (**c**) Specimen exhibiting daily unimodal rhythmicity

which is about 2 h longer than the free-running period of the daily bimodal rhythm. The activity onsets of the animals occur shortly before the time of the maximum hydrostatic pressure. Correspondingly, the onset of the first bout of activity in the free-running rhythm occurs 0.5 ± 2.1 h before the expected high tide in all seasons (Akiyama 2004). Thus, a hydrostatic pressure cycle can clearly entrain the circatidal rhythm of this species. It therefore appears that the entrained bimodal activity pattern is characterized by simple 12.5-h rhythmicity, with roughly equal amplitude and duration of the two conjugated activity bouts.

A small portion of individuals exhibit a daily unimodal activity rhythm while they are exposed to the hydrostatic pressure cycles (Fig. 4.3c). The activity period is 25.9 ± 1.6 h ($N = 11$), which is close to the free-running period at 10 °C, suggesting that the pacemaker that governs the unimodal activity pattern is not phased by hydrostatic pressure stimulus.

Phase responsiveness of circatidal rhythms to single pulses of zeitgebers has been examined and summarized as phase-response curves (PRCs) in an isopod *Excirolana* (Enright 1976), an estuarine amphipod *Corophium* (Holmström and Morgan 1983b), the rocky shore crab (Naylor and Williams 1984), a shore fish (Northcott et al. 1991b), the mangrove cricket (Sato et al. 2009), and a few additional marine species. The results seem to be rather simple; that is, PRCs of circatidal rhythms are similar to those of circadian rhythms, but on a 12.4-h time base.

In *D. asiatica*, phase responsiveness of the swimming rhythm to a 4-h hydrostatic pressure pulse (sudden increase and decrease of hydrostatic pressure of 0.3-atm amplitude) was examined. A unique characteristic of the phase responsiveness was that two conjugated activity bouts occasionally respond to the 4-h pulse of the hydrostatic pressure stimulus differently. The specimen shown in Fig. 4.4a shows a different magnitude of phase delay, and another specimen (Fig. 4.4b) shows phase advance and phase delay for each conjugated activity bout, respectively. These data suggest that the approximately 12-h rhythm, in appearance, of *D. asiatica* is composed of two 24-h pacemaker components (Fig. 4.4b).

PRCs of *D. asiatica* specimens (indicated on a 24-h time base; the onsets of daytime activity on the first day of recording are determined as circadian time 12), in which daily bimodal rhythm persisted after administration of a hydrostatic pressure stimulus have an obscure form (Fig. 4.4e). Both phase advance and delay occur around circatidal times 8 and 20, in contrast with the entraining ability of 12.5-h cycles of 'sinusoidal' change of hydrostatic pressure on the daily bimodal rhythm within 1–2 days of treatment (Fig. 4.3a). Apparently, the animals perceive a gradual increase or decrease of hydrostatic pressure as the dominant tidal time cue. Thus, the obscure PRC pattern may be a consequence of the sudden increase or decrease of hydrostatic pressure in this experiment.

Another possible factor causing the obscure form of the PRC is the unstable phase relationship between the entrained activity and the hydrostatic pressure pressure cycle: some specimens entrain the swimming activity to the phase of increasing pressure, whereas other specimens entrain to the phase of decreasing pressure, and rarely were both phase relationships observed sequentially in a single specimen (Fig. 4.3b). Such a flexible phase relationship between entrained rhythmicity and environmental cycles is also possibly attributed to the weak phase-setting effect of

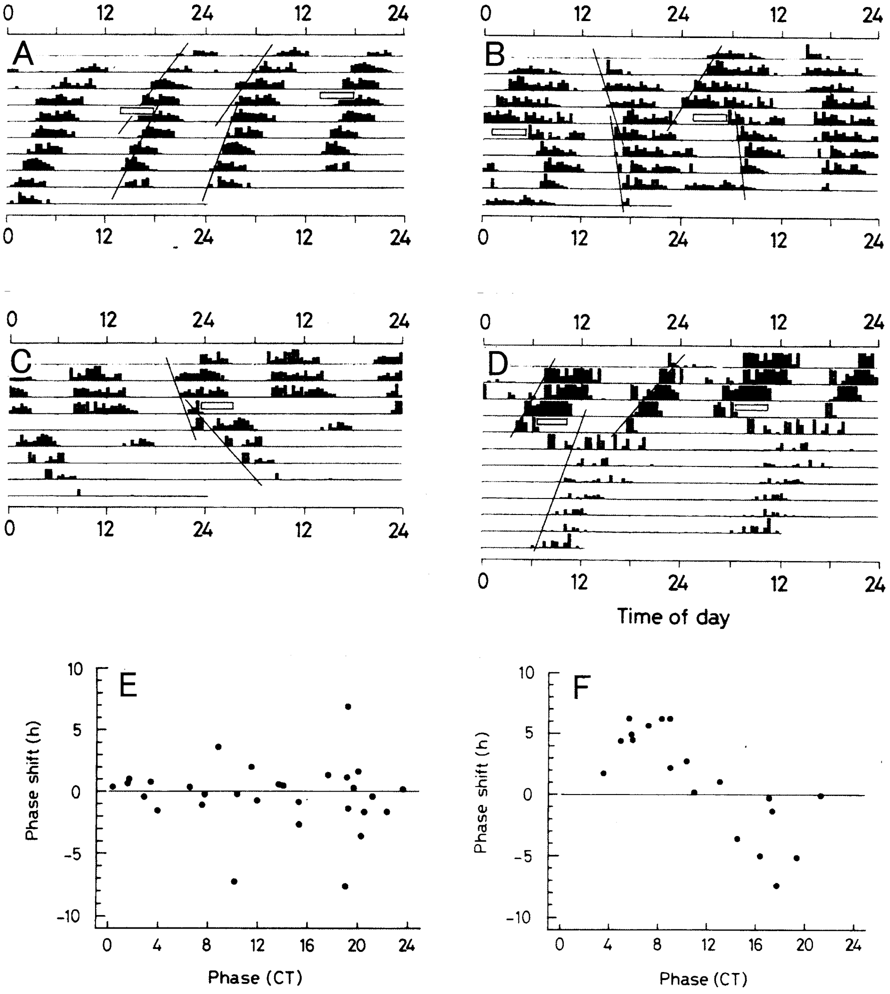

Fig. 4.4 Phase-setting effect of a 4-h pulse of hydrostatic pressure with 0.3-atm amplitude on the swimming activity rhythm of *Dimorphostylis asiatica* (Akiyama 1997). (**a**–**d**) Activity pattern of four specimens. *Rectangles* indicate times of treatment. *Oblique lines* are linear regression lines on the successive onsets of swimming activities. (**e**, **f**) Phase–response curves (*PRCs*) of the daily bimodal rhythms to the hydrostatic pressure pulses. CT circadian time. Time of activity onsets near the time of treatments was determined as CT12. (**e**) PRC of specimens that retained a daily bimodal rhythm after treatment (**a**, **b**). (**f**) PRC of specimens that exhibited a daily unimodal rhythm after treatment (**c**, **d**)

the hydrostatic pressure stimulus, as reported in the diurnal–nocturnal conversion of the circadian rhythm of fishes (Sánchez-Vázquez et al. 1995; Iigo and Tabata 1996).

In nearly half the treated specimens, daily bimodal activity rhythms changed to daily unimodal patterns immediately after administration of hydrostatic pressure pulses (Fig. 4.4c, d). For these specimens, phase advance and delay occur around circadian times 8 and 18 (Fig. 4.4f), respectively, showing that the PRC form is on a 24-h time base (Fig. 4.4f). A similar change of activity between daily unimodal and bimodal patterns, associated with treatment with a single pulse of tidal time cue, was also observed in *Excirolana*. However, in *Excirolana* the PRC pattern was simply 12.4-h intervals (Enright 1976). The PRC form of *D. asiatica* may be the first case of a PRC pattern of circatidal rhythm on a 24-h time base, suggesting that the animals recognize daytime or nighttime high water, even when the animals are exhibiting only circatidal rhythms. In view of adaptive significance, the PRC of the 24-h time base suggests that the animals recognize 'daytime' high tides as the more reliable time cue. During nighttime, swimming in the water and settling on the sea bottom at different depths would result in sudden change of environmental hydrostatic pressure, which would disturb the sensing of tidal time cue(s).

In conclusion, in contrast to the daily bimodal activity rhythm, the daily unimodal rhythm is not entrained by a single pulse of a 4-h hydrostatic pressure stimulus, which agrees with the fact that the unimodal rhythms are not entrained by 12.5-h hydrostatic pressure cycles (Akiyama 1997).

4.5 Administration of Light Stimulus

Current knowledge on phase-setting effects of light stimuli on circatidal pacemakers is limited, except for a few species. In the horseshoe crab *Limulus*, light–dark cycles entrained the daily bimodal circadian rhythm in a small portion of specimens (Chabot et al. 2007; Chabot and Watson 2010). Shift of light cycles causes phase shift of the larval release rhythm in the terrestrial crab *Sesarma* (Saigusa 1986, 1988), and this phenomenon is possibly related to their nontidal habitat, where the phase relationship of moonlight and solar day cycles can be a useful time cues.

Many *D. asiatica* specimens kept under light–dark (LD) cycles of LD 12:12 (about 1,000 lx for light phase; complete darkness for dark phase) exhibit daily unimodal activities during the dark phase, even during the first few days of recordings (Akiyama 2004). In contrast, the temporal activity pattern of the specimens kept under light cycles with a shorter photoperiod, such as LD 6:18, shows that the daily bimodal activity free-runs with periodicities shorter than 24 h. In the specimen shown in Fig. 4.5a, the activity rhythm free-ran on days 1–10, and entrained to the hydrostatic pressure cycle thereafter, with inhibition of activities during light phase throughout the recording. Thus, the unimodal activity pattern under LD 12:12 is the result of a masking effect that is caused by direct inhibition of activity by light. Specimens exposed to the 12.5-h hydrostatic pressure cycles and light cycles with longer photoperiod, such as LD 12:12, exhibit a temporal activity pattern similar to the tide-synchronized emergence pattern in the field, suggesting that the animals use these two cycles as important time cues in the field. Small bursts of activity shortly after lights off (on days 7–15 in Fig. 4.5b, also seen in Fig. 4.5a) also agree with the emergence just after sunset in the field (Fig. 4.1b).

The masking effect by light has been well documented in circadian rhythms (Page 1989). Nocturnal activity by direct suppression of the swimming activity by

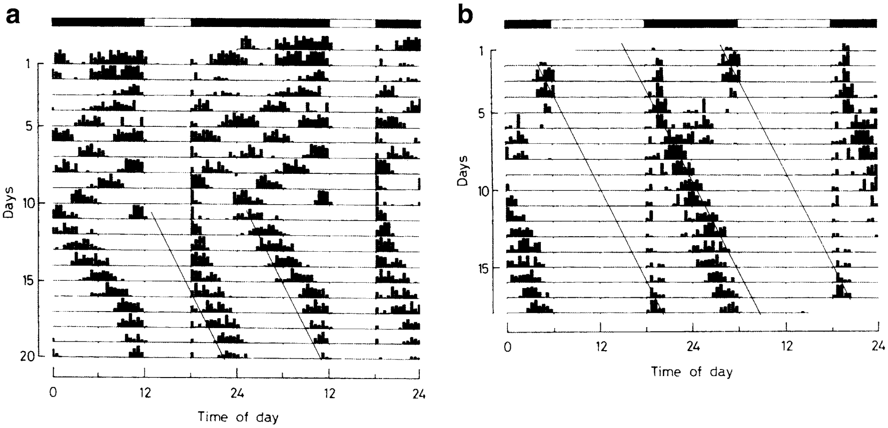

Fig. 4.5 Activity records of *Dimorphostylis asiatica* exposed to different light cycles and the 12.5-h hydrostatic pressure cycle. (**a**) A specimen kept under light regime LD 6:18; on day 10, the hydrostatic Zeitgeber was added. (**b**) A specimen kept under LD 12:12. *Oblique lines* indicate the times of maximum hydrostatic pressure (Akiyama 2004)

light stimuli during daytime is also observed in the diurnal vertical migration in shallow-water planktonic animals (Enright and Hamner 1967).

A problem in studies on circatidal rhythms is their noisy, short-life nature (Neumann 1981). Such noisiness may not reflect the output of an inferior clock mechanism but rather internal desynchronization of clock components in single specimens. In the eel pout *Zoarces viviparus*, desynchronized, arrhythmic swimming activity spontaneously changes to rhythmic daily unimodal or bimodal activity (Cummings and Morgan 2001). Tide-synchronized endogenous pacemakers in some species may have evolved by reorganizing such independent, desynchronized clock components.

For the daily bimodal rhythm in *D. asiatica*, no phase-setting effect of a single 4-h light pulse was observed (Akiyama 1997). Administration of the light pulses did not cause a change of the activity pattern into daily unimodal, which was occasionally observed by administration of the hydrostatic pressure pulses.

In contrast to the daily bimodal rhythm, a single 4-h light pulse administered to the specimens exhibiting a daily unimodal activity rhythm produces distinct phase advances (Fig. 4.6a) and delays (Fig. 4.6a, b). The PRC pattern (Fig. 4.6c) indicates that the 4-h light pulse completely reset the rhythm so that the onset of the activities occurred just after lights-off of each pulse. Considering no phase-resetting ability of hydrostatic pressure stimuli, the *D. asiatica* daily unimodal rhythm is definitely a circadian, light-phased rhythm. Occurrence of the unimodal circadian rhythms under nontidal conditions is similar to that in the shore crab *Carcinus* (Naylor 1958). However, the underlying pacemaker structure of these species would be rather different (see Sect. 4.6).

When the specimens were exposed to the hydrostatic pressure cycle under light–dark cycles (LD 6:18, 12:12), an increased number of specimens were active during

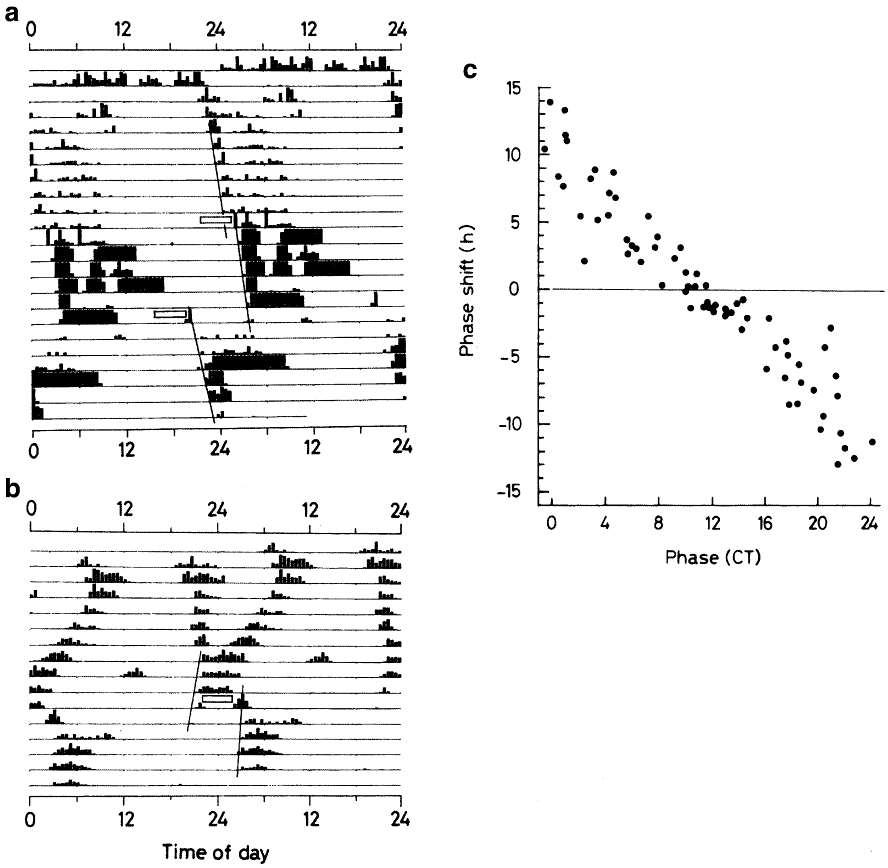

Fig. 4.6 Phase-setting effect of a 4-h light pulse on the daily unimodal swimming rhythm of *Dimorphostylis asiatica* kept in constant darkness. (**a**, **b**) Activity records of two specimens. *Rectangles* indicate time of treatment. *Oblique lines* are linear regression lines on the successive onsets of swimming activities. (**c**) Phase-response curve of the daily unimodal swimming rhythm to 4-h light pulses (Akiyama 1997)

the pressure-increasing phase compared to the number of specimens kept under constant darkness (Akiyama 2004). This observation suggests that light also affects the *D. asiatica* circatidal rhythm, in addition to responsiveness to hydrostatic pressure pulses, as indicated by the 24-h time base of the PRC (Fig. 4.4f).

4.6 Tidally Synchronized Circadian Rhythm?

The periodicity of circatidal clocks is closely related to that of circadian, solar day clocks. In addition, circadian clocks are occasionally phased by time cues other than light–dark cycles. Enright (1976) proposed that the locomotor activity rhythm of

Excirolana is "a tidally-synchronized circadian rhythm," because the "locomotor activities repeat with circadian intervals." Thereafter, few studies have focused on this hypothesis.

The 'splitting' phenomenon, that is, a gradual change of circadian temporal activity between daily unimodal and bimodal rhythms, has been reported for circadian rhythms of some terrestrial animals (Pittendrigh and Daan 1976; Underwood 1977). In these cases, the split and fused rhythms are governed by identical pacemaker(s) with a different phase relationship. In circatidal rhythms, a few genera of intertidal crabs, *Helice*, *Macrophthalmuas*, and *Uca*, and the horseshoe crab *Limulus*, showed two (or more) conjugated activities. Their free-running periods were slightly different from each other, and occasionally fused or split (Palmer and Williams 1986a, b, 1988; Chabot and Watson 2010). Interpreting the splitting in locomotor rhythms, Palmer and Williams (1988) explained that these rhythms were governed by two (or more) coupled pacemaker(s) with about a 24.8-h period, rather than a 12.4-h period, and the periodicity was called 'circalunidian.' Although the approximately 24-h period of the 'circalunidian' clocks is the same as the period of circadian clocks, evolutionary relationships of these clocks have not been investigated or discussed sufficiently.

Naylor and colleagues demonstrated that independent circatidal and circadian clocks govern locomotor rhythms in the shore crab *Carcinus* (Reid and Naylor 1989; Naylor 2010). The essential period of the tidal pacemaker was supposed to be simply 12.4 h. The rigid 12.4-h periodicity may be partly related to the regular semidiurnal tide of their habitat in the eastern North Atlantic (Barnwell 1976). Further comparative studies of circatidal rhythms with various environmental tidal patterns, including animals distributed in the geographic regions characterized by diurnal tide, would be helpful to reveal the rigidness of rhythmicity and the underlying mechanisms of circatidal rhythms.

In the swimming activity of *D. asiatica*, circatidal and circadian rhythms do not occur simultaneously. In addition, the daily bimodal, circatidal rhythm composed of two pacemaker components of about 24 h occasionally fuse to become one daily unimodal circadian rhythm for a few or several days (Fig. 4.6b). Rarely, the unimodal rhythms further split again to become a daily bimodal rhythm (Akiyama 2004). It is plausible that those two rhythms are governed by identical pacemaker(s), as well as in the cases of splitting in rodents and crabs, suggesting that *D. asiatica* has developed a 'tidally synchronized circadian clock' or 'solar day-synchronized circatidal clock.'

Diurnal vertical migration of marine organisms dominantly exhibits solar day-synchronized rhythmicity rather than tide-synchronized rhythmicity. In cumaceans, habitats of most species are rather deep, exceeding 100 m in depth (Băcescu 1988, 1992). However, certain genera such as *Dimorphostylis* might have been adapted to their life to shallow, tide-affected area. Thus, considering cumacean habitats, the biological clock of *D. asiatica* seems to be a 'tidally synchronized circadian clock.'

Recently, molecular analysis, using RNAi of the *per* gene, on the locomotor activity rhythm of the mangrove cricket composed of both circatidal and circadian components, revealed that the molecular oscillation system of the cricket's 12.5-h recurring circatidal clock does not include *per* products, showing that it is different from the general insect circadian clock (Takekata et al. 2012). This finding and succeeding results on the circatidal rhythm of mangrove crickets (Takekata et al. 2014) suggest that at least some marine organisms may have developed a 12.5-h period clock system, composed of molecular parts that are different from circadian clocks. However, considering various temporal patterns of environmental tidal cycles, and circatidal rhythms synchronized with those complicated time cues, further studies should be necessary to elucidate the evolution of traits and the divergence of circatidal clocks.

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Chapter 5
Coral Spawning Behavior and Timing

Michal Sorek and Oren Levy

Abstract One of the most prominent examples of synchronized behavior in corals is spawning, the enormous reproduction event known as the “spawning event,” where the moon choreographs sex among many coral species at the same night, once a year. The timing of annual coral spawning varies geographically around the earth but is consistent and predictable at each location. Several environmental factors such as sea temperature, salinity, tidal periodicity, and daylength have been suggested as inducers for gametogenesis and spawning in reef-building corals, whereas the actual spawning event appears to be triggered by the level of lunar irradiance. The repeated episodes, associated with broadcast spawning year after year, is a classic periodic biological rhythm, which is controled by exogenous *zeitgebers* and by an endogenous biological clock. A key imperative cue is the moon phase and moonlight. Nevertheless, the specific cellular mechanisms mediating this annual synchronization of behavior by reef-building corals has remained elusive, and the fact that cryptochromes may be involved in this process may suggest a role for the circadian clock in this unique phenomenon. Sexual reproduction is one of the most important processes for the persistence of reefs, yet worldwide reef systems are being rapidly degraded and face a multitude of threats, including global climate change and the anthropogenic stressors of artificial light pollution.

Keywords Circadian clock • Circadian masking • Coral reproduction • Cryptochromes • Mass spawning event • Moonlight

5.1 Coral Reefs

Coral reefs are one of the most impressive natural ecosystems in the world and are home to one quarter of the world’s marine biodiversity (Reaka-Kudla et al. 1997). In addition, coral reefs have an extremely important ecological role in the marine

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habitat. They are considered one of the most productive ecosystems on earth. Scleractinians (stony corals) are the major reef-building species and are classified ecologically as hermatypic corals (reef-building corals). These corals are the basis and framework for the entire ecosystem. They are responsible for the calcification process, which contributes calcium carbonate to the reef. Stony corals create homes for a wide variety of organisms and provide other important resources, such as shelter and food, for thousands of species of fish and invertebrates.

5.1.1 Coral Biology

Corals, class Anthozoa within the phylum Cnidaria, exist as solitary polyps or, more commonly, as colonies of many identical individuals. Each polyp consists of the following three basic tissue layers: the epidermis, which is the outer layer; an inner layer of cells lining the gastrovascular cavity, which acts as an internal space for digestion; and the mesoglea layer, which is located between the outer and inner layers (Barnes 1987). Scleractinian corals first appeared during the Triassic period in tropical shallow waters; however, the origin of these coral groups has remained an unsolved mystery of paleontology (Stanley 2003). Corals are known to be predators, and they use their tentacles to trap prey, such as zooplankton and small invertebrates, with stinging cells or nematocysts. However, the majority of their food is supplied by their symbiotic counterparts, endosymbiotic dinoflagellates (Fig. 5.1). The coevolution of corals with endosymbiotic algae, which are also known by the common name zooxanthellae, is responsible for the domination of reefs by corals. This symbiosis is primarily based on metabolic requirements in which the algae provide the corals with photosynthetic products and energy for the rapid calcification of the corals. In return, the algae obtain essential nutrients and shelter provided by the coral hosts. The coral life cycle is simple and includes a long sessile stage in which the coral colony is attached to the substrate and a short planktonic stage consisting of planulae or larvae. After several days, the planula eventually attaches and settles permanently on a hard substratum. It then metamorphoses from the larval form into a juvenile polyp, which produces a skeleton composed of calcium carbonate. Subsequent growth during the juvenile stage leads to the adult form, which can reproduce sexually. This form completes the life cycle (Harrison and Wallace 1990).

5.1.2 Coral Reefs Situation

During the past three decades, coral reefs have been under increasing pressure as a result of dramatic environmental changes (Gardner et al. 2003; Hughes et al. 2003). Acute global climate changes, ocean acidification, temperature increase in the marine environment, disease, and human activities, such as overfishing and coastal development, have destroyed 20 % of the world's 285,000 km^2 of known reefs

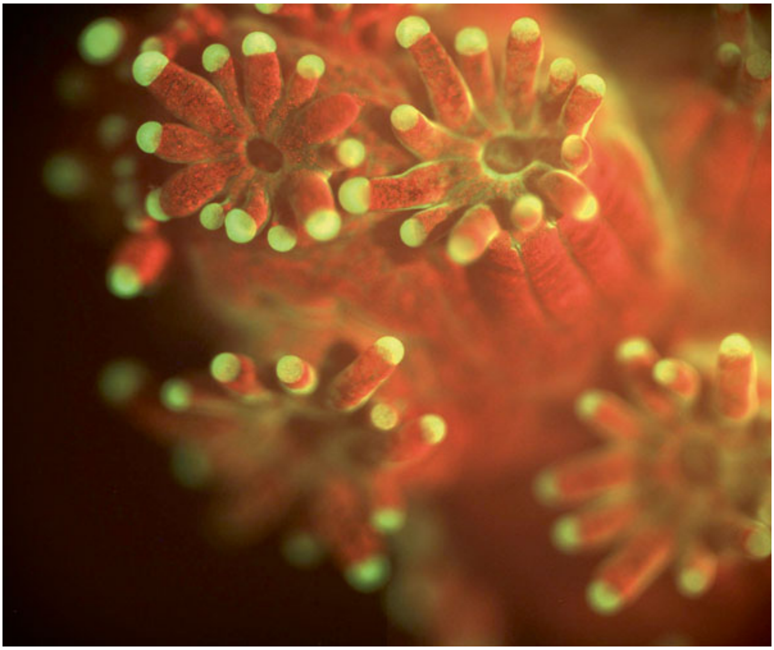

Fig. 5.1 Symbiotic coral *Pocillopora damicornis* polyps, photographed in the laboratory, using a long working distance high-magnification fluorescence imaging setup. (Picture by © Tali Treibitz, Andy Mullen, Jules Jaffe, UC San Diego)

(Pennisi 2007). This worldwide decline in coral reefs has raised major concerns regarding the future of these marine ecosystems and has encouraged additional research to provide a better understanding of coral biology and the conservation of this unique environment. The coral reproductive process holds the key for adjustment to these dramatic global changes, because it appears that coral populations depend almost entirely on the formation of sex cells or gametes along with DNA recombination to form more thermally tolerant genotypes that can adapt to warming ocean waters (van Woesik 2010).

5.2 Coral Reproduction

Coral reproduction has been a major research topic for many years. Much research has attempted to characterize the processes and modes of reproduction for different coral species in a wide variety of geographic locations. Corals are primarily classified based on their sex. They are classified as hermaphroditic, either simultaneous or sequential, with males and females in the same colony, or gonochoric, with all polyps being unisex, either female or male. Hermaphroditism is considered advantageous over gonochorism if the probability of finding members of the opposite sex is low and self-fertilization is possible. Corals are further classified based on two reproductive modes. The first mode is broadcast spawning, in which external fertilization occurs in the water column after the gametes are shed. The second mode is brooding, in which fertilization occurs in the maternal colony (Harrison and Wallace 1990; Richmond and Hunter 1990). The four main patterns of sexuality in corals are hermaphroditic broadcast spawners, hermaphroditic brooders, gonochoristic broadcast spawners, and gonochoristic brooders. Information on sexual reproduction in more than 400 scleractinian species confirms previous data suggesting that hermaphroditic broadcast spawning is the dominant pattern among coral species, whereas there are relatively few hermaphroditic or gonochoric brooding species (Harrison 2011). Characteristics of sexuality are primarily conserved within species, genera, and even families, although exceptions occur for different locations and species. However, not all coral species are readily classified into these four basic patterns because mixed sexual patterns or mixed modes of sexual development have been observed for certain species (Harrison 2011).

Corals can reproduce both asexually and sexually. Asexual reproduction in corals produces genetically identical modules of the original colony and can occur through fragmentation (Highsmith 1982) resulting from storm and wave impacts or other damage to corals; polyp bailout (Sammarco 1982), which occurs when new clonal polyps bud off from the parent polyps after they reach a certain size and expand or begin new colonies (Sumich 1996); or the asexual production of planulae (Stoddart 1983), which may occur during periods when sexual reproduction has ceased (Nakano and Yamazoto 1992; Lam 2000). This process continues throughout the life of the organism (Barnes and Hughes 1999) and primarily occurs when the environment is unable to support sexual reproduction. Another advantage of asexual reproduction is that the increased size of the initial stage reduces the risk of juvenile mortality. Also, this initial stage occupies substantial space on the reefs. In certain cases, this stage may disperse widely and preserve the original genotype.

Sexual reproduction involves the production of gametes, fertilization, embryo development, and a larval phase that is usually planktonic (Harrison and Wallace 1990). Fertilization may occur either within the maternal polyp (brooding) or externally in the water column after the gametes are shed (broadcast spawning). Sexual reproduction in corals may occur yearly, seasonally, monthly, or not at all. The timing of coral sexual reproduction depends on the species and environmental conditions. Sexual reproduction occurs when female gametes and male gametes combine to create fertilized eggs. Two different methods of sexual reproduction exist in corals. The first method is spawning, in which two parents of different sexes release their gametes into the water and fertilization occurs. The second method is brooding. This method of sexual reproduction is characterized by the presence of embryos and planula larvae within the coelenteron or expanded tentacles of the coral. In this case, the release of the planulae occurs during an advanced stage of larval development. In addition to brooding, corals can also spawn sperm, which fertilizes either the same colony or another colony nearby (Gleason et al. 2001; Okubo et al. 2007; Ayre and Miller 2006). The planulae released from brooding corals into the water column settle within a few hours after release. One important advantage of sexual reproduction in corals is the ability to broadcast gametes, particularly during mass spawning events. This process may promote hybridization among parallel or different pairings. If hybridization is an important feature of coral reproduction, then population-level consequences could rapidly generate new genetic combinations with the potential for increasing the diversity of responses to environmental change. Also, our ideas about the integrity of coral species might be radically altered because experimental laboratory studies of corals have demonstrated that hybrids can be formed (Richmond 1992; Willis et al. 1993, 1997). Sexual reproduction in corals is possibly the most important ecological process for the replenishment of degraded reefs. Sexual reproduction also maintains the coral population and supports evolutionary processes, such as genetic recombination and the production of new coral genotypes, which may enhance fitness.

5.3 The Chronobiology of Coral Spawning and Its Variability in Different Geographic Locations

The phrase "Timing is everything" is often correct and accurate. For coral reproduction, timing can be the key to reproductive success for all reproductive phases. The most tightly synchronized stage of reproduction is the final stage, that of fertilization. If corals use broadcast spawning and release both eggs and sperm into the water column, timing and synchronization are extremely important to ensure successful fertilization. Eggs that are released into the water are available for fertilization for only seconds to minutes before they drift away. For brooding corals, the precise timing of planulae release into the water column may be crucial in that it allows the planulae to find a suitable place to settle. The mechanism by which corals fine tune their spawning and the planulae release times remains unknown. Many environmental factors and stimuli are known to influence the reproductive timing of corals. The key factors are the sea surface temperature, the moon phase, and the daily light cycle. All three factors have been suggested as inducers for gametogenesis and spawning in reef-building corals (Harrison et al. 1984; Babcock et al. 1986, 1994).

These factors are apparently involved in three or more reproduction cycle patterns. The first pattern, the seasonal or annual rhythm, is related to the seasonal variation in sea temperature. Changes in sea temperature are most likely involved in triggering the maturation of eggs and sperm inside the adult (Yonge 1940; Kojis and Quinn 1981; Harrison et al. 1984; Stoddart and Black 1985; Willis et al. 1985; Babcock et al; 1986). The second cycle is the lunar or monthly rhythm, which coordinates the timing of mass spawning. The tidal cycles are important, and it is common for corals to spawn during low-amplitude neap tides (Oliver et al. 1988; Simpson 1991; Babcock 1995; Mendes and Woodley 2002). Spawning during neap tides is an advantageous strategy that reduces gamete dilution because the amount of water movement is low. Related to predicting neap tide, another important cue for corals is the moon phase and moonlight (Jokiel 1985). It is probable that these cues have the largest monthly influence. The third cycle pattern involving the timing of gamete release is the diel light cycle, which is thought to trigger spawning after a precise period of darkness (Harrison and Wallace 1990). An endogenous circadian clock is known to control and entrain several diel phenomena in certain coral species. These phenomena include calcification (Chalker 1977; Chalker and Taylor 1978) and tentacle diel behavior (Sweeney 1976; Sebens and Deriemer 1977; Lasker 1979). However, whether an endogenous circadian clock governs coral reproduction has not yet been clearly demonstrated. The fine tuning that results from the three major cycle patterns is highly important. Although many coral species spawn on the same night, the final spawning time can differ between species. It is possible that these differences in spawning time act as temporal barriers to avoid hybridization between closely related species (Szmant et al. 1997). Corals that occupy different habitats in areas with different climate conditions and latitudes show different life cycles, reproduction modes, and reproductive timing. Currently, it remains impossible to define a universal factor that can provide a good single explanation for the differences in reproductive timing between different geographic localities.

The majority of the data concerning coral reproduction modes, synchronization, and timing come from the tropical Pacific. The sources of these data include the Great Barrier Reef in Australia and reefs in Western Australia. Data also exist for Guam, Palau, Hawaii, Okinawa, and Panama, as well as the Red Sea and Caribbean. The timing and synchronization of coral reproduction have been most often studied for the Great Barrier Reef. These studies represent an adequate source for understanding the timing of coral reproduction. The major pattern observed in the Great Barrier Reef studies is the remarkable similarity and synchronization of reproductive activity among coral species. The majority (90 %) of species studied broadcast spawn gametes annually during the week following the full moon during the spring (Harrison et al. 1984; Willis et al. 1985). Many individuals of each broadcast spawning species release their gametes during a time window that is approximately 30 min long (Fig. 5.2). On the Great Barrier Reef, spawning synchronization is the most common reproductive mode and occurs for more than 140 species of coral. This type of massive synchronized spawning is familiar from the mass spawning events

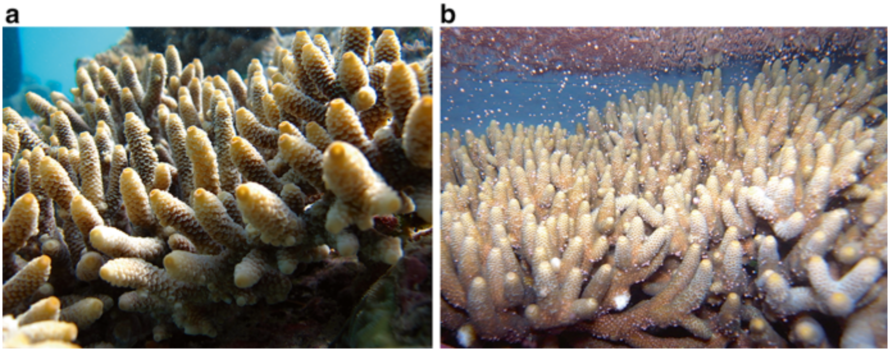

Fig. 5.2 *Acropora millepora* colonies before (**a**) and during (**b**) spawning time at Heron Island, Great Barrier Reef Australia. Spawning was documented around 21:30 during November 2011

that occur for certain reefs on the Great Barrier Reef (Willis et al. 1985; Harrison et al. 1983, 1984; Harrison 1993; Babcock et al. 1986; Wallace et al. 1986; Oliver et al. 1988; Harrison and Wallace 1990) and in western Australia during the austral autumn (Simpson 1985, 1991; Simpson et al. 1993; Babcock et al. 1994; Rosser and Gilmour 2008; Gilmour et al. 2009; Rosser and Baird 2009). These occurrences represent the largest scale synchronous mass spawning events of corals in the world, although the mass spawning that occurs in western Australia is smaller. Other smaller-scale synchronized spawning events involving fewer species or colonies of one or a few coral species have been reported primarily from the Gulf of Mexico and Caribbean region (Gittings et al. 1992; Hagman et al. 1998; Beaver et al. 2004; Bastidas et al. 2005). Several of these events were reported as mass spawning events, but most are considered multispecific spawning events (Harrison and Booth 2007) because they include a much lower number of species. More recently, many reefs in the Indo-Pacific region have been reported to exhibit coral spawning synchronization on a medium scale. These locations include Japan, Taiwan, the Philippines, Singapore, Indonesia, Papua New Guinea, the Solomon Islands, French Polynesia, and the Egyptian Red Sea (reviewed in Harrison 2011).

In contrast to the synchronized spawning events on any scale, the major reproductive activities of the most abundant species in the Red Sea occur during different seasons, different months, or different lunar phases within the same month (Shlesinger and Loya 1985; Rinkevich and Loya 1979). The coral species in the Red Sea exhibit temporal reproductive isolation. Broadcasting species release their gametes during periods of up to six nights, once or twice a year, whereas brooders release their planulae for 3–7 months (Shlesinger and Loya 1985) when the temperature rises or during the summer, when the water temperature is the warmest (Fadlallah 1984). Asynchronous reproduction is also exhibited by most coral species in the Central Pacific, primarily in Okinawa and Hawaii. For the latter corals, the spawning period peaks during the summer months, but spawning continues year round (Kolinski and Cox 2003) for certain brooders. More recently, a reproductive period of 9 months has been reported for a few *Acropora* species in Kenya (Mangubhai and Harrison 2006, 2009; Mangubhai 2009). This lack of synchrony was attributed to a reduction in environmental seasonality and low variability in the ranges of certain environmental parameters (Richmond and Hunter 1990), particularly annual sea surface temperatures and tidal amplitudes (Oliver et al. 1988).

Several observations show differences in reproductive timing patterns within the same species in different locations. For example, *Stylophora pistillata*, the most dominant stony coral on the reefs of the Red Sea, was found not to be controlled by the lunar periodicity in this area (Rinkevich and Loya 1979), although later research (Zakai et al. 2006) indicated a degree of lunar cycle influence on planulae release. This same species was controlled by the lunar periodicity of planulation in Palau (Atoda 1947). Another example of different timing of planulae release in *S. pistillata* within the Great Barrier Reef was found on Heron and Lizard Islands. This species releases planulae during the summer, the timing of which does not follow the lunar cycle for planulation (Tanner 1996; Loya 1983). Recent reports from the Egyptian Red Sea have described the highly synchronous maturation of gametes among many

Acropora species (Hanafy et al. 2010) with multispecific spawning on a medium scale, whereas *Acropora* in the northern part of the Red Sea, for example, in Eilat, does not show a similar pattern (Shlesinger and Loya 1985; Shlesinger et al. 1998). These two examples of *S. pistillata* coral and the *Acropora* corals demonstrate variations in reproductive timing modes at different locations. Differences in timing among allopatric populations of a species may represent adaptations to both local environmental factors and specific cues (Richmond and Hunter 1990). Differences between the synchronized and unsynchronized timing of spawning among corals at the equator compared to high and low latitudes may be caused by a lower variability in temperature close to the equator. Analyzing the existing data regarding latitudinal trends may explain the differences in timing behavior among the same species in different locations for some but not all reefs.

During recent years, global changes have affected coral reef conditions dramatically. The decline in the condition of the environment has produced accelerated research into coral reproductive behavior and timing. The current information on this subject is based on more than 400 corals from many different locations and includes new sites and research topics.

5.4 Environmental Factors Affecting Synchronized Coral Reproduction

We have shown that an important feature of coral reproduction is synchronization. Many proximate factors have been hypothesized to influence spawning and planulae release at different levels and times, including the aforementioned key factors of sea temperature (Glynn et al. 1991; Hayashibara et al. 1993), tidal regime (Wyers et al. 1991) and lunar phase (Guest et al 2002), and onset of darkness (Harriott 1983; Babcock et al. 1986; Hunter 1989). Additionally suggested factors include a near-zero solar insolation derivative (van Woesik et al. 2006), the duration of regional calm periods of wind that may enhance fertilization and synchronization (van Woesik 2010), food availability (Fadlallah 1981), the amount of rainfall combined with temperature (Mendes and Woodley 2002), twilight chromaticity (Sweeney et al. 2011), and salinity (Jokiel 1985). It is not yet clear whether and how all these factors work with each other or with internal components of the endogenous clock in corals, serving as input signals from external stimuli during synchronization.

The most common hypothesis (Glynn et al. 1991; Babcock et al. 1986) points to water temperature as the most influential factor, with rising temperatures stimulating the production and maturation of gametes in many invertebrates. The second cue is the full moon, with the process of spawning occurring primarily a few days after the full moon at a precise time after sunset, which is considered the third cue. However, the principal difficulty with this hypothesis is that it cannot provide a good explanation for any reef in any region. For years, attention was centered on reefs in the mid-latitudes, where changes in temperature are significant and can provide a good explanation for the determination of the timing of spawning. In the tropics, however, the temperature varies very little and cannot be considered a major cue for coral spawning. On certain reefs, coral spawning does not occur when the water is the warmest. For example, the corals on the west coast of Australia reproduce during the late summer and autumn (Simpson 1985, 1991; Simpson et al. 1993; Babcock et al. 1994), whereas reproduction of the corals on the east coast occurs during the spring. However, the water temperature begins to increase in both regions during the summer. Richmond and Hunter (1990) have demonstrated a correlation between the percentage of synchronized coral species and the annual variation in the seawater temperature, although there are exceptions. The attempt to find the ultimate cue for synchronization has revealed many differences between corals at high and low latitudes, with corals in the lower latitudes showing spawning synchrony that appears to be weaker compared to corals at high latitudes (Richmond and Hunter 1990).

5.5 Experiments Under Controlled Laboratory Conditions

Over the years, many researchers have classified and characterized areas of coral reefs for timing and synchronization of reproduction characteristics. To understand the mechanism of reproductive timing, corals were subsequently studied under controlled laboratory conditions. Under laboratory conditions, it is possible to isolate specific factors and to determine the influence of each factor on reproductive timing in corals. It is also possible to determine the influence of these factors on different cycle periods. Several researchers have attempted to determine whether spawning and planulae release in corals is a circadian behavior or is controlled directly by light. Manipulation of daylight and moonlight in the coral *Pocillopora damicornis* was conducted by Jokiel (1985) to investigate the influence of light on the timing of coral spawning. Corals were kept under natural light during the day. By night, one part of the experiment corresponded to artificial full moon light and another to new moon light (darkness). Corals in both treatments showed a loss of synchrony in monthly larval production. These results showed that spawning is synchronized by night irradiance cues that are essential for synchronization. Published evidence (Babcock 1984, 1988; Hunter 1989) indicates that coral spawning timing can be shifted by an early artificial sunset a few days before spawning occurs. This finding demonstrates two important principles. The first is that the light–dark (LD) cycle is responsible for the fine tuning of spawning at the level of hours and minutes, and the second is that light (not particular endogenous components that measure time) regulates the timing of spawning. Brady et al. (2009) demonstrated earlier spawning in *Montastraea franksi* under an earlier artificial sunset on the day of spawning. These results indicate that in this case either that the timing of spawning is directly controlled by the local solar light cycle, at least for the precise tuning of the hours of spawning, or that light masks an endogenous circadian rhythm. In support of the idea of weak circadian clocks, Levy et al. (2007) showed that the expression of two circadian clock genes (*cry1* and *cry2*) did not show sustained circadian rhythms under continuous darkness (Fig. 5.3) (see following).

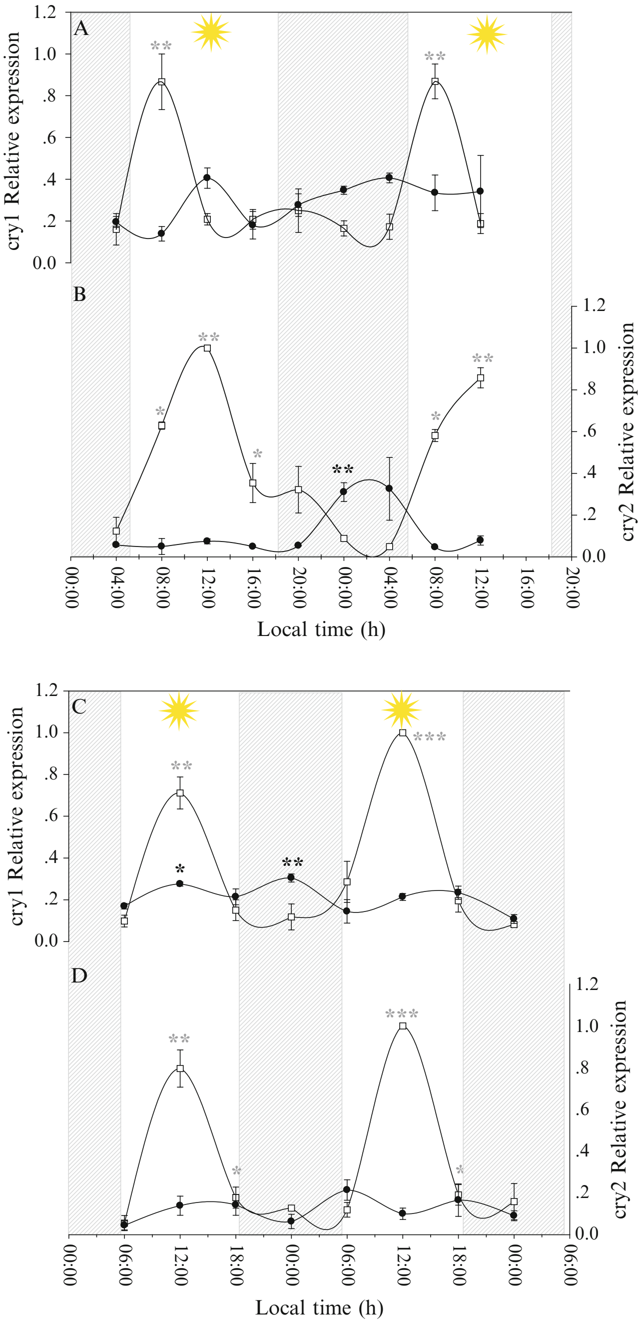

Fig. 5.3 Temporal expression patterns of *cry1* and *cry2* in *A. millepora* under light–dark (LD) (*open squares*) and DD (*filled circles*) cycles using quantitative polymerase chain reaction (PCR). (**a**, **b**) A 32-h cycle with sampling intervals of 4 h. (**a**) Quantitative analysis of *cry1* revealed a

Boch et al. (2011) analyzed the contributions of separate components of light dynamics because the effects of twilight and lunar light on coral spawning synchrony have previously been conflated. The alternative hypothesis, so far untested, was that twilight and lunar light have differential contributions as proximate cues. Boch et al. (2011) showed that under controlled conditions lunar photoperiod cues are most likely the major driver of spawning synchrony on a given night of the lunar cycle and at a specific time of night. The differences in spectral dynamics have secondary effects on spawning. Petersen et al. (2007) identified specific differences between broadcast and brooding corals. Twenty-four coral species from nine families under artificial conditions in an aquarium system were observed to show reproductive behavior. Broadcast spawners reproduced primarily in open systems under natural light conditions, whereas brooders showed less sensitivity toward certain environmental factors that are known to trigger reproduction in field populations, such as moonlight and temperature fluctuations. It has been suggested that spectral light composition affects the timing of gamete release, particularly during twilight. Under laboratory conditions, when the daylength was artificially extended by 6 h for two coral species, *Acropora millepora* and *Acropora aspera*, from the Great Barrier Reef, the corals demonstrated a phase shift in their spawning time. Coral colonies that were illuminated by red light did not show any delay in the spawning time compared to the same species under natural conditions in situ. In contrast, colonies irradiated by blue, green, and white (PAR) light spectra had a phase shift in their spawning time relative to the spawning time of the control colonies and corals of the same species found on the reef. These findings suggest that artificial light contamination in the blue and green spectral regions can mismatch and delay spawning, whereas red light has no effect on spawning behavior (Levy et al., unpublished data). These results suggest the presence of blue-light photoreceptors known as cryptochromes that could mediate this spawning behavior (Levy et al. 2007).

These findings could improve the understanding of the threats to coral reefs. Sexual reproduction is one of the most important processes for the persistence of reefs; yet worldwide coral reef systems are being rapidly degraded and face a multitude of threats. The interplay between an endogenous clock and external light cues for the timing of reproduction may be compromised in the era of industrialization and global change, where the moon is not the only source of light at night. Buildings, street lamps, lighthouses, cars, boats, and oil platforms provide obvious evident examples of artificial sources of light at night that can eventually affect reproductive timing and fertilization success in broadcasting species.

◄

Fig. 5.3 (continued) significant effect of light (*L*) and dark (*D*) (L/D, $P = 0.035$), as well as a significant effect of 'sampling time' (time, $P < 0.001$). (**b**) Expression of *cry2* (L/D, $P = 0.026$, time $P < 0.001$). Each value was normalized to **β**-actin and converted to percentage of maximal level for each gene. Values (mean ± SE) were tested by ANOVA with linear contrast method within groups to distinguish between the LD/DD rhythm amplitude of *cry1* and *cry2*. *cry1* DD ($P > 0.01$), *cry1* LD ($P < 0.01$). *cry2* DD ($P > 0.05$), LD ($P < 0.01$). Time points with asterisks are significantly different (*grey asterisks* LD, *black asterisks* DD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Sample size = 3. (From Levy et al. 2007)

5.6 What Do We Know About Molecular Aspects of Reproductive Timing?

The phenomenon of synchronized spawning of corals in different locations, species, and scales, particularly in the context of unique large-scale mass spawning events, remains an issue with many unanswered questions. It is not yet known whether and how corals sense the environment and how internal components of the endogenous clock assist the timing and synchronization of external stimuli. Continuous progress in molecular methods for studying corals is very important for understanding the timing of coral reproduction. Understanding the molecular mechanism will contribute to our knowledge of reproductive chronobiology and will provide a general mechanism for many reefs throughout the world. Gorbunov and Falkowski (2002) have suggested that detection of the blue region of moonlight by corals may cue the specific night of spawning because several species of corals are extremely sensitive to the blue region of the light spectrum. Molecular research based on the coral *A. millepora* has identified the central gene components of corals that are part of the central clock loops. The major photoreceptor genes known as cryptochromes (*cry*) have also been identified and are hypothesized to mediate the moonlight signal in the environment for reception by the central clock (Levy et al. 2007). As shown above Fig. 5.3, *cry1* and *cry2* genes were found to be rhythmic under light–dark conditions but not under constant darkness. *cry2* may entrain the intrinsic clock on a monthly basis because its expression increases massively on full moon nights compared to new moon nights (Levy et al. 2007; Fig 5.4). However, the expression of this gene has not yet been determined during mass spawning events.

Sweeney et al. (2011) have also determined that shifts in twilight color and intensity during nighttime and between nights immediately before and after the full moon, as well as an increase in the blue-light spectrum underwater during twilight after the night of the full moon, correlated significantly with observations of spawning in corals. In contrast, molecular studies of the coral *Favia fragum* regarding the *cry1*, *cry2*, *clock*, and *cycle* genes showed a diel oscillation for *cry1* and *cry2* but did not identify any relationship between the elevated expression of one of the *cry* genes and a monthly cycle specifically connected to the day of spawning (Hoadley et al. 2011). This finding suggests that the *cry* genes are not involved in the entrainment of reproductive cycles to lunar light cycles in *F. fragum*. Because of the ongoing debate and the deficiency of solid data related to the role of circadian clock in cueing broadcast spawning synchronization, as an alternative option "masking" should also be investigated. The term "masking" (Aschoff and Vongoetz 1989) describes an immediate effect of a stimulus that overrides the expression of an animal's endogenous clock. Under natural conditions, masking has the adaptive value of confining animals to their appropriate temporal niche and may complement the circadian clock in fine-tuning activity patterns in response to environmental stimuli (Redlin 2001). Thus, masking might be an important mechanism in the response of species to moonlight. The possible detection of a masking response under field conditions will provide evolutionary insights into its true adaptive value, which may or may not occur in coral reef spawning events. To define endogenous rhythms, the rhythm

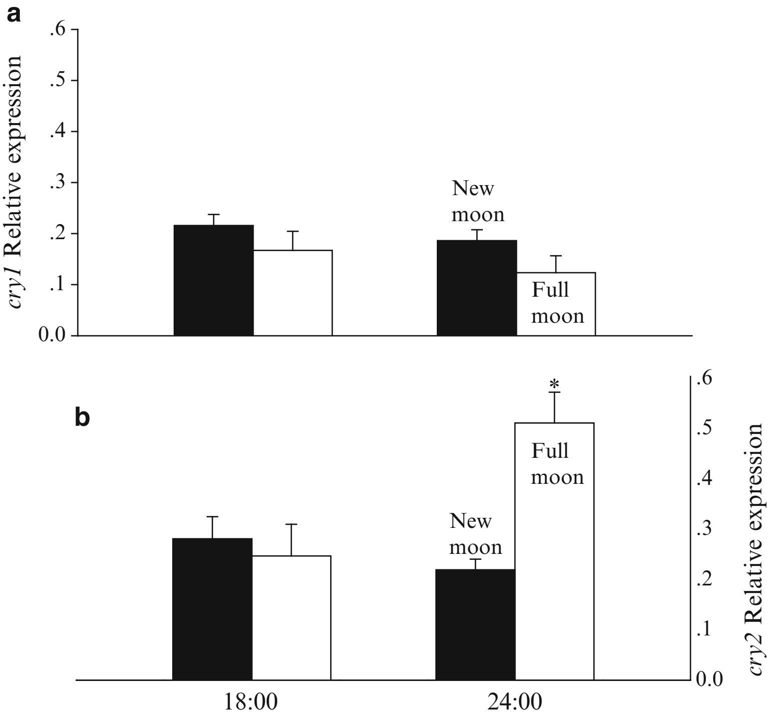

Fig. 5.4 (**a**, **b**) Quantitative analysis of *cry1* and *cry2* of two consecutive months, August and September 2005, comparing new moon nights (5 August and 4 September) to full moon nights (20 August and 18 September) at time points of 18:00 and 00:00. Each value is the average time point of the two respective sampling events (mean ± SE). ANOVA RM, $P > 0.05$ *cry1*, $*P < 0.05$ *cry2*; sample size = 4. (From Levy et al. 2007)

itself must continue under constant conditions in the absence of stimuli. The published molecular studies regarding lunar rhythms, so far, cannot necessarily be attributed to an endogenous biological clock. Whether the phenomenon of synchronized spawning is environmentally triggered or controlled by endogenous mechanisms remains an unanswered question. Understanding how corals perceive and integrate information regarding environmental cues to regulate their reproductive cycles may solve one of earth's biggest mysteries and help maintain the coral reef environment in marine ecosystems.

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Chapter 6
Lunar Periods in the Annual Reproductive Cycles of Marine Invertebrates from Cold Subtidal and Deep-Sea Environments

Annie Mercier and Jean-François Hamel

Abstract Moon-related rhythms of reproduction in the sea were originally evidenced and studied in selected species from tropical or subtropical nearshore or tidal environments. They are now being recorded across a widening range of taxa, habitats, and depths. Although still poorly understood, lunar components in the cyclic breeding of boreal subtidal and deep-sea organisms contribute to extend the putative influence of celestial forces to the most remote regions of the globe. Such occurrences deserve more attention if we are to develop a comprehensive understanding of biological rhythms. The present chapter reviews and discusses putative drivers of lunar periodicities and their adaptive significance in previously neglected taxa. It shows that the interplay between the moon and the sun seems to provide a diversity of marine organisms with a means to coordinate their reproductive events. However, universal underlying pathways have yet to be identified.

Keywords Cold-water corals • Echinoderms • Gametogenesis • Larval release • Spawning periodicity

6.1 Introduction

Reproductive activity is one of the most important and widely studied life history features in aquatic animals. It is therefore not surprising that some of the earliest phenomena to be tied to a lunar periodicity revolve around the reproduction of marine invertebrates. Swarming of the Pacific palolo worm, *Palola* (=*Eunice*) *viridis*, on a specific lunar phase was first recorded more than a century ago (Friedlaender

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1898). A large body of literature has accumulated since then to show that a number of species exhibit lunar-related rhythmicity in at least some components of their reproduction. The best known examples include semilunar and tidal spawning in coastal fishes (Takemura et al. 2010; see chapter by Ikegami et al., this volume) and shallow-water invertebrates (Harrison et al. 1984; Babcock et al. 1986, 1992a; Pearse 1990; Bentley et al. 2001; see chapters by Neumann, Chabot and Watson, Sorek and Levy, and Ikegami et al., this volume).

Lunar periodicities outside shallow nearshore and tidal habitats are comparatively understudied, especially in species inhabiting cold seas (Mercier and Hamel 2010; Aguzzi et al. 2011; Mercier et al. 2011a), where the focus has traditionally been on annual reproductive events that appear to follow seasonal (solar) cycles (Giese et al. 1991; Mercier and Hamel 2009a). The present chapter first provides a brief overview of seasonal reproduction in boreal and deep-sea invertebrates and then summarizes current knowledge on lunar reproductive periods recently evidenced in taxa that colonize nontidal and nontropical environments. It will also tease out pertinent information from the occurrence of synchronized multispecies breeding events that occur annually, on specific days of the lunar month, emphasizing the complexity in the already multifaceted roles played by the moon and sun on the perennity of marine species.

In chronobiology, investigations typically try to link lunar breeding periodicities with clock-based internal mechanisms and to examine how external cues are transduced through physiological or molecular pathways. Although subtropical and tropical species have been studied in greater depth and tend to be used as models (e.g., fishes, corals, worms; see this volume for examples), an equally broad diversity of temperate and boreal species have been reported to exhibit lunar components in their annual and seasonal reproduction (e.g., echinoderms, mollusks, annelids, cnidarians; present chapter). However, research on the species reviewed here (from cold and deep-sea habitats) is comparatively young. Most studies are at the stage of identifying putative drivers of lunar-related rhythms relative to the nature of the breeding event that exhibits a lunar component (e.g., gamete synthesis, gamete and larval release).

As a preamble, we must make an important distinction between the various levels at which the moon's influence can be examined. The proximate factors, which serve as cues to maintain synchronous breeding within a population are the most commonly investigated, compared to the ultimate factors, which serve as selective forces that give survival value to the adaptation. In other words, the adaptive significance of reproducing under certain conditions at a certain time can be distinguished from the mechanism by which this is achieved.

Reproductive rhythms in marine organisms, whether seasonal or lunar, have been proposed to derive from a need to optimize reproductive success by synchronizing male–female cycles or enhancing larval survival. The former adult-biology hypothesis postulates that breeding periodicity increases mating success and fertilization rates; the latter hatchling-biology hypothesis that it promotes faster larval development under optimal conditions and lower predator pressure on progeny via swamping or concealment (Robertson et al. 1990). For example, pelagic propagules may be hidden by the darkness or brightness afforded by the new/full moon, or may be retained or dispersed by appropriate tidal currents. Adult-biology hypotheses have been tested in reef fishes (Robertson et al. 1990), and optimal fertilization was studied in reef corals (Levitan et al. 2004); these notions are somewhat less relevant for sessile invertebrates that do not exhibit external fertilization or which rely on spermcasting (Bishop and Pemberton 2006). As for current hatchling-biology hypotheses, they apply chiefly to planktonic feeding propagules or the dynamics of tidal environments (Morgan 1995a). Moreover, there is presently no unequivocal support for the role of temperature-optimized larval development (Reitzel et al. 2004) or the importance of larval food availability (Przeslawski 2008). Hence, there is apparently no universal framework that can clearly explain why so many marine species release progeny in spring or early summer, irrespective of their life histories or the habitat they occupy, and why many of them favor a particular lunar phase (Reitzel et al. 2004; Mercier and Hamel 2010; Mercier et al. 2011a). In an effort to clarify our current understanding of seasonal and lunar reproductive periodicities in species that appear to transgress common paradigms, this chapter reviews available evidence and attempts to elucidate general concepts.

6.2 Seasonal Reproduction in Marine Invertebrates

6.2.1 Subtidal Boreal Species

Seasonality is typically defined as a component of a time series exhibiting repetitive and predictable changes that occur over a period of one calendar year or less. For macrobenthic species that live in cold waters, spring and early summer are the most intense breeding periods, irrespective of reproductive strategy (free-spawning or brooding) and larval type (feeding or nonfeeding) (Reitzel et al. 2004; Mercier and Hamel 2010). Seasonal cycles of gamete development in benthic organisms have been correlated with large-scale environmental conditions, primarily photoperiod (see reviews by Giese 1959; Giese et al. 1991; Olive 1995; Himmelman 1999; Mercier and Hamel 2009a). Most studies on reproductive periodicity have focused on gamete release, although cyclic release of postzygotic propagules (e.g., larvae) has been recorded in tropical (Tanner 1996) and boreal species (Mercier and Hamel 2010; Mercier et al. 2011a). Similar to gametogenesis, seasonal spawning events have been correlated with large-scale environmental factors, although temperature and phytoplankton blooms are the most commonly proposed drivers for them (Olive 1995; Mercier and Hamel 2009a). Of note is the fact that photoperiod and sea surface temperature are closely linked and difficult to tease apart.

It is widely accepted that periodicities in reproduction (or portions thereof) can either be driven directly by the cyclic change in a given factor or involve the action of a biological clock entrained by external stimuli. For practical reasons, studies of reproductive rhythms under free-running conditions in marine organisms have largely centered on tropical and subtropical taxa with rapid cycles of reproductive activity (see chapter by Neumann, this volume); for example, the marine midge *Clunio mari-*

nus (Neumann and Heimbach 1984) and the ragworm *Platynereis dumerilii* (Fischer and Dorresteijn 2004). In benthic invertebrates from cold regions, such studies can be arduous to undertake for reasons of the annual (or longer) period of reproduction; documenting free-running cycles would require several years of monitoring in controlled settings, making them much less popular. The endogenous mediation of gamete synthesis over the annual/seasonal cycle is believed to be under hormonal control in most major groups of marine invertebrates (Andries 2001; Lawrence and Soame 2009; Mercier and Hamel 2009a; Nagaraju 2011). Exactly how cyclic environmental signals are perceived (e.g., visually, chemically) and transduced into a suite of reactions via the endocrine system, ultimately leading to gamete maturation, remains unclear. The full cycle was proposed to be governed by an endogenous oscillator (endocrine cascade of sexual maturation) entrained by environmental cues (photoperiod, temperature), allowing phase shift in gamete maturation and responsiveness to late-acting triggers (lunar or chemical signals) (Olive 1995).

The interplay between biological rhythms and social interactions is rarely a focus in the study of timing programs, with the exception of sociable schedules in birds (Helm et al. 2006) and debates on estrous synchrony in mammals (Weller and Weller 1993; Schank 2002). However, this topic has received a fair degree of attention among invertebrate biologists preoccupied with the level of synchrony among individuals and populations over the annual cycle of gamete production. Studies have revealed that environmental and social cues act synergistically to synchronize gamete synthesis and release in some species of free-spawning boreal invertebrates. The occurrence of chemical communication during gametogenesis in boreal holothuroids (sea cucumbers) was evidenced on the basis that increasing daylength triggers the onset of gametogenesis but that (1) interindividual exchanges are necessary to synchronize gamete development within the population (Hamel and Mercier 1996); (2) gametogenic activity can be induced in less mature individuals by the proximity of those more developed (Hamel and Mercier 1996); and (3) ectodermic mucus is a carrier of chemical information (Hamel and Mercier 1999). Furthermore, the internal fluid that bathes the organs, which is called the perivisceral coelomic fluid (PCF), when taken from mature spawning individuals can trigger gamete release in mature nonspawning individuals either when injected or spread in the seawater (Mercier and Hamel 2002). A broad interpretation of these results is that substances present in the PCF and mucus help holothuroids coordinate epidemic spawnings, which are typical in free-spawning marine organisms. Although the endocrine control of reproduction was well studied in polychaetes (Andries 2001; Lawrence and Soame 2009) and steroids have been measured during the reproductive cycle of asteroids (Schoenmakers and Dieleman 1981; Hines et al. 1992; Barker and Xu 1993), it remains unclear whether these compounds disperse during gametogenesis, similar to the occurrence in some freshwater fishes (Stacey et al. 2003).

From this we may conclude that various rhythms and corresponding cues (annual, lunar, diel) are superimposed to achieve breeding synchrony, under the following assumptions: (1) the most sensitive members of a population (sentinels) cue gametogenesis on environmental factors; (2) gametogenic activity unfolds and gonadal maturation in the population is phased via external signals and a waterborne dialogue; (3) at the peak of gamete maturity, spawning is triggered by a proximate cue (temperature, light, phytoplankton), whereas epidemic gamete release is coordinated through a waterborne signal. Whether clock-based mechanisms are involved at any of these stages remains unresolved.

6.2.2 Deep-Sea Species

Of the deep-sea species that have been investigated so far, relatively few have been found to display convincing evidence of reproductive periodicity, although examples now include most major phyla and span bathyal and abyssal depths (Schoener 1968; Lightfoot et al. 1979; Tyler 1988; Tyler et al. 1994; Young 2003; Watling et al. 2011). It should be emphasized that gathering such evidence is difficult for deep-sea species for logistical and economic reasons. Live animals can only be observed for brief periods in situ and are rarely held in conditions suitable for long-term study. The sporadic and multidisciplinary nature of deep-sea expeditions also means that preserved samples are typically collected across wide temporal and spatial scales (e.g., having to mix several years to obtain samples from most months; mixing samples collected hundreds of kilometers apart). This limitation can prevent the observation of a reproductive peak or a spawning event, should it be brief or restricted to a small portion of the population (Baillon et al. 2011). Other potential difficulties in detecting reproductive periodicities include spatial variations in the reproductive status of colonial species (e.g., oocytes developing asynchronously along the different section of coral colonies) and the simultaneous development of multiple cohorts of oocytes within individuals or colonies (Baillon et al. 2014, in press). Therefore, assumptions of ‘continuous’ or aperiodic reproduction must be treated with caution when they are based on a few sporadic gonad samples that do not cover most of one single or two consecutive annual cycles. To date, some of the clearest evidence of reproductive periodicity in deep-sea taxa has been obtained either from relatively complete time-series of gonad samples or from the study of live specimens that underwent successive breeding events in the laboratory.

A recent field study of reproduction in deep-sea corals from the Northwest Atlantic showed that one brooder (*Anthomastus grandiflorus*) and one free-spawner (*Keratois grayi*=*ornata*) both exhibited clear evidence of seasonal reproduction (Mercier and Hamel 2011). The onset of gametogenesis apparently occurred early in the year, no later than March, when daylength and seawater temperature were starting to increase. The putative spawning in late summer and fall coincided with the warmest seawater temperature of the cycle and with high rates of detritic matter deposition. Similarly, various studies of live deep-sea invertebrates revealed seasonal cycles of gamete release in asteroids (Mercier and Hamel 2008), scleractinian corals (Mercier et al. 2011b), and sea anemones (Mercier and Hamel 2009b), as well as seasonal larval release (planulation) or spawning in octocorals (Sun et al. 2010b, 2011; Baillon et al. 2014, in press). All these cycles were correlated with broad environmental variables, chiefly primary production and resulting deposition of phytodetritic matter. Pulses of food supply to the deep seafloor have long been considered putative drivers of periodic or seasonal reproduction (Tyler et al. 1982).

Although many patterns of reproduction have so far been documented in deep-sea taxa, including periodic (annual/seasonal), aperiodic (sometimes coined 'continuous'), and opportunistic (Tyler and Young 1992), we must reiterate that a large portion of the supporting data is incomplete and far from ideal. Remarkably, all the deep-sea organisms we have been able to monitor over long periods to obtain direct evidence of reproduction (i.e., gamete or larval release) have displayed relatively discrete breeding periods, many of which were determined to be cyclic and exhibit both seasonal and lunar components (see Sect. 6.3.2).

6.3 Lunar Cycles of Reproduction in Cold Nontidal Environments

6.3.1 General

Organisms that dwell in the intertidal zone experience daily modulations in water level that reach maximum amplitude every 14.8 days. They also experience marked cycles in the amount of light reflected by the moon on a period of 29.5 days (lunar month); at the sea surface, illuminance at the full moon is approximately 25 times greater than at the quarter moon and 250 times greater than on moonless nights. It is therefore not surprising that biological rhythms synchronized with lunar phases have mostly been documented in environments directly exposed to moonlight or submitted to tidal regimes (Fox 1924; Neumann 1981; Naylor 1982; Chandrashekaran and Sharma 2008). Lunar periodicities in reproductive events are particularly conspicuous in coastal habitats, notably in tropical reef fishes (Takemura et al. 2010; Chap. 10) and corals (Harrison et al. 1984; Babcock et al. 1986; Tanner 1996; Chap. 5). Several free-spawning corals release gametes annually during the week following the full moon, both on the Great Barrier Reef in the austral spring (Richmond and Hunter 1990) and in the Caribbean Sea in the fall (Levitan et al. 2004). Certain brooding corals also exhibit peaks in the release of planula larvae around the new or full moon (Harrison and Wallace 1990). Moreover, spawning events attuned to the lunar cycle have been documented in several shallow-water tropical or subtropical species of echinoderms (Kubota 1981; Lessios 1985; Kubota and Tomari 1998; Mercier et al. 2007), mollusks (Yoshioka 1989; Counihan et al. 2001), crustaceans (Skov et al. 2005; Flores et al. 2007), and annelids (Caspers 1984; Bentley et al. 2001). In contrast, lunar breeding cycles are much less frequently evidenced in marine taxa that live in regions above 40°N (Korringa 1947; Bentley et al. 2001; Mercier and Hamel 2010), and least of all in deep-sea species (Mercier et al. 2011a). Limited as it may be, this evidence is crucial in devising comprehensive frameworks for future studies of lunar-related rhythms.

6.3.2 Subtidal Boreal Species

Why is lunar periodicity so rarely examined in the huge body of literature that concerns the reproduction of boreal and polar benthic marine species? The most likely explanation is that lunar components can be obscured or tend to be overlooked when reproduction shows marked solar-related (annual/seasonal) periods, as is commonly the case at higher latitudes. Another explanation is that recurrent data are not easy to gather when spawning or release of progeny occurs infrequently (roughly once a year) in unwelcoming environments (cold, turbid, rough seas). It remains difficult to identify lunar components when reproductive state is monitored either indirectly (e.g., using gonad indices) or punctually (e.g., during dive surveys).

Evidence of circalunar cycles is slightly more common in temperate than in cold (boreal and polar) species. For example, Korringa (1947) documented lunar-based (tidal) maxima in seasonal larval abundances of the commercial oyster *Ostrea edulis* and in nuptial swarms of annelids. Lunar rhythms of reproduction have now been evidenced in other temperate bivalves (Caddy 1967) and annelids (Bentley et al. 2001) living in tidal environments. Apart from anecdotal and correlative reports (Mercier and Hamel 2009a), cold-water species living below the tidal zone had not received much attention until recently, when a study of fine periodicities and coordination within and among species showed remarkably synchronized seasonal breeding with a strong lunar component in 23 benthic species belonging to six phyla (Mercier and Hamel 2010). Thirteen free-spawning species of echinoderms (holothuroids, asteroids, echinoids), cnidarians (sea anemones), gastropod mollusks, and tunicates released gametes in March or April between the first and last moon quarter, with peaks around the full moon (Figs. 6.1a and 6.2). Another ten species of

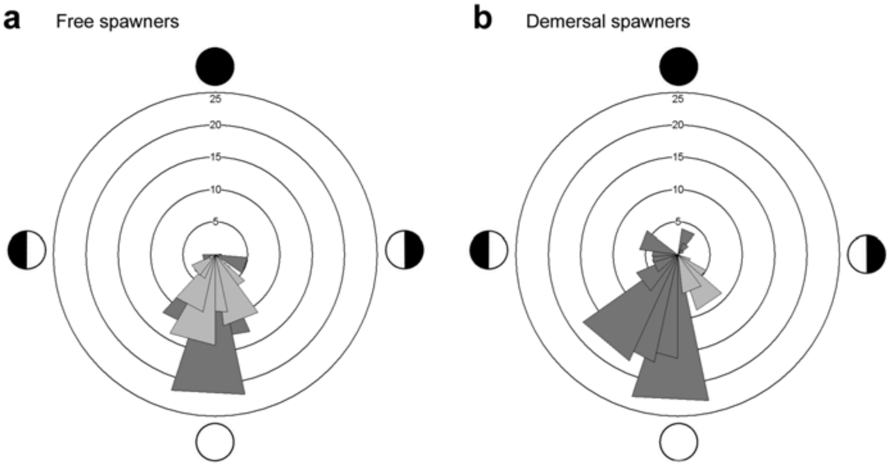

Fig. 6.1 Circular histograms of breeding events recorded in March (*dark gray*) and April (*silver*) for (**a**) free spawners ($n=13$) and (**b**) demersal spawners ($n=10$). Data shown as frequency of occurrence on each lunar day. *Filled circles*, new moon; *open circles*, full moon. (Adapted from Mercier and Hamel 2010, with permission)

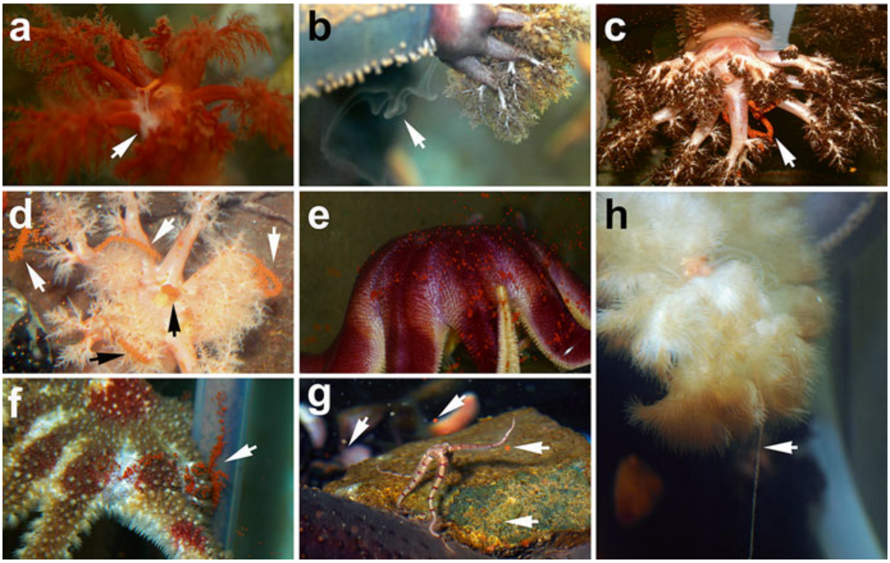

Fig. 6.2 Seven of the boreal species shown to exhibit lunar cycles of reproduction (see Fig. 6.1) in a mesocosm study. (**a**) Male *Psolus fabricii* (Holothuroidea) releasing a thick stream of sperm (*arrow*). (**b**) A thin wisp of sperm (*arrow*) escaping from the tentacle crown of male *Cucumaria frondosa* (Holothuroidea). (**c**) Female *C. frondosa* releasing a string of eggs (*arrow*). (**d**) Female *Psolus phantapus* (Holothuroidea) releasing a string of eggs that is breaking apart (*arrows*) from tentacular movements. (**e**) Female *Solaster endeca* (Asteroidea) releasing a cloud of red buoyant eggs. (**f**) Female *Crossaster papposus* (Asteroidea) releasing a stream of buoyant eggs (*arrow*). (**g**) *Ophiopholis aculeata* (Ophiuroidea) spawning while the water is filled with eggs of different origins (*arrows*). (**h**) Female *Metridium senile* (Anthozoa, Actiniaria) releasing a trickle of eggs (*arrow*). (From Mercier and Hamel 2010, with permission)

echinoderms, crustaceans, mollusks, and annelids displayed a lunar rhythm in the deposition of demersal eggs or the release of brooded progeny, with peak activity centered on the full moons of March and April (Figs. 6.1b and 6.2). Furthermore, distinct species-specific events occurred at scales of hours or minutes during same-day multispecies free spawnings (Mercier and Hamel 2010). The study included a review of in situ accounts of spawning events. In a field study (Gulf of St. Lawrence, North Atlantic) by Himmelman et al. (2008), the lunar phase underlying the spawnings was generally consistent, and the spawnings observed in the Northeast Pacific by McEuen (1988) all occurred a few days before and after the full moon. Another study conducted in Maine (USA) on the echinoid *Strongylocentrotus droebachiensis* used fertilization assays to determine in situ sperm emissions and observed that they were significantly greater around the full moon (Gaudette et al. 2006). Circalunar spawning rhythms have also been suggested in other cold-water echinoderms, for example, *Ophiothrix fragilis* in the United Kingdom (Smith 1940), *Ophiopholis aculeata* and *Ophiura robusta* in the White Sea (Hendler 1991), and *Evechinus chloroticus* in New Zealand (Lamare and Stewart 1998).

Lunar rhythms are commonly distinguished on the basis of being entrained (generally by tides) or directly cued (generally by light) (Palmer 1973; Naylor 1999; Baird et al. 2009; Takemura et al. 2010). Free-running lunar cycles that are retained in the absence of moonlight or tides have been reported in tropical and subtropical mollusks (Yoshioka 1989; Counihan et al. 2001) and temperate annelids (Bentley et al. 2001), among others. Conversely, time of larval release was shown to be directly tied to nocturnal light in the reef coral *Pocillopora damicornis*, as colonies exposed to constant darkness or light were unable to maintain synchronized lunar periodicity for long (Jokiel et al. 1985). The transition from a moonlit to a dark night was found to act as a zeitgeber for the lunar swarming rhythm of the temperate marine polychaete *Syllis* (=*Typosyllis*) *prolifera* (Franke 1985), similar to observations in laboratory populations of *P. dumerilii* (Hauenschild 1960). Gamete release in the tropical holothuroid *Isostichopus fuscus* also appeared to be cued by moonlight because blocking this component interrupted the predictable monthly spawnings (Mercier et al. 2007). In boreal benthic invertebrates that reproduce only once a year, studies of gamete or larval release under controlled conditions are difficult to conduct over more than one cycle. To date, it remains unclear whether their lunar reproductive periodicities are entrained by moonlight, tides, or other less frequently evoked variables (e.g., hydrostatic pressure, gravitational pull, geomagnetic fields). In a laboratory investigation of multispecies breeding events (Mercier and Hamel 2010), lunar cycles were linked to moonlight rather than tides because windows allowed penetration of light, and tidal oscillations were not reproduced (with the potential caveat that subtle tidal shifts in plankton levels, organic load, and general water chemistry could still be perceived).

The broad assumptions that emerge from the accounts of annual mass coral spawnings on tropical reefs are that the month of the year is set by temperature variations, the day of the month by lunar rhythms, and the time of the day by sunset (Vize et al. 2005). Boreal species appear to set the month(s) of the year by phytoplankton abundance, the day(s) by the lunar phase, and the time of day by sunrise (or perhaps its indirect effect on seawater temperature) (Mercier and Hamel 2010). The occurrence of predictable species-specific daily spawning windows also strongly suggests the presence of a circadian component in the reproduction of most species (Mercier and Hamel 2010). Currently proposed proximate cues for the occurrence of reproductive activity on a particular lunar phase and time of day/night include temperature (Neumann and Heimbach 1984; Olive 1995), lunar irradiance (Harrison et al. 1984), lunar photoperiod (Hauenschild 1960), tidal levels (Babcock et al. 1986), seasonal photoperiod (Babcock et al. 1994), and twilight chromaticity (Sweeney et al. 2011). Again, studies have largely focused on reef corals, which are increasingly believed to respond directly to local solar cues (Penland et al. 2004; Brady et al. 2009). The presence, phase, and position of the moon in the sky, which modulates the twilight color, was recently put forward as a plausible cue for mass lunar-related spawning events on tropical reefs (Sweeney et al. 2011). Boch et al. (2011) proposed the existence of a threshold response to differential periods of darkness after twilight that is primarily influenced by lunar photoperiod and secondarily by discrete optical components of early nocturnal illumination. Blue-light-sensing photoreceptors (cryptochromes) have been detected in reef-building corals (Levy et al. 2007). Behavioral responses to faint light fluctuations (in the order of nautical twilight and full moonlight intensity; ~1 lux) have been documented in other marine invertebrates, including sea urchins (Pearse 1972) and polychaetes (Bentley et al. 2001).

How these external lunar stimuli may be translated into reproductive processes remains unclear. Although annual/seasonal fluctuations in sex steroids have been examined in boreal marine invertebrates (Andries 2001; Lawrence and Soame 2009; Mercier and Hamel 2009a; Nagaraju 2011), to our knowledge lunar (monthly) periods in the concentration of these compounds have never been investigated such as they have in certain tropical fishes and corals (Gassman 1992; Twan et al. 2003; Takemura et al. 2010). Hence, the mechanisms that underlie lunar-related periods in the reproduction of most marine invertebrates are incompletely understood. In contrast to tropical fishes, where lunar spawning cycles have been shown to involve successive monthly cycles of plasma steroid levels and ovarian development (Takemura et al. 2010), boreal invertebrates typically exhibit annual cycles of gamete development; the lunar component appears only at the culmination of the reproductive cycle, that is, upon release of gametes or zygotes on a particular moon phase. Although this might indicate that gametogenesis is not under lunar control, fragmentary evidence suggests that the final phase of gamete maturation may be quite rapid in some marine invertebrates, perhaps occurring within the last month leading to spawning (Mercier and Hamel 2009a; Baillon et al. 2011; Doyle et al. 2012). Interestingly, experiments on cultivated populations of the marine polychaete *Typosyllis prolifera* have revealed that periodic stolonization (prelude to reproduction) is linked to lunar rhythm secretion of a prostomial hormone (Franke 1983a, b). A circalunar oscillator within a photo-(neuro)endocrine pathway was thus proposed to define rhythmic periods of time during which hormone secretion from the prostomium is enabled, setting in motion the complex system of physiological processes culminating in the release of a mature swarm-ready stolon (Franke 1985).

6.3.3 Deep-Sea Species

In deep-sea environments (>200 m), which make up more than 66 % of our planet's surface, lunar rhythms are virtually overlooked. Reasons for this include problems in monitoring organisms in situ, low resolution of sampling protocols (below that required to identify lunar or semilunar periodicities), and scarcity of observational and experimental studies on live specimens. As opportunities multiply for sampling in the deep ocean and with recent breakthroughs in the maintenance and study of live deep-sea organisms, we are beginning to elucidate the finer aspects of their reproductive periodicities (Mercier and Hamel 2008, 2009b; Sun et al. 2010b). Furthermore, the observation of thin rings in the skeleton of deep-water gorgonian corals, which appear to follow monthly periods, evokes the possibility of lunar growth rhythms (Roark et al. 2005).

Unprecedented evidence of the occurrence of lunar reproductive cycles in deep-sea taxa was recently published (Mercier et al. 2011a). Both gamete release in free spawners and larval release in brooders were determined to exhibit a significant lunar trend in five species studied in the laboratory over several lunar months and breeding periods (Figs. 6.3 and 6.4). Furthermore, indirect evidence of reproductive activity from field samples collected in two different years also suggested a lunar component in the annual spawning of a sixth species (Mercier et al. 2011a).

Lunar periodicity in the deep sea is a rather counterintuitive concept at first glance, given the quickly decreasing light and weakened influence of tides as depth increases (Fig. 6.5), which may explain why this factor is essentially overlooked in deep-sea biology. A similar reluctance to consider the occurrence of seasonal cycles of reproduction in the deep ocean was common until recently. Deep-sea species living below the permanent thermocline were presumed to experience little to no variations in physicochemical factors susceptible to drive periodic reproduction. However, downfalls of phytodetritus to the deep seafloor were shown to exhibit cyclic pulses that are now believed to mediate seasonal reproduction in a variety of deep-sea invertebrates (e.g., Young 2003; Sun et al. 2010b; Mercier et al. 2011b; Baillon et al. 2014).

Factors that might drive lunar cycles at depth are even more difficult to identify, but evidence is gathering in favor of a few hypotheses, including (1) lunar fluxes of surface-produced material to the deep sea; (2) direct perception of lunar light; and (3) deep tidal currents. Lunar cycles in zooplankton abundance are known to occur in freshwater and saltwater environments (Gliwicz 1986; Hernandez-León et al. 2002), and peak deposition rates of planktic foraminiferans at 2,700 m depth (Fig. 6.6) were shown to occur about 12.5 days after each full moon (Lončarić et al. 2005). Growth bands in deep-sea gorgonian corals lend support to the detritic flux hypothesis. Counts of fine skeletal rings evoking monthly/lunar periods were suggested to be driven by influx of sedimentary organic matter at spring tides (Roark et al. 2005; Tracey et al. 2007). Apparent tidal periods in turbidity fluctuations and in behavior of vestimentiferan worms documented in hydrothermal ecosystems have been suggested to coordinate the reproductive cycle of vent species (Zal et al. 1995). Although a direct response to lunar light is only conceivable above the limit of downwelling illumination (~200–1,000 m; Fig. 6.5), evidence of sensitivity to low light or moonlight has been documented in marine taxa. Coral polyps can sense the blue portion of lunar irradiance (Gorbunov and Falkowski 2002), deep-sea shrimp can detect near-UV light remaining in the downwelling field at a depth of 600 m (Frank and Widder 1994), and rabbitfish exhibit changes in plasma melatonin levels in response to moonlight intensity (Rahman et al. 2004). Furthermore, Norway lobsters were shown to exhibit a light-entrained circadian cycle of emergence down to 300–400 m (Chiesa et al. 2010; Aguzzi et al. 2011), and modulations in melatonin release by isolated pineal glands of deep-sea fishes were proposed to follow semidiurnal tidal oscillations (occurring every ~12 h) at ~2,700 m depth in the Northeast Atlantic (Wagner et al. 2007). Although daily shifts cannot explain lunar cycles of reproduction, Wagner et al. (2007) referred to unpublished data of spring-neap lunar periodicity in deep currents. Other putative lunar cues that have

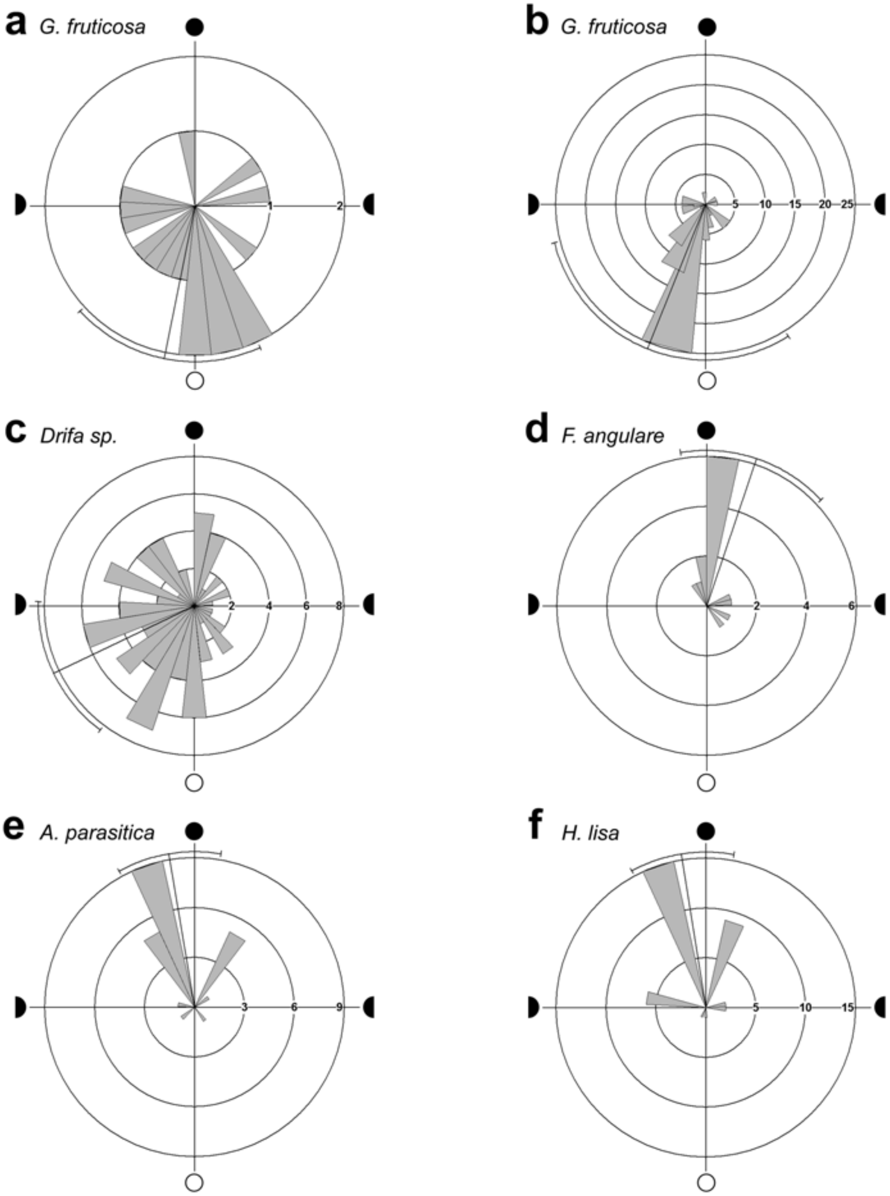

Fig. 6.3 Circular histograms depicting reproductive events (*wedges*) of deep-sea species monitored in the laboratory, with mean angle (*line*) and 95 % confidence interval (*bracket*). Occurrences of planulation events (**a**) and number of planulae released (**b**) in *Gersemia fruticosa*. (**c**) Occurrences of planulation events in *Drifa* sp. (**d**) Occurrences of spawning events in female *Flabellum angulare*. (**e**) Occurrences of spawning events in male and female *Allantactis parasitica*. (**f**) Occurrences of spawning events in male and female *Henricia lisa. Filled circles*, new moon; *open circles*, full moon. (From Mercier et al. 2011a, with permission)

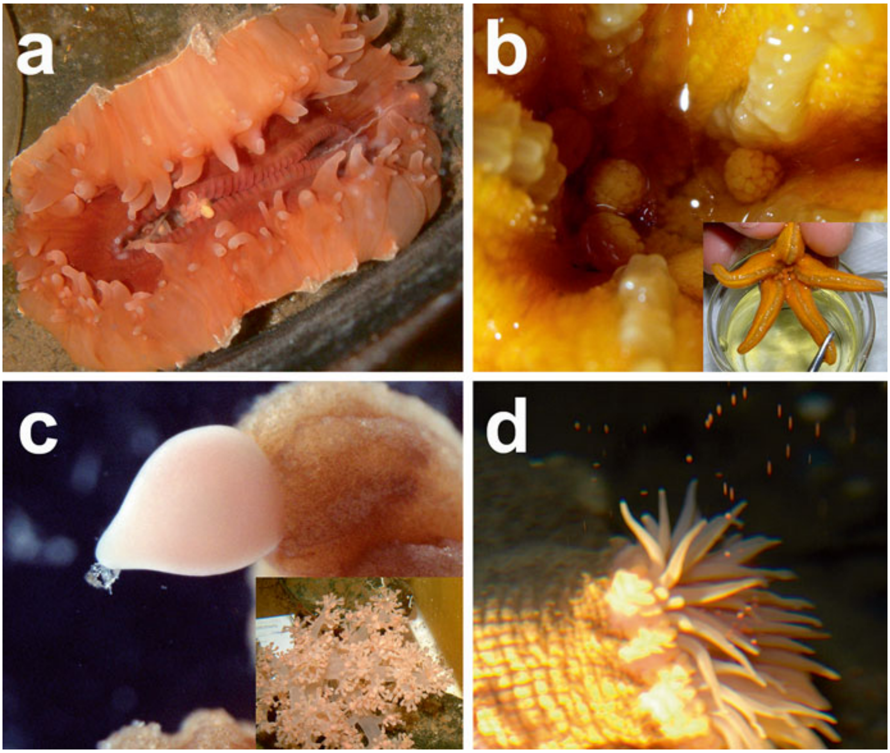

Fig. 6.4 Four of the deep-sea species shown to exhibit lunar-related reproductive activity (see Fig. 6.3) by Mercier et al. (2011a): the solitary coral *Flabellum angulare* (Anthozoa, Scleractinia) emitting bundled oocytes (**a**); the sea star *Henricia lisa* (Asteroidea) brooding embryos under its bell-shaped body (**b**) (*insert* shows entire female); the soft coral *Drifa* sp. (Anthozoa, Alcyonacea) emitting a planula larva (**c**) (*insert* shows entire colony); and the epibiotic sea anemone *Allantactis parasitica* (Anthozoa, Actiniaria) releasing oocytes into the water column (**d**)

yet to be thoroughly investigated include gravitational and geomagnetic forces. Overall, the perception and transduction of moon-associated cues by marine organisms remain largely unresolved, even in fishes in which lunar cycles have been investigated for more than a half century (Takemura et al. 2010).

The fact that lunar cycles were evidenced in the laboratory, as well as in the field, during the study by Mercier et al. (2011a) suggests that they are either persistent lunar-entrained endogenous rhythms or, more likely, that they are cued by lunar cycles in deposition rates of planktic material. The latter hypothesis is in line with previously documented seasonal reproduction in deep-sea taxa that followed peak rates of sedimentation in the laboratory (Mercier and Hamel 2008, 2009b; Sun et al. 2010a; 2010b). However, it leaves us wondering if and how reproductive events could be precisely timed to celestial events via indirect proxies, given the lag between surface production and settling of planktic matter at depth. This question will remain a mystery until we develop our capacity to conduct in situ investigations in the deep sea.

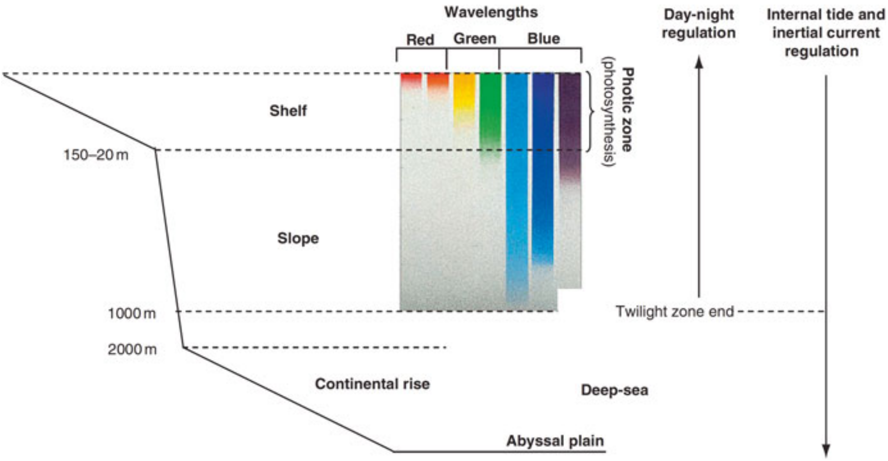

Fig. 6.5 Schematic representation of continental margin depth zonation. Light undergoes a double modification over depth as photons travel within the water column body: intensity decreases and spectral diversity diminishes. The photic zone represents the depth range (0–200 m) where photosynthesis occurs. The twilight zone end border represents the upper limit of the aphotic deep-sea realm, where the last few remaining photons can be detected. (From Aguzzi and Company 2010, with permission)

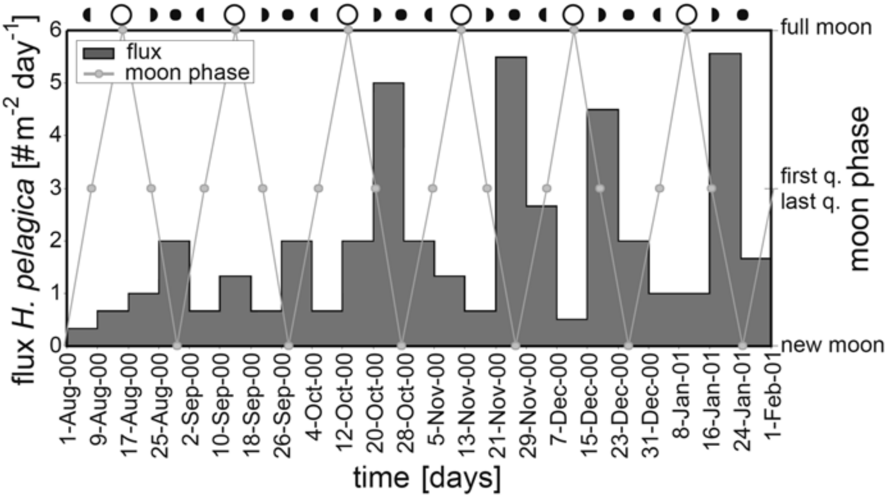

Fig. 6.6 Deposition flux of the foraminifera *Hastigerina pelagica* (*dark grey bars*) relative to phases of the moon (*light grey line*) from August 2000 to February 2001, recorded at 2,700 m by the Walvis Ridge sediment trap. (From Lončarić et al. 2005, with permission)

6.4 Adaptive Significance of Lunar Breeding Periodicities

Although many marine organisms display a lunar or semilunar component in some phase of their reproductive process (Naylor 1999), the adaptive significance of lunar periodicity in reproduction is still unclear, especially in environments that are not perceptibly influenced by tidal factors (currents, desiccation). One of the simplest explanations is that lunar cues provide a reliable way of coordinating reproductive activities. Rhythmic reproductive cycles, and lunar periodicity in particular, have been proposed to favor population persistence under conditions of female-biased or low population size (Omori 1995). Reproductive synchrony has its own set of selective advantages (reviewed by Ims 1990), including fertilization success and protection of adults and offspring against predators. Hatchling-biology hypotheses are particularly well suited to intertidal invertebrates, in which tidal or semilunar periodicities are said to maximize survival, settlement, and dispersal of planktonic offspring (Pearse 1990; Morgan 1995b). However, their pertinence is questionable for deep-sea organisms. As for adult-biology hypotheses, they have mainly been tested in reef fishes, for example, gregarious spawning might improve defense against egg predators (Robertson et al. 1990).

Although the broader within-season synchrony has been questioned as an antipredatory strategy (Ims 1990), same-day mass spawnings such as those discussed in Mercier and Hamel (2010) certainly evoke a means of diluting predation risks. Counterpredation is a particularly attractive explanation when spawning or larval release coincides with full or new moons, both of which can add an element of concealment to predator swamping. Breeding activities in various taxa often coincide either with the brightest or darkest nights of the lunar cycle (see reviews by Baird et al. 2009; Mercier and Hamel 2009a; Takemura et al. 2010). The deep-sea taxa examined by Mercier et al. (2011a) essentially showed the same dichotomy (Fig. 6.3), and a trend appeared on the basis of reproductive strategy. One of the brooding corals predominantly released larvae at the full moon and the other planulated over the waning phase (between the full and new moon). In contrast, the four broadcast-spawning species synchronized gamete release with the new moon (Mercier et al. 2011a). These findings are in apparent contradiction to the full-moon mass events reported in broadcast-spawning reef corals, although it might be caused by the respective environmental and predatory pressures of each habitat.

When lunar phases synchronize annual breeding events, many species may ultimately release gametes within the same period (e.g., spring full moons), leading to mass-spawning events (Mercier and Hamel 2010). Synchronized release of heterospecific gametes evokes the risk of wasting gametes in unfertile or nonviable mating crosses for those species lacking prezygotic mechanisms of gamete recognition. Studies have shown that interspecific fertilization is possible among cross-generic species, and even among cross-phyletic species, when the gametes are artificially mixed (Hamel and Mercier 1994; Harper and Hart 2005). In this context, two nonexclusive hypotheses can be proposed to account for the maintenance of reproductive isolation in sympatric species that breed simultaneously. (1) The occurrence of species-specific spawning cues whereby each species responds to different signals, or somewhat differently to the same signals. Reproductive isolation would then become the ultimate factor shaping reproductive periodicities. (2) The existence of an interspecific communication mechanism (e.g., allomones) that would stimulate or inhibit gamete release in given species on various time scales. The level of coordination evidenced in the mesocosm study of boreal invertebrates (24 species from six genera; Mercier and Hamel 2010) has very rarely been demonstrated, except in reef-building corals (seven species from three genera; Vize et al. 2005). In three hermaphroditic species of the coral genus *Montastraea*, a combination of gametic, temporal, and spatial prezygotic isolating mechanisms was found to help reduce the likelihood of hybridization (Levitan et al. 2004). Similarly, phylogenetic analyses suggest that slightly different spawning times form the basis of reproductive isolation among *Acropora* corals (Fukami et al. 2003).

Although parallel evolution of spawning cues may be suggested to account for general heterospecific breeding synchrony, the occurrence of discrete diel spawning windows in free spawners (Mercier and Hamel 2010) evokes some degree of coevolution among sympatric species. The simultaneous involvement of free-spawning species and species that lay encapsulated or demersal eggs is puzzling because the offspring of the latter are presumably not under the same selective pressures. However, it could be further indication of cross-specific cueing. In free spawners, the latter assumption is only sustainable if mechanisms exist to prevent the wastage of gametes through heterospecific interactions, as discussed earlier.

6.5 Summary and Outlook

There are significant geographic and bathymetric biases in studies of lunar-related marine biological rhythms and clocks. Understandably, taxa from tropical and near-shore environments that display brief life cycles or short breeding periods have so far provided most of the currently available data (Naylor 2010; Takemura et al. 2010; Tessmar-Raible et al. 2011). Boreal and deep-sea marine organisms occupying habitats between 20 and 2,500 m deep presumably receive less direct tidal and moonlight cues; however, the fact that many of them have now been shown to exhibit lunar-related breeding periodicities may help broaden our understanding of both the adaptive significance and underlying mechanism of such rhythms. As it stands, several questions are still not resolved: (1) which levels of the breeding cycles (seasonal, lunar, diel) are clock based versus directly cued by external signals? There is evidence for both types of circadian and lunar/tidal rhythms in marine taxa (Naylor 2010). (2) Why/how do so many species spawn concurrently? (3) How can seasonal/lunar cues operate in deep waters (in and below the disphotic zone)? (4) How do ultimate and proximate drivers of periodicities compare in species with different life histories?

Beyond well-known aspects of breeding synchrony among conspecifics of a population or between populations of a species over annual or monthly periods, synchronous breeding in marine invertebrates can encompass single-site same-day multispecies spawnings that leave us wondering: (1) How are these mass spawnings orchestrated, and (2) What is the adaptive value of such a strategy? Same-day mass spawnings have mainly been documented in reef corals (Harrison et al. 1984; Babcock et al. 1986; Penland et al. 2004; Guest et al. 2005; Carroll et al. 2006) and other tropical marine taxa (Babcock et al. 1992b; Van Veghel 1993; Hagman and Vize 2003; Scheibling and Metaxas 2008). However, they have also been recorded in temperate and cold benthic animals, both in the field (McEuen 1988; Pearse et al. 1988; Minchin 1992; Sewell and Levitan 1992; Himmelman et al. 2008) and under naturally fluctuating laboratory conditions (Mercier and Hamel 2010).

The latter work was the first to present clear evidence of coordinated seasonal mass spawning events outside coral reefs, in cold-water species, thus showing that an unsuspected number of free-moving macroinvertebrates may not only exhibit synchronous breeding but undergo finely coordinated spawning events. The excellent fertilization and development rates obtained in mesocosm settings emphasize the efficacy of these coordinating mechanisms in maintaining reproductive isolation during mass spawnings. Further investigation of species-specific spawning windows and their adaptive value might include manipulative trials (i.e., selectively isolating/mixing various taxa and experimentally testing putative spawning cues) as well as field video-monitored mesocosm studies to corroborate laboratory results. It would be particularly informative to contrast ambient mesocosms and mesocosms removed from putative cues (e.g., sunlight, moonlight) to test whether spawning events would remain rhythmic and synchronous.

Finally, the recent discovery of lunar rhythms of reproduction in various deep-sea taxa (Mercier et al. 2011a) serves to highlight our limited understanding of the drivers and advantages of lunar rhythms in marine organisms, emphasizing the need for further research on a broader scale than previously anticipated. There is increasing support for the interacting roles of the sun and the moon in generating specific luminance and chromacity that may drive lunar rhythms in the photic and disphotic zones. The same or similar interactions may well produce specific gravitational and magnetic fields perceived everywhere on the planet's surface, and these deserve to be investigated as putative universal drivers of lunar-related reproductive events at various scales.

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Chapter 7
Local Adaptations of Circalunar and Circadian Clocks: The Case of *Clunio marinus*

Tobias S. Kaiser

Abstract Biological clocks are considered to increase an organism's fitness in that they allow the synchronization of reproduction, physiology, and behavior with environmental conditions. Indirect evidence for the biological fitness value comes from the existence of local timing adaptations within a species. Some of the most clear cut examples of adaptations in circalunar and circadian clocks are found in the intertidal midge *Clunio marinus*. In adaptation to the local tidal regime, populations of this insect differ in the phase of the circadian clock, as well as in the phase, period, and zeitgeber sensitivity of the circalunar clock. These differences allow comparative genetic and molecular studies that may both shed light on the evolutionary forces shaping biological clocks and unravel the first known molecular components of a circalunar clock.

Keywords Evolution • Fitness • Population genetics • Quantitative trait locus (QTL) • Temporal isolation

7.1 Introduction: Selected Evolutionary Aspects of Circatidal and Circalunar Clocks

7.1.1 The Adaptive Value of Circatidal and Circalunar Clocks

Biological clocks are at the core of some of the most fascinating phenomena in biology, ranging from the time-compensated sun-compass orientation of the migratory monarch butterfly to the swarming of the palolo worm during a few predictable nights a year. With astonishing precision, circatidal, circadian, circa(semi)lunar, and circannual clocks capture the environmental cycles that are driven by the earth's rotation around its axis, the earth's revolution around the sun, and the revolution of

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the moon around the earth. It is reasonable to assume that biological clocks have evolved because they enhance an organism's *fitness*, that is, its longevity and reproductive success, by allowing the organism to coordinate its physiology or behavior with environmental cycles. However, experimental validation of this hypothesis has turned out to be challenging. Even for the adaptive value (i.e., the 'fitness advantage') of circadian clocks, convincing evidence has been presented in only a few tractable systems (O'Donnell et al. 2011; Saunders 1972; Ouyang et al. 1998; Dodd et al. 2005) (for a review of other evidence, see Yerushalmi and Green 2009).

Circatidal and circalunar clocks are primarily found in marine organisms, and two major hypotheses have been discussed with respect to their adaptive value (Christy 1978; Pearse 1990; Naylor 2001).

On the one hand, circatidal and circalunar clocks may time an organism's activity or reproduction to the most suitable times of the tidal cycle. This capacity is most important in intertidal zones, which experience strong fluctuations in physical conditions. Circalunar clocks come into play here because the tidal cycle is modulated across the lunar cycle (see Chap. 1 by Neumann, this volume, Sect. 1.2.1). This hypothesis may hold true for many crustaceans (e.g., *Eurydice pulchra*: Reid and Naylor 1985; Zhang et al. 2013; *Excirolana chiltoni*: Klapow 1972; Enright 1972; *Sesarma haematocheir*: Saigusa 1986; Saigusa 1980), and the marine midge *Clunio marinus* (see Chap. 1 by Neumann, this volume, and this chapter).

On the other hand, circalunar clocks in particular may synchronize reproduction within a population, which may be especially important for organisms with external fertilization, such as corals (Harrison et al. 1984) and marine annelids (*Eunice viridis*: Hauenschild et al. 1968; *Platynereis dumerilii*: Hauenschild 1960; Zantke et al. 2013; *Typosyllis prolifera*: Franke 1985). Lunar cues may also serve to synchronize reproduction in tropical regions where distinct seasonal cues are not available. A striking example may be the lunar-synchronized mass emergence of midges from tropical lakes (Corbet 1958; Hartland-Rowe 1955). There are many other ways in which lunar cycles can influence an organism's fitness (Kronfeld-Schor et al. 2013), but in most cases evidence for the involvement of an endogenous circalunar clock is lacking.

There has been very limited experimental testing of the adaptive value of circalunar and circatidal clocks. For the marine midge *Pontomyia oceana*, which times its reproduction to distinct tidal situations by combining circalunar and circadian clocks (similar to *C. marinus*; see Chap. 1 by Neumann, this volume, and the following sections of this chapter), it was tested whether there is a fitness difference between the midges emerging in the full moon peak versus the new moon peak of a semilunar rhythm. The authors found no difference and concluded that emergence during both new moon and full moon spring tides is a *bet-hedging strategy*, which allows the organism to cope better with stochastic variation in the tides (Soong and Leu 2005). The same insect served to test the two hypotheses on the adaptive value of tide-related clocks in terms of the synchronization of reproduction versus timing to the tides. As already mentioned, *P. oceana* does not have a circatidal clock but instead relies on a circadian clock in combination with a circasemilunar clock to time emergence to the tides. The precision of circadian reproductive timing within the population was found to be higher than the accuracy of coordination with the tides (which also depends on the circalunar clock). This observation suggests that in *P. oceana* the adaptive value is rather synchronization of reproduction within the population (Soong et al. 2006).

7.1.2 Local Adaptation

Indirect evidence for the adaptive value of circatidal and circalunar clocks comes from the existence of local adaptations in these clocks, that is, from clock-controlled differences in tidal and lunar rhythms within one species that correlate with differences in the environmental cycles which the species experiences across its geographic range. Generally, *adaptations* are defined in terms of fitness, that is, as characteristics that enhance an organism's survival or reproduction, relative to alternative character states. They are caused by natural selection and therefore must have a genetic basis (Futuyma 2009). *Phenotypic plasticity*, an environmentally induced change of a character during an organism's lifetime, also enhances an organism's performance in a specific environment (cf. Naylor 1989 with respect to tidal and lunar clocks). These changes are not embraced by the term adaptation in its evolutionary sense.

7.1.3 Temporal Isolation

Local adaptations in timing are particularly interesting if they affect reproduction. In these cases they may entail temporal isolation between populations, representing a prezygotic reproductive barrier (Dobzhansky 1937; Mayr 1942, 1970). Most studies on temporal isolation are concerned with seasonal differences in reproductive timing, be it in terrestrial (Alexander and Bigelow 1960; Tauber and Tauber 1977; Smith 1988; Filchak et al. 2000; Abbot and Withgott 2004; Levin 1978), riverine (Palstra et al. 2004), or marine habitats (Knowlton 1993; Palumbi 1994). There are some studies on diel differences in reproductive timing (Lloyd 1966), including in *Drosophila*, where the isolating effect of different circadian timing of activity and reproduction has been shown in the laboratory and could in one case be attributed to a single gene (Tauber et al. 2003). Studies on temporal isolation in tidal or lunar rhythms are rare: differing lunar and diel rhythms of reproduction are reported in closely related damselfish species (Foster 1987), and differing lunar rhythms of spawning were observed in sea urchins (Lessios 1984; Muthiga 2003). Organisms from the intertidal zone have hardly been studied with respect to local timing adaptations and temporal isolation, which is surprising because the fluctuation of environmental conditions is strong in the intertidal zone and tidal regimes differ tremendously between different geographic locations (see Sect. 1.2.1 by Neumann, this volume). Hence, these organisms can be expected to have pronounced and site-specific tidal and lunar rhythms.

7.1.4 Examples of Local Adaptation in Circatidal and Circalunar Cocks

Local adaptation in a circatidal clock is reported for the sand beach isopod *Euridyce pulchra*. This crustacean displays a tidal rhythm of swimming activity that is modulated across the lunar cycle (Reid and Naylor 1986). Entrainment of the tidal rhythm by cycles of mechanical disturbance was found to be most effective when the 2-h mechanical stimulus was presented during the daytime of high tide on spring tide days. For isopods from two different geographic locations, the time of day when most effective mechanical entrainment occurred, was found to differ according to the tidal regimes at their place of origin (Fig. 7.1).

Local adaptations in a circalunar clock are found in the marine midge *Clunio marinus*, for which the components of the circalunar and circadian timing system have been progressively characterized throughout the 1960–1990s (see Chap. 1 by Neumann, this volume). In the past years this species has become accessible to

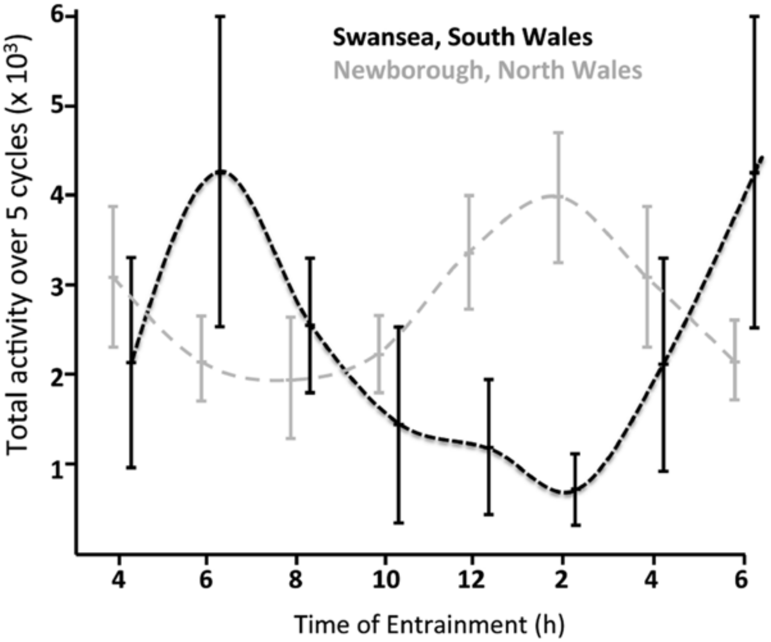

Fig. 7.1 Mean total activity (±SD) over five tidal cycles of the isopod *Eurydice pulchra* after exposure to a regime of 2-h agitation every 12 h for 4 days. The *x*-axis gives the time of day of the entraining 2-h agitation pulse. Each point on the *x*-axis is an independent experiment with 4–10 groups of 20 specimens. The last two points are repetitions of the first two for ease of comparison. The *y*-axis gives mean total activity, which is an approximation for the quality of entrainment of the tidal rhythm by the 2-h agitation pulse, plotted for populations from Swansea (*black*) and Newborough (*grey*). Spring tide high tides occur around dusk and dawn in Swansea and around noon and midnight in Newborough. For both locations, entrainment is most effective around the time of local spring high tides. (Adapted from Reid and Naylor 1986)

genetic and molecular studies. The remainder of this chapter focuses on the timing adaptations of this re-emerging model system, which may inspire both chronobiologists and evolutionary biologists.

7.2 Local Timing Adaptations in *Clunio marinus*

7.2.1 Introduction to C. marinus

An overview on the life cycle of *C. marinus*, its endogenous clocks, and their experimental corroboration is given in Chap. 1 by Neumann (for more details see Neumann 1966, 1986, 1995). Briefly, *C. marinus* (Diptera, Chironomidae) is a marine midge that is found in the rocky intertidal zone along the European Atlantic Coast (Fig. 7.2). The larvae build their tubes in pads of small algae and sand, which are found in the lower midlittoral on protected rocks that are almost permanently submerged. However, the adults need these larval substrates to be dry for oviposition, as they literally "glue" the egg masses onto suitable sites. To overcome the problem, *Clunio* has an extremely reduced adult lifespan of 1–2 h, and adult emergence is timed to coincide with the spring tide low tides, that is, the low tides on days around new moon and full moon, when the water levels are generally lowest. Upon emergence, the midges mate and oviposit immediately, then die in the rising tide. A circalunar clock controls development to ensure that pharate pupae, that is, pupae ready to emerge, are only present during spring tide days. A circadian clock controls the daily timing of adult emergence. Notably, in *C. marinus* there is no evidence for a circatidal clock. The midge achieves synchronization of its life cycle with the tides only by the combination of the circadian clock with the circalunar clock. Both clocks have been shown to be free running and temperature compensated, and the entraining cues ('zeitgebers') are known: the circadian clock is entrained by the light–dark (LD) cycle, whereas the circalunar clock responds to moonlight, tidal turbulence, or tidal temperature fluctuations. Along the European coastline, *Clunio* populations differ in at least four aspects of these timing systems: (1) phase of the diel emergence rhythm with respect to the zeitgeber cycle, (2) phase of the lunar emergence rhythm with respect to the zeitgeber cycle, (3) zeitgeber sensitivity of the circalunar clock, and (4) period of the circalunar clock. These aspects are discussed in detail below.

7.2.2 Phase of the Diel Emergence Rhythm

Differences in diel emergence time were the first variable clock properties to be observed in *C. marinus* (Neumann 1966). As delineated in Sect. 1.2.1 by Neumann (this volume), the daytime of low tide on spring tide days is close to constant for a given geographic location, but it varies tremendously between geographic locations. Laboratory strains of *Clunio* from different geographic locations emerge at different times relative to the artificial LD cycle, and these differences correlate well with the time of low tide on spring tide days at their place of origin (Fig. 7.2).

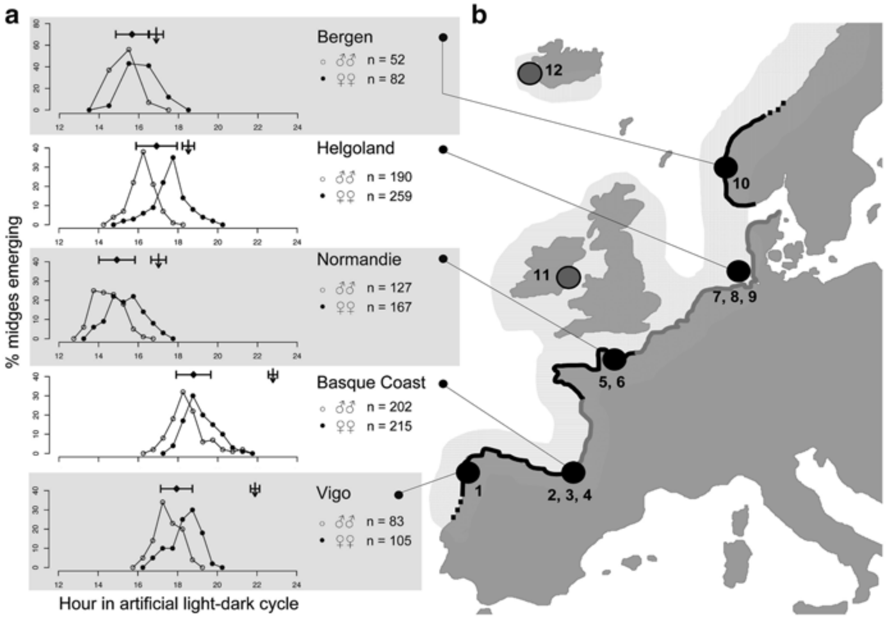

Fig. 7.2 Geographic range and locally adapted diel emergence times of selected laboratory strains of *Clunio marinus*. (**a**) Diel emergence peaks of males (*open circles*) and females (*filled circles*) are plotted against time in the artificial LD cycle in the laboratory. *Diamonds with whiskers* are mean emergence times ± SD. *Arrows with whiskers* are time of low tide on spring tide days ± SD at the place of origin of the respective laboratory strains. (**b**) The places of origin of the five laboratory strains as indicated by *black circles* correspond to the five regions analyzed in the population genetic study reviewed in Sect. 7.4. *Grey shaded area* is the known distribution of *Clunio marinus. Black lines* along the coast indicate predominantly rocky coast; *grey lines* indicate sandy coast, which is generally not inhabited by *Clunio marinus. Numbers* indicate sampled subpopulations: *1* Punta Helena; *2* St. Jean-de-Luz; *3* Erromardie; *4* Guéthary; *5* Grandcamp; *6* Port-en-Bessin; *7* Westwatt, *8* Vorhafen; *9* Düne; *10* Kviturdvikpollen. Other samples (*11* Greystones/Ireland; *12* Reykjavík/Iceland) are only used in the haplotype network (Fig. 7.6). (Adapted from Kaiser et al. 2010)

There is no evidence for involvement of a circatidal clock, given there is no daily delay of emergence according to the tides, and in each strain adults emerge only during one of the two daily low tides. The adaptive value of these diel timing differences has not been tested experimentally. However, it is intuitively clear that, given the short lifespan of *Clunio* adults, individuals emerging only a few hours early or late will find neither mating partners nor suitable substrates for oviposition, which must severely impair their fitness. The genetic basis of these adaptations in the circadian clock is discussed in Sect. 7.3.

7.2.3 Phase of the Lunar Emergence Rhythm

It was noticed early that *Clunio* laboratory strains of different geographic origin emerge at different times of the artificial moonlight cycle in the laboratory (Neumann 1966). This observation remained enigmatic for a long time, as *Clunio* populations in the field emerge around the spring tide days, that is, at the same time of the lunar cycle, irrespective of geographic location. Only recently were the different lunar emergence times shown to represent local adaptations to the tidal regime (Kaiser et al. 2011).

The conceptual background to understanding these lunar timing adaptations was established in two experiments, which are described in detail in Sect. 1.5.4 by Neumann (this volume; see Figs. 1.6 and 1.7). Briefly, one experiment showed that because of the circadian gating of *Clunio* moonlight sensitivity, moonlight is only an effective zeitgeber around midnight. The other experiment brought forth the idea that the tides may modulate the intensity of moonlight that reaches the submerged larval substrates of *Clunio*. In the light of these findings, it seems reasonable to assume that the time point of moonlight perception in the field depends on three conditions (Fig. 7.3). (1) *Clunio* must be sensitive to moonlight, which is only the case around midnight. (2) The moon must be in the sky around midnight, which is true during the moonlit quarters of the lunar cycle from waxing half moon to waning half moon. (3) The larval habitat in the lower midlittoral must be effectively exposed to moonlight by a low tide around midnight. The latter condition dramatically narrows the window for moonlight perception to a few days and is fulfilled during specific days in the lunar cycle for each geographic location, depending on the local tidal regime (compare site 1 and site 2 in Fig. 7.3a). Thus, the days of moonlight perception in the field are site specific, whereas the days of *Clunio* emergence during the spring tides are the same for all populations; this may explain the strain-specific phase relationship between the artificial moonlight stimulus and the emergence peak in the laboratory.

The testable prediction resulting from this hypothesis is that, for each *Clunio* population, the number of days between the artificial moonlight stimulus and the adult emergence peak in the laboratory should correspond to the number of days between the days with low tides around midnight and the spring tide days in the field, as has been tested for 11 laboratory strains; the results depict a perfect correlation (Fig. 7.3b). This finding has two general implications for the study of moonlight perception in circalunar clocks. First, it argues for caution when interpreting the artificial moonlight stimulus given in laboratory experiments; it may not necessarily represent full moon. Second, the modulation of light intensity by the tides makes moonlight basically a ‘tidal stimulus.’ Its perception depends on a circadian modulation of receptor sensitivity, and the very same mechanism has been discussed for the perception of other tidal stimuli, such as tidal turbulence cycles or tidal temperature cycles (see Sect. 1.5.5 by Neumann, this volume).

The genetic basis of the strain-specific phase relationship between moonlight zeitgeber and lunar emergence time is described in Sect. 7.3.

7.2.4 *Zeitgeber Sensitivity of the Circalunar Clock*

Three different stimuli have been shown to act as zeitgebers for entrainment of the circalunar clock in *C. marinus*: moonlight, tidal cycles of water turbulence, and tidal cycles of water temperature (for details of the mechanisms, see Sect. 1.5.5). The geographic distribution of the sensitivity to these zeitgebers suggests a certain degree of local adaptation (Table 7.1). Whereas all laboratory strains from southern

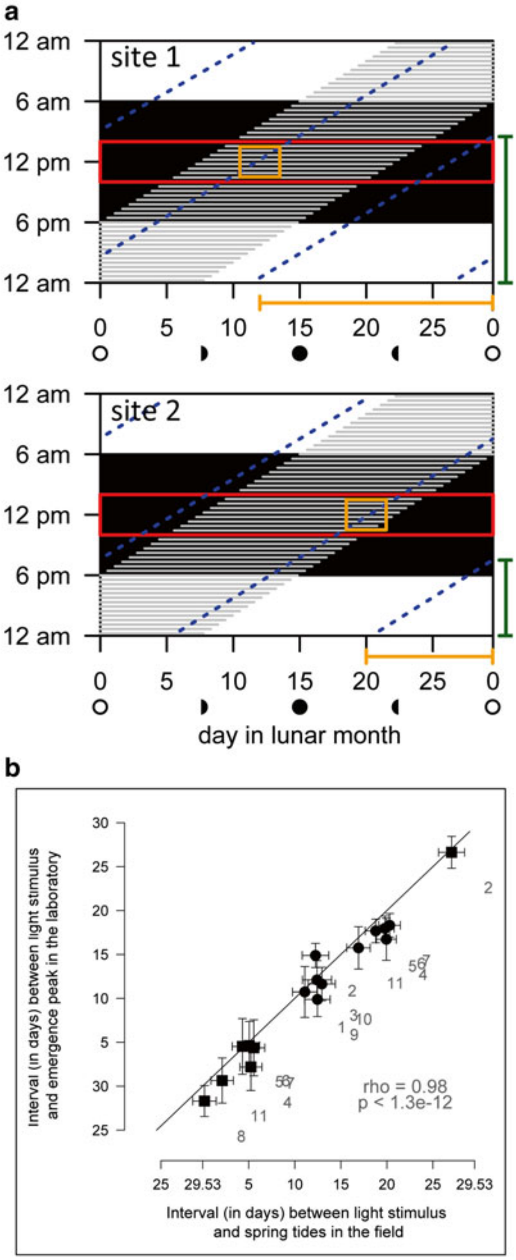

Fig. 7.3 Local adaptation in lunar emergence times of *Clunio marinus*. (**a**) Schematic relationship of the moon phases, tides, *Clunio's* moonlight sensitivity, and the locally adapted diel and lunar emergence times for two localities with differing tidal regimes (site 1, site 2). Time of day is plotted against days of the lunar cycle. *Black area* represents dark phase; *grey shading* indicates when the moon is in the sky. *Red box* marks the circadian period of sensitivity of *Clunio* to moonlight.

Table 7.1 Zeitgeber sensitivity of selected laboratory strains of *Clunio marinus*

Strain	Origin	Effective entrainment by zeitgeber		
		Moonlight	Turbulence	Temperature
Vigo	Vigo, Spain	+	+	Not tested
Jean	St. Jean-de-Luz, Basque Coast/France	+	–	Not tested
Qui	Quiberon, Bretagne/France	+	+	Not tested
Ros	Roscoff, Bretagne/France	+	–	Not tested
Por	Port-en-Bessin, Normandie/France	+	+	Not tested
Stud	Studland, UK	+	+	+
He	Helgoland, Germany	–	+	+
Ber	Bergen, Norway	–	o	+

+ strong response; o intermediate response; – very weak, irregular, or no response
Data from Neumann (1989), Neumann and Heimbach (1979, 1984), Neumann (1966), Heimbach (1976)

regions respond to moonlight, the *Helgoland* strain from the North Sea does not; similar to other strains from northern regions, it responds strongly to tidal cycles of water turbulence or temperature. The suggested adaptive value of the lack of moonlight sensitivity in the *Helgoland* strain is that moonlight may not be a reliable time cue at northern latitudes, where the moon is low above the horizon and nights are short or even bright in summer may thus not penetrate the water surface effectively. The *Bergen* strain, which comes from a sheltered fjord location, responds much more strongly to temperature cycles than to turbulence cycles, which may correlate to the general lack of surf or waves that characterizes its habitat.

Notably, most populations responded to all tested zeitgebers (compare Table 7.1), suggesting that *Clunio* generally relies on the combination of several time cues to assure entrainment of the circalunar clock. In this sense, local adaptation may not involve the acquisition of habitat-specific zeitgeber sensitivities, but rather the loss of sensitivity to zeitgebers that are unreliable or absent or not required in a particular habitat.

The genetic basis of zeitgeber sensitivity has so far not been investigated.

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Fig. 7.3 (continued) Hypothetically, moonlight is best perceived when the time of low tide (*blue dotted lines*) falls to midnight (*yellow box*). As tidal regimes differ for other places along the coast (compare *blue dotted lines* in site *1* and site *2*), the time when moonlight is best perceived differs. Nevertheless, all known *Clunio* populations emerge during the spring tides (around new moon and/or full moon). Thus, they must respond to the moonlight stimulus with a different delay of their emergence peak (*yellow bars below the graph*). Note that the required strain-specific phase of circalunar emergence time (*yellow bars*) is correlated with the required strain-specific phase of circadian emergence time relative to LD (*green bars*) because both are adaptations to the tidal regime. (**b**) Lunar emergence times of *Clunio* laboratory strains relative to the artificial moonlight stimulus, plotted against the time between the *days with low tide at midnight* and *spring tide days* in the field (according to the model in **a**). *Error bars* are standard deviations. *Squares* mark emergence peaks that fall to full moon, *circles* mark emergence peaks that fall to new moon in the field. Correlation coefficient and *p* value of the correlation are given in the graph (Pearson's product–moment correlation). Strain identities: *1* Vigo; *2* Santander; *3* St. Jean-de-Luz; *4* Port-en-Bessin; *5* Lulworth; *6* Studland; *7* Bembridge; *8* Roscoff; *9* Concarneau; *10* Camaret-sur-Mer; *11* St. Briac-sur-Mer. (Adapted from Kaiser et al. 2011)

7.2.5 Period of the Circalunar Clock and Emergence During New Moon Versus Full Moon

With respect to the period of the circalunar clock, two classes have been described in *C. marinus*. There are strains with a *semilunar rhythm* that emerge during both full moon and new moon spring tides in the field. Two of these strains were tested in the laboratory and showed a free-running period of the circasemilunar clock of 11 days (*He* strain from Helgoland, Germany) or 13–14 days (*Por* strain, from Port-en-Bessin, Normandie/France), corresponding closely to half a lunar cycle of 14.77 days (Neumann 1966). There are also strains that have a *lunar rhythm*. Most of these strains emerge during new moon spring tides only (e.g., *Jean* strain, from St. Jean-de-Luz, Basque Coast/France), but one of them was found to emerge during full moon spring tides only (*Ros* strain, from Roscoff, Bretagne/France; see Neumann 1989). The free-running period of the circalunar clock in the *Jean* strain was measured to be 26–27 days (Neumann 1966).

It is unclear whether these differences represent local adaptations. The level of new moon versus full moon low tides can differ by 1–2 m along the European Atlantic Coast. However, magnitude and direction of the effect change gradually and are not consistent throughout the years. In some years full moon low tides are lower during *Clunio* emergence season in summer, and in other years new moon low tides are lower. In this respect, emergence in a semilunar rhythm may represent a bet-hedging strategy, as is reported for *Pontomyia oceana* (Soong and Leu 2005). Most strains with a lunar rhythm emerge during dark phase, and an explanation for emergence during new moon versus full moon in strains with a lunar rhythm might be found in factors associated with the increased nightly illumination during full moon nights, for example, an increased risk of predation.

There are hints to a genetic basis for the differences in the period of the lunar rhythm (see Sect. 7.3).

7.2.6 Adaptive Loss of the Lunar Rhythm

In two geographic areas, *C. marinus* is reported to have lost the lunar rhythm. The arctic population of Tromsø in northern Norway has completely abandoned both the circalunar and the circadian rhythm of adult emergence and instead relies on a tidal hourglass mechanism (see Sect. 1.4.4 by Neumann, this volume). These changes can be considered an adaptation to arctic summer with more than 2 months of permanent daylight, which precludes entrainment of the circadian clock and thereby also zeitgeber perception for the circalunar clock.

Clunio midges from the Baltic Sea were first recorded as *C. marinus*, but were later described as an independent species, *C. balticus* (Heimbach 1978). It is likely that *C. balticus* was derived from *C. marinus* within the past 10,000 years, as subfossil *Clunio* head capsules can be found in Baltic Sea sediments only within the past 8,000–10,000 years (Hofmann and Winn 2000). The two species can still be interbred in the laboratory (Heimbach 1978) and cannot be distinguished by standard phylogenetic markers (T. Kaiser, unpublished data). Supposedly, in adaptation to the virtual absence of tides in the Baltic Sea, *C. balticus* has lost the lunar rhythm of emergence. In contrast to *C. marinus*, the adults of *C. balticus* do not rely on exposure of the larval habitat but instead swarm and lay their egg masses on the open water surface, sometimes far offshore. Unlike the egg masses of *C. marinus*, egg masses of *C. balticus* penetrate the water surface and sink to the larval substrates (Endraß 1976), which are red algae at a depth of 2–20 m. Presumably to ensure sufficient aggregation of mating partners in the open sea, the diel emergence window of *C. balticus* is extremely narrow (30–60 min); it is timed to and modulated by dusk. While these adaptations make *C. balticus* independent from the tides, they do not impede the species from settling in places with tides, beyond the Baltic Sea. Indeed, sympatric populations of *C. marinus* and *C. balticus* are described from Bergen in Norway, where heterospecific matings are prevented by their differing circadian emergence times (Heimbach 1978). The stable co-occurrence of both species in this site as a consequence of temporal isolation was the basis for giving species status to *C. balticus*. This interesting evolutionary scenario may represent a case of ongoing ecological speciation and certainly deserves further investigation.

In summary, local adaptations in circalunar and circadian timing occur at a multitude of levels in *C. marinus*. Two of them—namely, phase of the lunar rhythm and phase of the diel rhythm relative to the respective zeitgeber cycles—have been studied at the genetic and molecular level. These experiments are described in the following sections.

7.3 The Genetic Basis of Circalunar and Circadian Emergence Times of *Clunio marinus*

7.3.1 Crossing Experiments

As early as 1967 Dietrich Neumann reported genetic control of the strain-specific diel emergence times in *C. marinus* (Neumann 1967). Recently, these results were confirmed, and beyond that, genetic control of the strain-specific lunar emergence times was discovered (Kaiser et al. 2011). Both studies relied on crossing experiments between two specific laboratory strains, which differ considerably in lunar and diel emergence time (Fig. 7.4a, b; panels 1 and 2). The two strains originate from the shores of St. Jean-de-Luz (Basque Coast, France) and Port-en-Bessin (Normandie, France) and are therefore referred to as *Jean* and *Por* strains (see Fig. 7.2). F_1 hybrids of these two strains emerge at intermediate times between the parents with respect to both the lunar and the diel emergence time; the backcross progeny are intermediate between the backcross parents (Fig. 7.4a, b; panels 3 and 4), which documents a genetic basis to both strain-specific diel and lunar emergence times. The lack of 1:1 segregation in the backcross progeny indicates that each trait is controlled by two or more independent genetic factors (Kaiser et al. 2011).

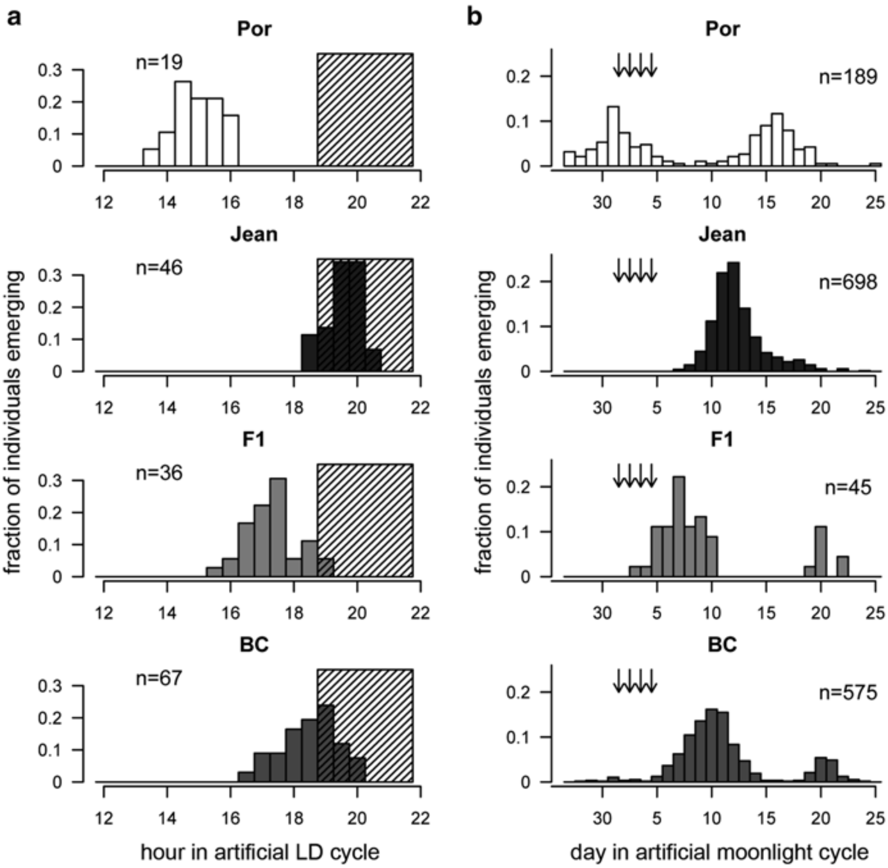

Fig. 7.4 Emergence patterns of parental strains, hybrids, and backcrosses of two populations of *Clunio marinus* under light-dark cycles (LD 14:10) and artificial moonlight entrainment. (**a**) Diel rhythm plotted as the fraction of individuals that emerged during 30-min intervals, for the parental strains of Port-en-Bessin (*Por*) and St. Jean-de-Luz (*Jean*), the F_1 generation, and the backcross generation (*BC*). Daytime is given in hours after the middle of dark phase ("hour 0"), which necessarily makes the middle of the light phase "hour 12." *Hatched area* marks dark phase. (**b**) Lunar rhythm plotted as the fraction of individuals that emerged during each day of the artificial moonlight cycle. *Arrows* mark days with artificial moonlight. Additive data of two lunar cycles are shown. (From Kaiser et al. 2011)

7.3.2 Linkage Mapping and QTL Analysis

One family of the backcross progeny from the aforementioned crosses served to construct a linkage map of the *C. marinus* genome (Kaiser and Heckel 2012). The map has three linkage groups (Fig. 7.5), corresponding to the three chromosomes known from light microscopy (Michailova 1980). It has a genetic length of 167–193 cM (centimorgans) and the genome has a physical size of 95–140 million base pairs (Mb), as estimated by flow cytometry. The map also contains the

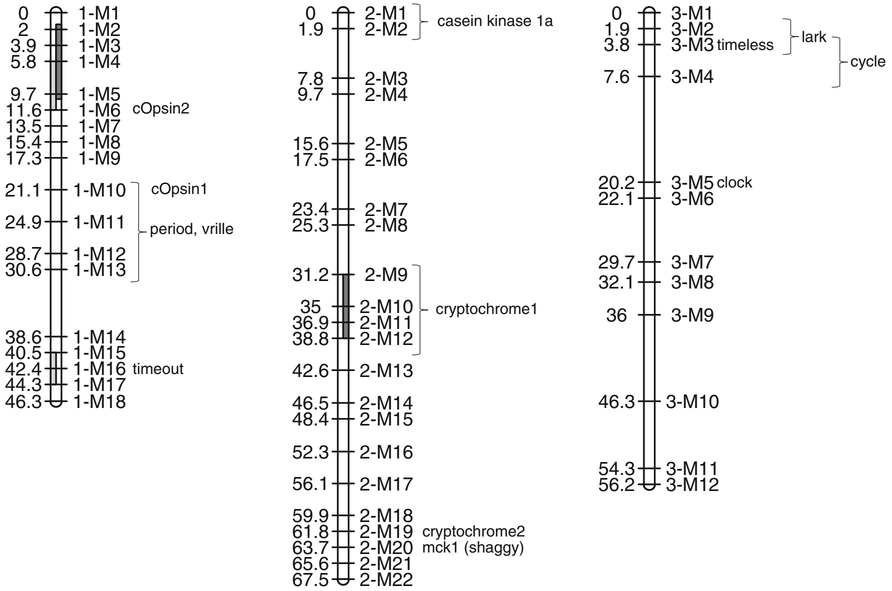

Fig. 7.5 Linkage map of the *Clunio marinus* genome including the quantitative trait loci (*QTLs*) for diel and lunar emergence times. There are three linkage groups corresponding to the three chromosomes. Map length in centimorgans (*cM*) is given to the *left*, marker names to the *right*. Locations of a number of light receptor and clock genes are given. For *period*, *vrille*, *casein kinase 1a*, *cryptochrome 1*, *lark*, and *cycle* only approximate positions are known. The QTLs for the differences in diel emergence time are *light grey*; QTLs for differences in lunar emergence time are *dark grey*. They overlap with the loci of *cOpsin2*, *timeout*, and *cryptochrome 1* respectively. (Adapted from Kaiser and Heckel 2012)

locations of most circadian clock genes known from insects, as well as a number of light receptor genes, which are considered candidates for being involved in the timing differences between the strains.

Quantitative trait locus (QTL) analysis, a statistical method testing the association of the patterns of inheritance of genetic markers with the pattern of inheritance of a given trait of interest, identified two QTLs controlling lunar emergence time, one on linkage group 1 and one on linkage group 2 (Fig. 7.5). For diel emergence time, two QTLs were identified, both on linkage group 1. The lunar QTL on linkage group 1 overlaps with one of the circadian QTLs. The estimated additive effects of these loci account for 85 % of the difference in diel emergence time and the full difference in lunar emergence time between the strains, indicating that these loci are the only major-effect loci for the two traits. The QTLs overlap with the location of the clock gene *timeout/timeless 2* as well as the locations of the light receptors (*insect type*) *cryptochrome 1* and *ciliary opsin 2*. These genes remain candidates for being involved in the timing differences. All other mapped candidate genes, including the well-studied circadian clock genes *period*, *cycle/bmal*, *clock*, *timeless*, and (*mammalian type*) *cryptochrome 2*, are not in the QTLs. For these genes, a major effect on strain-specific timing can be excluded.

7.3.3 Genetic Nonindependence of Diel and Lunar Emergence Times

In the backcross progeny of the crossing experiment, diel and lunar emergence times were correlated. Individuals emerging early in the artificial moonlight cycle also emerged early in the LD cycle, and individuals emerging late in the artificial moonlight cycle also emerged late in the LD cycle, suggesting a genetic nonindependence of both traits (Kaiser et al. 2011). This finding was underscored by QTL mapping, which showed that the QTLs with the largest effect on diel or lunar emergence time, respectively, are overlapping (Fig. 7.5). The resolution of the genetic map does not allow one to conclude whether the correlation is the result of independent but close-by circadian and circalunar genes (*genetic linkage*) or is caused by a single gene affecting both timing traits (*pleiotropy*). In the latter case we may further distinguish an effect of the circadian clock as a whole on the lunar rhythm (*modular pleiotropy*; e.g., the known involvement of the circadian clock in moonlight perception in *C. marinus*), from the effect of a single gene being involved in both circalunar and circadian clocks (*gene pleiotropy*). Deciding between these possible explanations will likely require the identification of the underlying genes.

Irrespective of the mechanism, the correlation of lunar and diel emergence times can itself be considered adaptive in the intertidal zone, as it stabilizes well-adapted combinations of diel and lunar emergence times. This correlation is beneficial, as each geographic location requires a specific combination of adaptations in lunar and diel emergence time to ensure emergence during the low tide of spring tide days. As both diel and lunar adaptations are adaptations to the tidal regime, they change in a correlated manner along the coastline (compare Fig. 7.3a). If lunar and diel timing are found to be controlled by independent but linked genes, it is conceivable that selection may have shaped genome architecture toward genetic linkage of those genes.

7.3.4 A Genetic Basis of the Period of a Lunar Rhythm?

The crossing experiment is in principle also informative on the nature of a lunar rhythm (*Jean* strain) versus a semilunar rhythm (*Por* strain). However, it is not clear whether the minor second peaks in the lunar rhythm of the F_1 and the backcross progeny (see Fig. 7.4) represent a genetic influence of the *Por* strain or an artefact of artificial moonlight entrainment (which sometimes produces small second peaks in the *Jean* strain as well). All individuals in the minor peak in the mapping family of the backcross progeny inherited a region of linkage group 2, ranging from marker groups 2-M16 to 2-M22, from the *Por* grandparent. If genetic control of the minor peak were to be confirmed, the genetic factors controlling the trait are expected to be in this genomic region.

7.3.5 Future Prospects

The reference genome sequence of *Clunio marinus* is close to completion, and population samples of the *Por* and *Jean* strains have been resequenced to identify which genes within the QTLs show strain-specific genetic variation (Kaiser, von Haeseler, Tessmar-Raible, unpublished). This step will reduce the number of candidate genes to a manageable level for analysis. The required molecular tools are currently being established for *C. marinus*.

The availability of a reference genome will not only facilitate molecular studies of *Clunio's* complex timing systems but also boost studies investigating the evolutionary causes and consequences of these timing adaptations. These topics are the subject of the following section.

7.4 The Evolutionary Background of Timing Adaptations in *Clunio marinus*

C. marinus has been be found at basically all rocky sites along the European mainland coast, on the British Isles, and in Iceland. At each geographic location, the populations are adapted to the local tidal regime in various aspects of the circadian and circalunar clocks (see Sect. 7.2 and Fig. 7.2). In the light of these findings, several questions must be raised with respect to the evolutionary history and population genetics of these populations: (1) When were the European coasts colonized? Namely, in which timeframe did the timing differences evolve? (2) Were the European coasts colonized from a single source or from several sources? Colonization from a single source would imply that the timing adaptations evolved after colonization. (3) Are these populations genetically isolated today? (4) If so, is genetic isolation the result of temporal isolation? And do the local timing adaptations drive genetic divergence between the populations? Answering these questions may also allow indirect conclusions to be drawn on the evolutionary mechanisms that shaped the timing adaptations. These issues were addressed in a recent study (Kaiser et al. 2010) that included a hierarchical sampling of ten populations from five regions along the European mainland coast (see Fig. 7.2b), at two very different scales of geographic separation. *Regions* were separated by 650–1,150 km. Within regions, *populations* were separated by 2–20 km only. From each population, 18 males were analyzed with nuclear and mitochondrial genetic markers: microsatellites, amplified fragment length polymorphisms (AFLPs), and mitochondrial DNA sequences (cytochrome oxidase I). The following conclusions could be drawn with respect to the afore-mentioned questions:

1. *Timeframe of colonization*: The haplotype network of COI sequences showed one major haplotype for each region, with a star-like expansion of minor haplotypes derived from the major haplotypes (Fig. 7.6). This is a signal of recent colonization, likely after the last Ice Age, within the last 20,000 years.

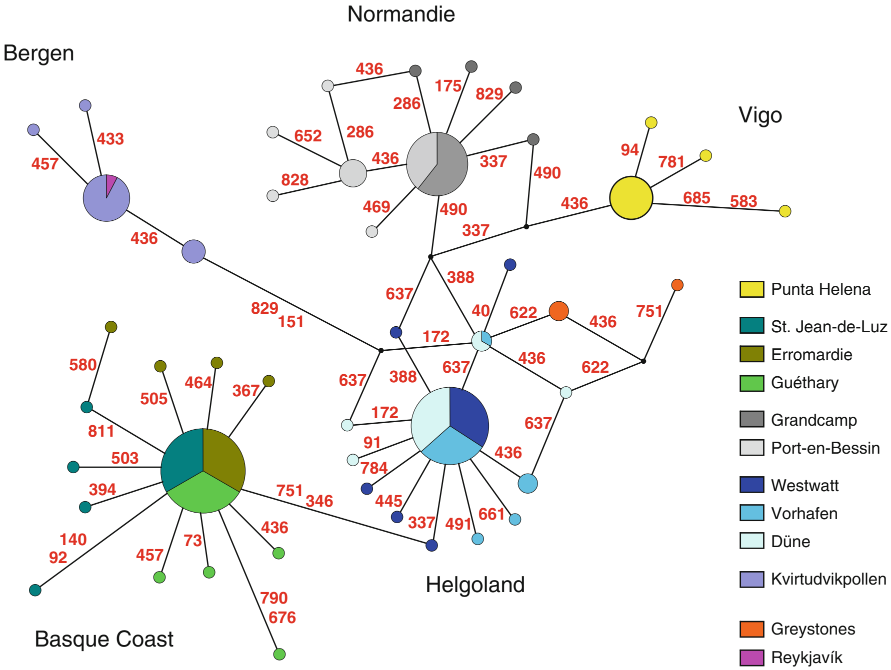

Fig. 7.6 Haplotype network for COI sequences. Each *circle* represents a haplotype; size of the circle equals haplotype frequency in the sample. *Lines* and *red numbers* indicate the mutations separating the haplotypes and the position of the mutation along the sequence. Geographic origins of the haplotypes are color coded (see *insert*). *Small black dots* are hypothetical haplotypes. Each region has its own set of haplotypes; only Iceland shares its haplotype with Norway. (From Kaiser et al. 2010)

The nuclear AFLP markers support this view, in that more than 80 % of the markers lack polymorphism all across Europe.

2. *Colonization sources*: The colonization source does not seem to be contained within the sampled regions, as all of them seem to be of recent origin. With respect to the number of colonization sources, the data are contradictory. Mitochondrial markers suggest colonization from five different sources (Fig. 7.6), but polymorphism in nuclear markers—if present—is shared and suggests a common source of all populations. As the identity and number of the colonization sources is unresolved, it remains unclear which of the timing adaptations were already present before colonization.
3. *Genetic isolation*: Within regions at a scale of 2–20 km, genetic differentiation is very low, indicating that these populations admix freely (Table 7.3). Between regions, on a scale of 650–1,150 km, mitochondrial haplotypes are perfectly private (Fig. 7.6). Genetic polymorphism in nuclear markers is shared, but allele frequencies differ tremendously, supporting strong genetic isolation (Table 7.2).

Table 7.2 Genetic differentiation in nuclear markers as measured by pairwise F_{ST} between regions

Region	Vigo	Basque coast	Normandie	Helgoland	Bergen
Vigo	–	0.225	0.332	0.438	0.393
Basque coast	0.060	–	0.276	0.387	0.349
Normandie	0.135	0.199	–	0.309	0.315
Helgoland	0.179	0.256	0.129	–	0.329
Bergen	0.144	0.137	0.085	0.226	–

Table 7.3 Genetic differentiation in nuclear markers as measured by pairwise F_{ST} between populations within regions

Basque coast	2	3	4
2	–	(0.008)	0.017
3	0.028	–	(0.011)
4	(0.002)	(0.013)	–
Normandie	5	6	
5	–	0.038	
6	0.022	–	
Helgoland	7	8	9
7	–	0.042	0.044
8	(0.004)	–	0.019
9	(0.005)	(–0.005)	–

Numbers correspond to population identities as given in Fig. 7.2; values for amplified fragment length polymorphism (AFLP) markers above the diagonal and for microsatellite markers below the diagonal; values in brackets are not significant *Source:* Data from Kaiser et al. (2010)

Most likely these regions have not had much genetic exchange since they were first colonized. Long stretches of sandy coast (which are not habitable for *Clunio*) seem to represent particularly strong barriers to gene flow: *Vigo* and *Basque Coast*, which are the only regions situated within the same stretch of continuous rocky coast, are less differentiated than the other regions (Table 7.2).

4. *Temporal isolation*: A test for temporal isolation was done at the scale of regions. If temporal isolation matters, genetic differentiation between the regions should be correlated to the timing difference between the regions. At the time of the study, local adaptation in the lunar rhythm was not yet fully understood, so that only the overlap of the diel emergence peaks (compare Fig. 7.2a) served as an approximation of timing differences. The diel timing differences show no correlation to the genetic differentiation, indicating that at the scale of regions temporal isolation does not matter. Geographic isolation seems to be the major isolating force at this scale.

In the light of the foregoing, the evolutionary background of the timing adaptations seems to differ depending on the geographic scales. At the scale of regions, the most likely scenario is that discrete stretches of rocky coast were colonized in rare and separate events. Strong geographic isolation between the regions likely facilitated the subsequent evolution of timing adaptations.

At the scale of populations within the same stretch of continuous rocky coast, geographic isolation is not very effective. If these populations have local adaptations in their circadian and circalunar clocks, these must have evolved or are being maintained, despite gene flow, only as a result of strong local selection. This idea represents a very different evolutionary mechanism compared to the one found at the scale of regions and deserves further investigation.

Concluding Remarks

The local timing adaptations of *C. marinus* in its circalunar and circadian clocks offer the unique opportunity to access and investigate the yet unknown circalunar clock and its interaction with the circadian clock by means of experimental and genetic dissection. At the same time, *C. marinus* provides a natural setup to study a multitude of evolutionary processes associated with biological clocks, ranging from the selective forces that act on biological clocks, to the temporal isolation that these clocks may impose on a species. In a very fruitful way, functional and evolutionary studies can shed light on each other in *C. marinus*. Evolutionary genomic scans for patterns of selection or differentiation between the locally adapted *Clunio* strains may help to identify genes that are involved in determining the local timing adaptations, possibly including the first genetic components of circalunar clocks known in any species. Conversely, knowing which genes are involved in circadian and circalunar clocks and determining their molecular interactions may foster our understanding of the evolutionary history of *Clunio* populations and of the evolutionary forces that shape local timing adaptations and temporal isolation.

Beyond this, the genus *Clunio* comprises a few dozens of species worldwide, and there are many more timing adaptations to be studied that may allow further experimental analysis of circadian and circalunar clocks, and perhaps circatidal and circannual clocks as well. Notably, the species *Clunio tsushimensis* from the seas of Japan was reported to display a tidal modulation in its diel emergence time (Saigusa and Akiyama 1995; Oka and Hashimoto 1959). As yet, it has not been tested whether this is a direct response to the tides, or whether there is an underlying circatidal clock. Furthermore, *C. tsushimensis* emerges year round and the tidal regime in its habitat is characterized by a distinct inequality between the levels of the two daily low tides. In summer, the morning low tides are lower, whereas in winter the evening low tides are lower. Consequently, *C. tsushimensis* was found to emerge during the morning low tides in summer and during the evening low tides in winter (Saigusa and Akiyama 1995; Oka and Hashimoto 1959). A preliminary laboratory study suggested that the seasonal switch in diel emergence times is directly controlled by photoperiod (Neumann 1983).

Clearly, there are many more enigmatic clocks to be unraveled in the unique model system *Clunio*.

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Chapter 8
Circadian and Circalunar Clock Interactions and the Impact of Light in *Platynereis dumerilii*

Juliane Zantke, Heinrich Oberlerchner, and Kristin Tessmar-Raible

Abstract The marine annelid *Platynereis dumerilii* coordinates its life in accordance to the daily sun cycle but also with the monthly changes of the moon. These rhythms are driven by internal molecular oscillators, both entrained by light. Here we provide an overview of our current knowledge on both circadian and circalunar clocks of the worms, as well as their interactions on molecular and behavioral levels.

In addition, this chapter also presents new data on the impact of nocturnal light (simulating moonlight) on circadian clock gene expression and locomotor behavior. Consistent with work in other species, nocturnal illumination impacts on both. Circadian clock gene expression profiles of worms at "full moon" (FM, i.e., dim nocturnal light) become arrhythmic. Similarly, worms at "full moon" are equally active during day and night, in contrast to their predominant nocturnality during "new moon" (NM, i.e., dark nights between full moon phases) and "free-running full moon" phases (FR-FM, i.e., dark nights when full moon would be expected). Although circadian clock transcript kinetics are different between FM and FR-FM, the circalunar clock-controlled spawning peaks are indistinguishable. This difference further confirms that circalunar clock function is independent of circadian clock transcript oscillations.

Keywords Annelid • Behavior • Bristle worm • Maturation • Molecular tools • Moon • Sun

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8.1 Monthly Rhythms in Reproduction

> "LUNA ALIT OSTREA ET IMPLET ECHINOS" Lucilius, ca. 150 BC

The above quote—*the moon nourishes the mussels and inflates the urchins*—illustrates the fact that classical authors had already seen a connection between the different phases of the moon and the size of certain marine invertebrates. Over the centuries, this point of view, which originally reflects a report in Aristotle's work "On the Parts of Animals," seems to have been transformed by generalization and popular mythology. As a result, Francis Bacon reports on a contemporary view holding that "the brains of rabbits, woodcocks and calves are largest at the full moon" (Sylva Sylvarum, 1627, cent. IX, sect.892). Although there are obviously no scientific data that would support such changes in brain size, zoological descriptions of the early and mid-twentieth century have clearly reestablished the connection between the apparent size of marine animals and different phases of the moon. More precisely, the studies revealed that the maturation of gonads in these animals depends on a lunar cycle (Fox 1924; Korringa 1947). In species in which the gonads contribute to a large proportion of the body mass, such as sea urchins, this effect is particularly prominent, but it is a very common phenomenon in diverse groups of animals (Fox 1924; Korringa 1947; Franke 1986; Naylor 2010). The lunar cycle provides a steady rhythm that can be used to synchronize gonadal maturation and hence spawning across a population, which is especially important for organisms that rely on external fertilization.

Polychaetes (bristle worms) are a particularly attractive group to study lunar periodicity (see Chap. 10 by Last and Hendrick, this volume). Not only are the mass spawnings of polychaete species, such as the bioluminescent fire worm of Bermuda (*Odontosyllis*), or the famous Palolo worms in the Southern Pacific (*Palolo viridis*), spectacular and fascinating natural phenomena, but in selected animals from this group, lunar reproductive periodicity already has a long history of scientific research (e.g. Korringa 1947; Ackermann et al. 2005; Hauenschild 1954; Franke 1985; Tessmar-Raible et al. 2011).

8.2 What Is This Chapter About?

In this chapter, we provide an overview on our current knowledge of the circalunar rhythm of the marine annelid *Platynereis dumerilii*, the underlying clock, and its relationship to the worm's circadian clock. We discuss our present understanding of the molecular composition of the *Platynereis* circadian clock and its anatomical location. We also include additional data on the impact of nocturnal light (given as 'full moon stimulus'/entrainment cue) on the worm circadian core clock gene expression and behavior. We compare three different conditions (Fig. 8.1): full moon [FM, i.e., over the course of 24 h the light regime changes between "day"light and "moon"light (LM)], alternating with new moon [NM, i.e., over the course of 24 h the light regime changes between "day"light and darkness (LD)] conditions. To discriminate between the direct impact of nocturnal illumination during the FM

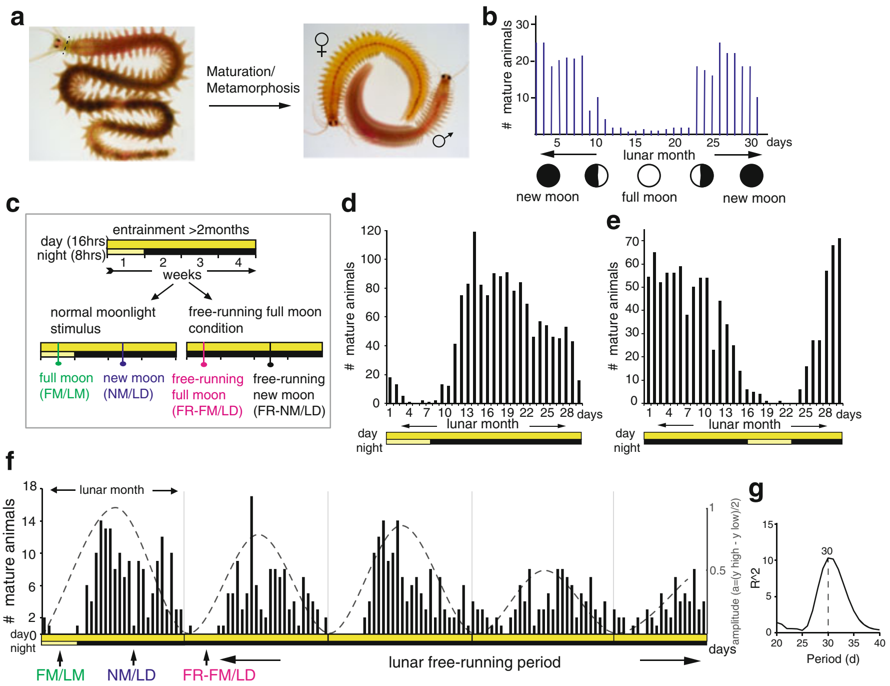


Fig. 8.1 *Platynereis* circalunar periodicity in gonadal maturation and reproductive behavior is entrained by nocturnal light and controlled by an endogenous clock. (**a**) *Left*: Adult worm before the start of sexual maturation. *Right*: Mature male and female at the time of spawning as counted for the quantification of the monthly synchronized spawning behavior shown in (**b**). (**d**–**f**) Worms were counted when exhibiting the nuptial dance behavior. (**b**) Maturation of *Platynereis dumerilii* is synchronized to the new moon phase of the lunar cycle. (Adapted from Hauenschild 1955.) (**c**) Illumination conditions: worms were entrained under normal daily and lunar light regime. Day, *yellow bars*; night, *black bars*; night with dim nocturnal illumination simulating full moon, *light yellow* marking on nocturnal bars. For free-running full moon experiments: dim nocturnal illumination is omitted. (**d**, **e**) The swarming peak depends on the position of the nocturnal light period and is shifted independently of the phase of the natural moon cycle. (**d**) Nocturnal illumination in phase with natural moon cycle. (**e**) Nocturnal illumination out of phase with natural moon cycle. (**f**) Monthly synchronized swarming rhythm is maintained for several months under lunar free-running conditions. *Dashed line*, amplitude. (**g**) Fourier analysis of free-running full moon spawning data shown in (**f**) reveals a period length of about 30 days. (See Zantke et al. 2013 for further details)

phases and the impact of the worm endogenous circalunar clock, we also analyze worms under “free-running full moon conditions” (FR-FM). For FR-FM conditions, worms are exposed to NM and FM conditions for at least 2 months. After this, the worms are exposed to darkness at the times when the nocturnal light stimulus would be expected to occur during the monthly cycle [i.e., over the course of 24 h the light regime changes between “day”light and darkness (LD)]. Thus, there is no difference in the illumination between NM and FR-FM times, but the phase of the monthly timer is different.

In the course of this chapter, we aim to highlight the particular suitability of *Platynereis* for the study of circalunar rhythms and the underlying clock.

8.3 The Lunar Model *Platynereis dumerilii*

8.3.1 Moonlight Synchronizes the Platynereis *Reproductive Cycle in Nature*

The marine polychaete annelid *Platynereis dumerilii* (Fig. 8.1a) was among the first organisms for which a lunar swarming rhythm in reproduction was scientifically reported (Fage and Legendre 1927; Ranzi 1931a, b). *Platynereis* has an indirect development from a free-swimming larval stage into a benthic adult stage (Fischer and Dorresteijn 2004; Fischer et al. 2010). During maturation, *Platynereis* transforms into a heteronereis form (Fig. 8.1a). At the end of metamorphosis the worms swarm at night near the surface of the sea, release their gametes into the water, and die. The synchronized swarming behavior of mature *Platynereis* and the simultaneous release of gametes are controlled by the lunar cycle in the worm's natural environment. In cultures kept in the laboratory next to a window or under light conditions mimicking the natural light timing, swarming of mature *Platynereis* occurs as in the sea, in clear correlation to the lunar cycle. Swarming peaks around the time of new moon, whereas around full moon only few to no mature worms appear (Fig. 8.1b; Hauenschild 1954, 1955, 1960).

8.3.2 Nocturnal Light Is Sufficient to Synchronize the Platynereis *Reproductive Cycle in the Laboratory*

Platynereis has been cultured successfully in the laboratory since the 1950s, and it has been shown that light at night is sufficient to synchronize the worm's monthly swarming behavior (Hauenschild 1954, 1955, 1960). We recently recapitulated these classical experiments by entraining our culture to a daily cycle (16:8 h light–dark cycle, = LD) and a lunar cycle [eight consecutive nights of dim nocturnal light per lunar month, simulating the full moon period (FM)] (Fig. 8.1c). As previously reported, *Platynereis* swarms under these laboratory conditions in clear correlation to artificial illumination, irrespective of the natural lunar phase, with a period of about 30 days (Fig. 8.1d, e) (Zantke et al. 2013). In cultures exposed to permanent artificial illumination or cultures that were never exposed to such periodic FM illumination, swarming is distributed randomly over the whole month (Hauenschild 1956, 1960; Zantke et al. 2013).

Experiments with artificial illumination using daytime light intensities ranging from 0.3 to 120 lux and nighttime light intensities (simulating moonlight) in the range of 0.02–2 lux suggest that the monthly periodicity in swarming is independent of light intensity, but rather depends on the periodic changes between dark and "moonlight" nights (Hauenschild 1956, 1960). Gradual changes in the duration of daytime illumination are also effective but must exhibit a duration difference of at least 4 h (Hauenschild 1960). Interestingly, also the wavelength does not appear to matter too much, as the monthly periodicity in swarming is equally well synchronized by white light or monochromatic red or blue light (Hauenschild 1956, 1960; Zantke and Tessmar-Raible, unpublished data). This finding suggests that either the illumination intensity in the different wavelengths was high enough to excite photoreceptors even at suboptimal wavelengths to sufficiently elicit synchronization, or that more than one type of photoreceptor is used for lunar (nocturnal) light perception.

Our experiments were conducted in the laboratory with inbred strains of *Platynereis* kept under controlled temperature, feeding frequency, and photoperiod conditions. Worms were maintained and cultured as previously described (Hauenschild and Fischer 1969). In our experiments with artificial illumination we used daytime intensities between 60 and 500 lux and nighttime intensities (simulating full moon) ranging from 1 to 10 lux. Our values are different from those of Hauenschild's experiments already mentioned as our whole illumination and worm culture setup is different. The variation in the values stem from the different position of the worm boxes relative to the lamps, so that for a given box, light intensities are correspondingly low or high at daytime and nighttime. For behavioral analyses, worms were grown under the aforementioned conditions. For the time of recording we placed them in a separate box to shield them away from disturbance, with daytime and nighttime illuminations in the range from 50 to 80 lux, that is, the light values for behavioral recordings under FM conditions equaled those of constant light conditions. For NM and FR-NM recordings, worms had received the monthly entrainment stimulus in the culture, that is, with the light regimes already mentioned. Behavioral analyses were conducted as described by Zantke et al. (2013).

8.3.3 **Platynereis** ***Possesses an Endogenous Circalunar Clock***

One question that had remained somewhat unclear was whether the observed lunar spawning rhythm in *Platynereis* is controlled by direct environmental light cues or by an endogenous clock mechanism? As this point was debated previously (Hauenschild 1956, 1960; Palmer 1974), we repeated the earlier lunar free-running experiments in our laboratory culture. We confirmed that worms clearly maintained their synchronized swarming behavior over several months even in the absence of the nocturnal light stimuli that simulate moonlight (Fig. 8.1f). The period length of the free-running rhythm is close to 30 days, resembling the length of a lunar month (Fig. 8.1f, g). The amplitude appears to slightly dampen over time and the peak to drift slightly relative to nocturnal light entrainment stimulus, suggesting that the endogenous rhythm might be slightly faster than the zeitgeber (Fig. 8.1f). This finding establishes that *Platynereis* possesses a circalunar reproductive periodicity that is entrained by nocturnal light and endogenously generated by a circalunar clock mechanism (Zantke et al. 2013).

8.3.4 **Platynereis***: An Old Model Reemerging for Marine Molecular Chronobiology*

As *Platynereis* has been used for laboratory experiments in zoology and evolutionary and developmental biology for decades, its culture conditions, the morphology and development are very well worked out (Hauenschild and Fischer 1969; Fischer and Dorresteijn 2004; Fischer et al. 2010). Recent work also suggests that *Platynereis* is slowly evolving and that its cellular, as well as molecular, makeup resemble evolutionary ancestral states (Tessmar-Raible and Arendt 2003; Arendt et al. 2004; Raible and Arendt 2004; Raible et al. 2005; Denes et al. 2007; Steinmetz et al. 2007; Tessmar-Raible et al. 2007; Tomer et al. 2010). Several molecular analysis tools, such as whole mount in situ hybridizations (Tessmar-Raible et al. 2005; Jekely and Arendt 2007), immunocytochemistry and immunohistochemistry (Arendt et al. 2004; Denes et al. 2007; Tessmar-Raible et al. 2007; Hasse et al. 2010; Rebscher et al. 2007), profiling by image registration (Tomer et al. 2010), laser-mediated specific cell ablations, and larval swimming assays (Jekely et al. 2008) are now complemented by a new set of molecular resources, functional and analyses techniques (Zantke et al. 2014):

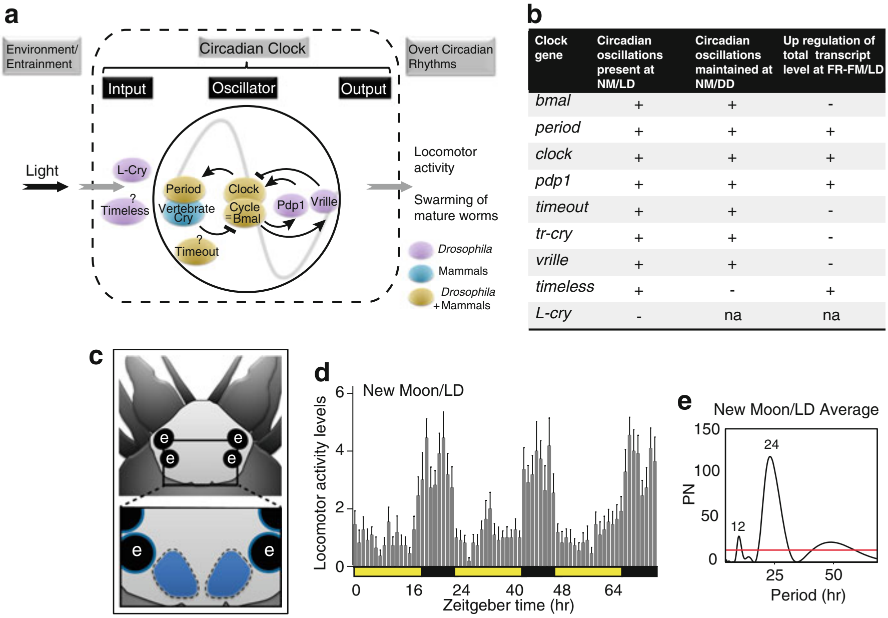
- Inbred strains (Zantke et al. 2013)
- Adult locomotor analyses setup (Zantke et al. 2013)
- Large sets of transcript and genomic sequences (Simakov et al. 2013)
- Morpholinos (Conzelmann et al. 2013)
- Transient and stable transgenesis (Backfisch et al. 2013, 2014)
- Metronidazole/nitroreductase-mediated conditional cell ablation (Veedin Rajan et al. 2013)
- TALE, nuclease-mediated targeted genome mutagenesis (Bannister et al. 2014)

Thus, currently *Platynereis* presents itself as possibly the functionally most powerful system for the elucidation of the molecular and cellular mechanisms that underlie circalunar rhythms.

8.4 The Circadian Clock of *Platynereis*

8.4.1 The Likely Basic Clock Mechanism

Circadian clocks generally utilize interlocking transcriptional-translational feedback loops as a mechanism to generate rhythms. Further levels of complexity in circadian clock regulation arise through posttranscriptional and posttranslational mechanisms (Zantke et al. 2014; Mehra et al. 2009), and through the involvement of redox states linking metabolism to the daily clock (Edgar et al. 2012; O'Neill et al. 2011). As transcription-based feedback loops are central to the generation and maintenance of circadian rhythms (Padmanabhan et al. 2012), we concentrated on genes orthologous to core circadian clock genes in other animals to gain a first insight into the *Platynereis* core circadian oscillator. *Platynereis* possesses a complete set of the *Drosophila* and mouse core circadian clock gene orthologues that are likely arranged in an autoregulatory positive/negative transcriptional/translational feedback loop (Fig. 8.2a, (Zantke et al. 2013). Consistent with a functional circadian clock we found that *Pdu*-BMAL and *Pdu*-CLOCK activated the transcription of a reporter construct containing E-box elements in a luciferase reporter gene assay. Furthermore, a functional analysis of *Platynereis* cryptochromes in S2 cells validated their function as light receptor (*Pdu-L-Cry*) and transcriptional repressor (*Pdu-tr-Cry*), and thus suggests a position in the input pathway and the core circadian oscillator, respectively (Fig. 8.2a) (Zantke et al. 2013). Moreover, we validated the presence and circadian transcript oscillations of *Platynereis vrille* and *par-domain protein 1* (*pdp1*). The orthologues of these genes form a second circadian regulatory loop regulating *clock* transcription in *Drosophila* (Cyran et al. 2003). Thus, our data indicate that such a secondary loop might also be present in *Platynereis dumerilii* (Fig. 8.2a, b; (Zantke et al. 2013). Transcript oscillations of other *Platynereis* circadian clock genes displayed a daily rhythm under LD (light–dark) condition with the



Clock gene	Circadian oscillations present at NM/LD	Circadian oscillations maintained at NM/DD	Up regulation of total transcript level at FR-FM/LD
bmal	+	+	-
period	+	+	+
clock	+	+	+
pdp1	+	+	+
timeout	+	+	-
tr-cry	+	+	-
vrille	+	+	-
timeless	+	-	+
L-cry	-	na	na

Fig. 8.2 Putative core transcriptional-translational circadian clock feedback loop of *Platynereis dumerilii*. (**a**) Tentative position of *Platynereis* circadian clock gene orthologues in the worm's transcriptional circadian oscillator. (**b**) Summary of *Platynereis* circadian clock gene regulation based on q-PCR analysis on adult heads over 24 h at new moon/light–dark (NM/LD), new moon/dark–dark (NM/DD), and free-running full moon/light–dark (FR-FM/LD). +, yes, −; no, *na* not assayed.(**c**) Circadian clock genes are coexpressed in the medial forebrain region of the adult *Platynereis* head (*blue oval-shaped* domains in magnified panel) and in the adult eyes as examined by WMISH (whole mount in situ hybridization). *e*, adult eye, anterior is to top. (**d**) Mean daily locomotor activity cycles (hourly average ± SEM) at NM/LD; $n = 14$. (**e**) Average periodogram of the 3-day experiment for NM/LD shows a dominant period of 24 h and an additional 12-h peak. *Red line* indicates significant p level = 0.05. *PN* normalized power. (See also Zantke et al. 2013 for further details)

exception of *Pdu-L-cry* (Fig. 8.2b). The daily transcript oscillations were maintained under constant darkness (dark–dark, = DD) with the exception of the expression of *Pdu-timeless* (Fig. 8.2b), suggesting that the changes in mRNA level of this gene are predominantly controlled by light (Zantke et al. 2013).

We furthermore analyzed daily locomotor activity patterns of *Platynereis* by actogram and periodogram analysis. Worms showed a nocturnal daily locomotor activity at NM/LD with a dominant 24-h period (Fig. 8.2d). This rhythmic daily activity pattern was maintained under DD condition (at NM), suggesting that the daily locomotor activity in *Platynereis* is controlled by a circadian clock. This interpretation is supported by the fact that the abolishment of circadian transcript oscillations by a CK1δ/ε inhibitor resulted in arrhythmic daily locomotor activity (Zantke et al. 2013).

8.4.2 The Location of Platynereis *Circadian Clock*

Previous work on the circadian clock gene *bmal* during early larval stages revealed that this gene is most prominently expressed in the medial forebrain of *Platynereis*, including its ciliary photoreceptor cells (Arendt et al. 2004).

By analyzing the expression of the whole complement of possible core circadian clock genes in the bristle worm during immature, premature, and mature adult stages, we confirmed this finding also for later stages (Fig. 8.2c). At these adult stages, the initial forebrain expression domain in the larval brain has developed to expression in paired oval-shaped brain nuclei, located between the second pair of adult eyes (Fig. 8.2c; Zantke et al. 2013). One possible exception might be *Pdu-tr-cryptochrome*, which has been difficult to localize reliably by whole mount in situ hybridization.

In addition to the already described medial brain domain, we also found expression of core circadian clock genes in the adult eyes, similar to the situation described for *Drosophila melanogaster* (Hunter-Ensor et al. 1996). Quantitative polymerase chain reaction (qPCR) analyses also show additional core circadian clock gene expression in the worm trunks (Arboleda and Tessmar-Raible, unpublished data).

8.5 Responses of Circadian Clock Genes to Nocturnal Light and the Circalunar Clock

An increasing number of studies have been investigating the effects of moonlight/nocturnal light exposure on circadian clock gene and/or protein transcript levels, as well as its effects on physiology and behavior.

In the coral *Acropora millepora*, the blue-light receptor *cryptochrome2* shows lunar light-dependent expression with an upregulation during full versus new moon nights, which has been taken as a possible indication for its involvement in mass coral spawning regulated by the moon (Levy et al. 2007; see also the chapter by Sorek and Levy, this volume). Moonlight has been shown to affect transcript levels of the circadian clock gene *period2* (Sugama et al. 2008) and *cryptochrome* (Fukushiro et al. 2011) in the reef fish *Siganus guttatus*. Effects of moonlight on the circadian clock system have been reported also for organisms that do not show any lunar periodicity, such as *Drosophila*, in which dim nocturnal light has been shown to shift the endogenous clock of fruit flies in the laboratory (Bachleitner et al. 2007), although this effect appears to be masked under natural conditions (Vanin et al. 2012).

However, so far there is no information about whether these distinct effects on the circadian system are solely generated by direct nocturnal light, or whether the circalunar clock is also involved. We therefore used the strength of our *Platynereis* system to determine how nocturnal light during the full moon phase can affect organisms with an endogenous circalunar clock.

8.5.1 Nocturnal Light Suppresses Daily Rhythms in Clock Gene Expression

In *Platynereis*, nocturnal illumination ("full moon stimulus") is required to entrain its circalunar clock. To determine possible corresponding effects of nocturnal light exposure on the circadian system, we measured clock gene mRNA levels in the heads of *Platynereis* under the presence of nocturnal light simulating full moon (FM/LM). The light regime used for circadian and circalunar entrainment is illustrated in Fig. 8.1c. *Platynereis* heads are sampled on the fourth day/night after the nocturnal light has been switched on, referring to the middle of a full moon period. q-PCR analysis on *Platynereis* core circadian clock genes *bmal*, *period*, *tr-cry*, *clock*, *pdp1*, *vrille*, *timeout*, and *timeless* reveal that these genes fluctuated apparently randomly at FM/LM, leading to overall flattened graphs after the pooling of single 24-h biological replicates [one-way analysis of variance (ANOVA), $p > 0.05$ for all genes] (Fig. 8.3a). We conclude that nocturnal light exposure directly affects the *Platynereis* circadian clock by random or damped circadian transcriptional oscillations. FM/LM experiments were performed in parallel to a set of experiments without a nocturnal light stimulus; these included "new moon" NM/LD and "free-running full moon" FR-FM/LD, in which the full moon stimulus was omitted (Fig. 8.1c). The comparison between FM/LM, FR-FM/LD, and NM/LD conditions enables us to differentiate the contribution of nocturnal light on the circadian system from that of the circalunar clock. We found that the random or damped clock gene expression at FM/LM differed clearly from the daily transcriptional oscillations observed at FR-FM/LD (Fig. 8.3b, pink graphs) and NM/LD (Fig. 8.3b, blue graphs) where robust circadian cycles were present (one-way ANOVA, $p < 0.05$ for all genes; Zantke et al. 2013). Thus, we concluded that the circadian clock of *Platynereis* is highly sensitive to nocturnal light at the level of transcriptional clock gene regulation and that the abolished transcriptional oscillations of *bmal*, *period*, *tr-cry*, *clock*, *pdp1*, *vrille*, *timeout*, and *timeless* result from a direct, clock-independent response to the nocturnal light (at "full moon time").

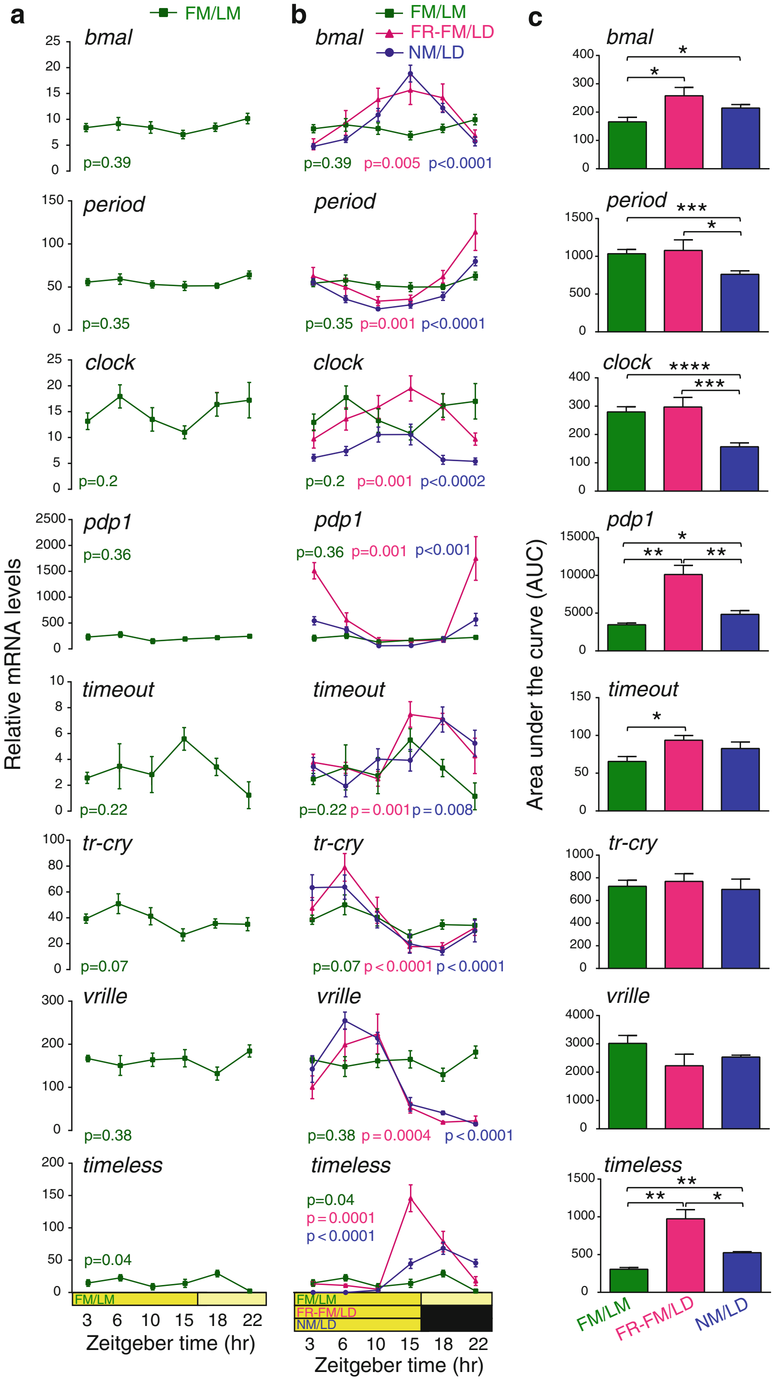


Fig. 8.3 Nocturnal illumination disrupts regular circadian transcript level oscillations. (**a**)Temporal profiles of clock gene RNA expression in *Platynereis* heads at the indicated *zeitgeber* time points sampled at full moon FM/LM (*LM* light–moonlight). Worms were entrained under a daily cycle

8.5.2 Nocturnal Light Reduces Overall Transcript Levels of **bmal, pdp1, timeout,** ***and*** **timeless**

As transcriptional oscillations of *Platynereis* clock genes appeared to be rather random or damped under the presence of nocturnal light, we next analyzed the overall level of clock gene expression by calculating the total amount of clock gene transcripts expressed over 24 h and compared these levels between FM/LM, FR-FM/LD, and NM/LD. We found that overall transcript levels of *bmal*, *pdp1*, *timeout*, and *timeless* were significantly reduced at FM/LM compared to FR-FM/LD (Fig. 8.3c green vs. pink bars). In addition, *bmal*, *pdp1*, and *timeless*, but not *timeout*, showed significantly lower levels of expression at FM/LM compared to NM/LD (Fig. 8.3c green vs. blue bars). These results imply that nocturnal light affects the core circadian oscillator of *Platynereis* by reducing overall expression levels in daily clock gene expression.

8.5.3 The Circalunar Clock Influences Transcript Levels of **period, clock, pdp1,** ***and*** **timeless**

In the reef fish *Siganus guttatus*, a lunar-synchronized spawner, *period2* mRNA levels were higher at full moon compared to new moon (Sugama et al. 2008). However, it remains unclear if the changes in *period* mRNA expression in the reef fish were generated by a circalunar clock mechanism or were a direct response to nocturnal light. Also in *Platynereis* we find that *period* (and in addition also *clock*) mRNA levels are significantly higher at FM/LM compared to NM/LD (Fig. 8.3c green vs. blue bars). Interestingly, we see the same elevation of *period* and *clock* mRNA levels under “free-running full moon” conditions (FR-FM/LD; Fig. 8.3c pink vs. blue bars). This observation indicates that the regulation of *period* and *clock* transcript levels is under circalunar clock control rather than a direct response to nocturnal light. Astonishingly, mRNA levels of *pdp1* and *timeless* also showed a higher expression in FR-FM/LD when compared to NM/LD but not under nocturnal light exposure at FM/LM (Fig. 8.3c). We conclude that monthly variations in *period*, *clock*, *pdp1*, and *timeless* mRNA levels depend on the regulation of a circalunar clock mechanism in *Platynereis*.

Fig. 8.3 (continued) (16:8 h light–dark cycle = LD) with a lunar light regime (8 consecutive nights of dim nocturnal illumination per lunar month) (see Fig. 8.1c for illumination scheme). Sampling was at the fourth day/night after nocturnal light was switched on. (**b**) Temporal profiles of clock gene RNA expression at FM/LM (*green graphs*) replotted from (**a**), FR-FM/LD (*pink graphs*) and NM/LD (*blue graphs*). Circadian transcriptional oscillations are eliminated at FM/LM compared to FR-FM/LD and NM/LD. *Light cycle* is illustrated in the *horizontal bars* at the bottom: *yellow bars*, light; *light yellow bars*, moonlight; *black bars*, dark. Values are means ± SEM, FM/LM (N = 3–10), FR-FM/LD (N = 3–10), NM/LD (N = 6–16); 4–5 heads/N. p-value determined by one-way ANOVA. (**c**) Overall transcriptional levels calculated as area under the curve (AUC) based on 24-h expression data shown in (**b**). Values are means ± SEM (t test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$)

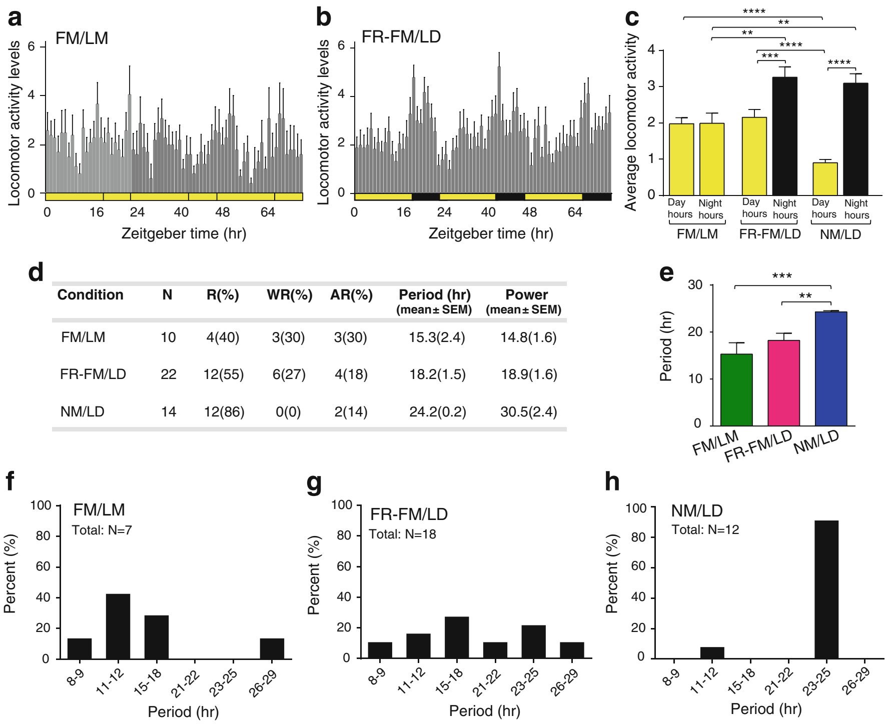
8.6 Regulation of Circadian Behavior

8.6.1 Nocturnal Light and the Circalunar Clock Influence Daily Locomotor Activity

Platynereis possesses a rhythmic, predominantly nocturnal behavior under a 16:8 LD cycle at new moon with a dominant 24-h period (Fig. 8.2d; Zantke et al. 2013). To evaluate the effects of nocturnal light on *Platynereis* daily behavior, we recorded locomotor activity at FM/LM. We found that daily rhythmic locomotor activity was eliminated in worms monitored at FM/LM, resulting in an activity pattern distributed all over the course of 24 h (Fig. 8.4a). Group analyses during the 3-day FM/LM experiment revealed that overall locomotor activity significantly increased in subjective day hours and decreased in subjective night hours compared to FR-FM/LD and NM/LD (Fig. 8.4c), which resulted in the loss of nocturnal activity in worms under the presence of nocturnal light. Interestingly, we find an increase in locomotor activity during day hours also at FR-FM/LD, but to a lesser extent, so that worms maintained their predominantly nocturnal activity (Fig. 8.4b, c). We conclude that the increase in activity during day hours at FR-FM/LD is caused by an internal circalunar clock mechanism, suggesting an impact of the circalunar clock on *Platynereis* circadian behavior. However, nocturnal light at FM/LM also directly alters *Platynereis* circadian behavior by reducing total nocturnal activity, leading to an overall arrhythmic daily locomotor activity. Similarly, nocturnal light exposure alters locomotor activity patterns in the Siberian hamster (*Phodopus sungorus*) by reducing total nocturnal activity (Fu et al. 2014; Schneider and Bowerman 2007). However, hamsters do not show an increase in locomotor activity during daytimes, which is a phenomenon that we observed in *Platynereis*, supporting our hypothesis that the circalunar clock that can indeed impact on daytime activity regulation in *Platynereis*.

8.6.2 Nocturnal Light and the Circalunar Clock Influence Rhythmic Period Length

To better understand the responses of locomotor activity to nocturnal light simulating moonlight in correlation to the circalunar clock, we next examined rhythmic periodicities under different light conditions. We analyzed rhythms in locomotor activity of individual worms for frequency components using Lomb–Scargle analysis. Worms at NM/LD synchronized to a 24-h cycle (mean period, 24.2 ± 0.2 h; Fig. 8.4d). Interestingly, worms at FM/LM showed a significant shortening in circadian period length to about 15 h (mean period, 15.3 ± 2.4 h). These much shorter rhythms in individual worms ranged from 8 to 18 h (Fig. 8.4d–f). The shortening of individual circadian period lengths was similar under FR-FM/LD (mean period: 18.2 ± 1.5 h; Fig. 8.4d, e, g) (Zantke et al. 2013). We conclude that the observed



Condition	N	R(%)	WR(%)	AR(%)	Period (hr) (mean± SEM)	Power (mean± SEM)
FM/LM	10	4(40)	3(30)	3(30)	15.3(2.4)	14.8(1.6)
FR-FM/LD	22	12(55)	6(27)	4(18)	18.2(1.5)	18.9(1.6)
NM/LD	14	12(86)	0(0)	2(14)	24.2(0.2)	30.5(2.4)

Fig. 8.4 Circalunar clock and nocturnal light affect locomotor behavior. (**a**, **b**) Mean daily locomotor activity cycles (hourly average ± SEM) at FM/LM ($N = 10$) and FR-FM/LD ($N = 22$). (**a**) Daily activity rhythms are disrupted at FM/LM (compare to FR-FM/LD in (**b**) and NM/LD in Fig. 8.2d). (**c**) At FM/LM worms are no longer nocturnal because of an increase in locomotor activity during the subjective day and a decrease during the subjective night, compared to FR-FM/LD and NM/LD (*t* test $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$); *error bars* represent ± SEM. (**d**) Summary of Lomb–Scargle periodogram analysis at FM/LM, FR-FM/LD, and NM/LD. Period and power were calculated for all rhythmic animals. *N* number of worms analyzed, *R* rhythmic, *WR* weakly rhythmic, *AR* arrhythmic. **e** Period length is significantly reduced at FM/LM and FR-FM/LD compared to NM/LD (*t* test $^{**}p < 0.01$, $^{***}p < 0.001$); error bars represent ± SEM. (**f**–**h**) Percentage of estimated period lengths of individual worms at FM/LM, FR-FM/LD, and NM/LD. Worms displayed additional shorter and longer periods at FM/LM (**f**) and FR-FM/LD (**g**) compared to NM/LD (**h**). Periods of about 24 h were no longer present at FM/LM (**f**). (For all data except FM/LM see also Zantke et al. 2013)

shortening of circadian period length depends on mechanisms underlying the circalunar system and is not a direct effect of nocturnal light exposure (Zantke et al. 2013). However, periodogram analyses of individual worms as well as group analysis confirmed a complete absence of 24-h locomotor rhythms at FM/LM (Figs. 8.4f and 8.5), likely reflecting an additional masking effect of nocturnal illumination. Taken together, the abolished circadian locomotor activity in FM/LM is generated by changes in both period length via the circalunar clock and additional direct effects of nocturnal light.

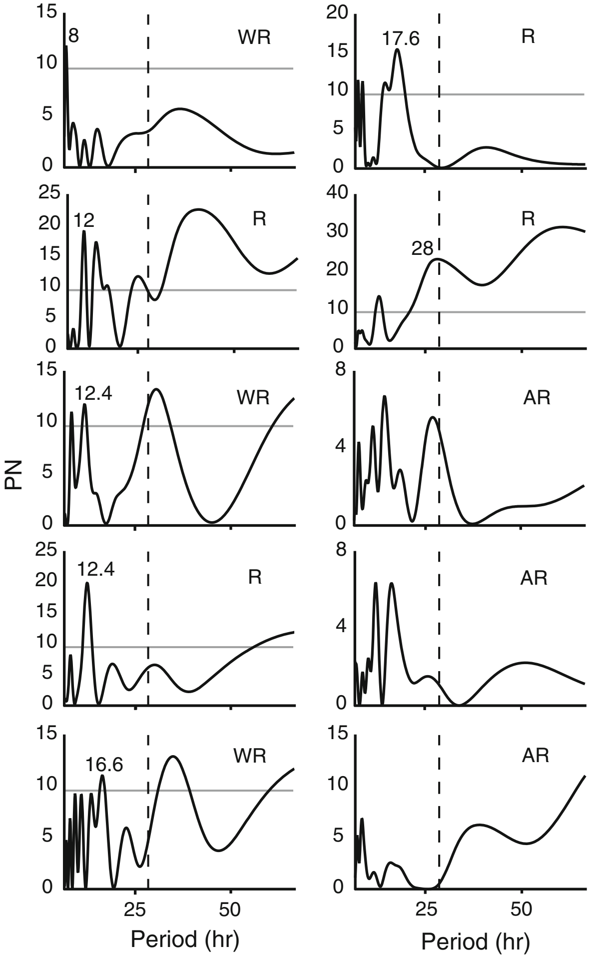


Fig. 8.5 Nocturnal light abolishes 24-h rhythms in locomotor activity in individual worms. Individual Lomb–Scargle periodograms at FM/LM arranged according to increased period length (from *top* to *bottom*). *Gray horizontal line* indicates single *p*-level = 0.05. Period lengths >29 were excluded from the 3-day analysis (indicated by *dotted line*); *R* rhythmic, *WR* weakly rhythmic, *AR* arrhythmic. Worms were defined as arrhythmic when period length was below the single *p*-level = 0.05

8.7 Is the Circadian Clock Required for Circalunar Clock Function?

One model that explains the generation of a circalunar rhythm is a dual oscillator model. In such a model a circadian oscillator (24 h) is coupled with a circalunidian oscillator (24.8 h, Fig. 8.6a), similar to what has been proposed for the generation of shorter circatidal rhythms (Palmer 2000). We thus tested the involvement of *Platynereis* circadian oscillator in the generation of the worms' circalunar clock. We interfered with *Platynereis* circadian clock gene oscillations and assessed the effects of this interference on the monthly spawning rhythm. For this, we used an inhibitor of the mammalian Casein Kinases 1δ/ε. Casein Kinases 1δ and 1ε have

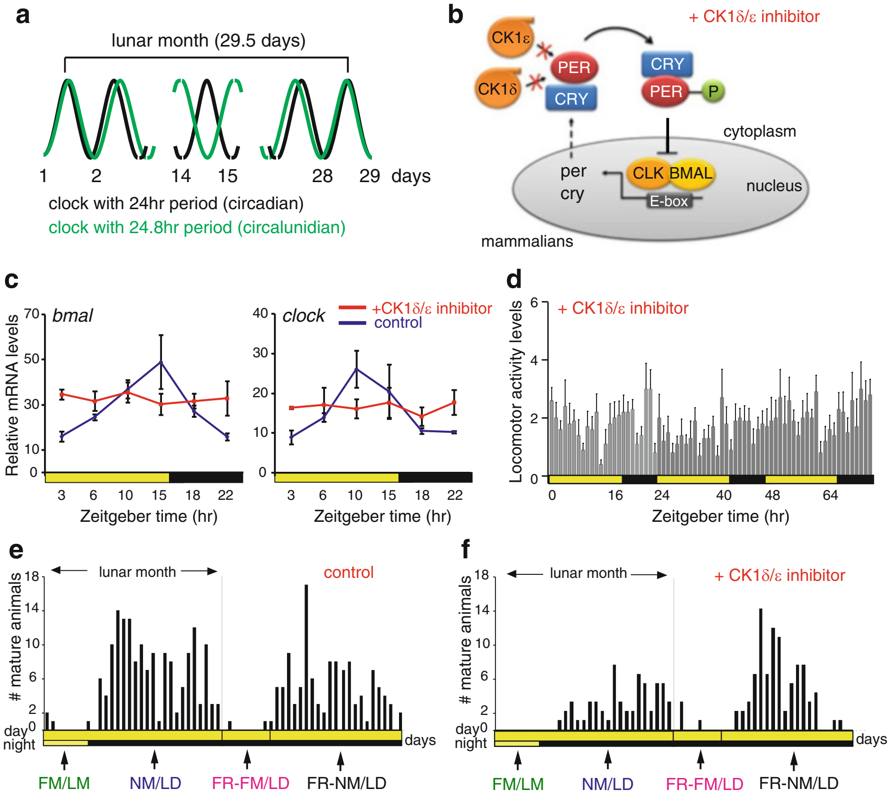


Fig. 8.6 Circalunar clock is independent of circadian clock gene oscillations. (**a**) Dual oscillator model that could explain circalunar rhythm generation. This model is based on a circadian oscillator (24 h, *green oscillations*) and a circalunidian oscillator (24.8 h, *black oscillations*), which are coupled together and coincide once per lunar month, generating monthly periods (29.5 days). (**b**) Casein Kinases 1**δ/ε** serve as important clock regulators of the mammalian circadian clock. Treatment with CK1**δ/ε** inhibitor interferes with Period phosphorylation (see main text for details). (**c**) Circadian transcript level oscillations are abolished under the presence of the inhibitor (PF-670462) (*red line*) compared to untreated control (*blue line*). Values are means ± SEM; ($n=3$); 4–5 heads/*n*. (**d**) Treatment with PF-670462 severely disrupts rhythmic daily locomotor activity. (**e**, **f**) Circalunar spawning periodicity is maintained under control (**e**) and under treatment with CK1**δ/ε** inhibitor (**f**). (Primary data from Zantke et al. 2013)

multiple functions in animal cells. Mammalian Casein Kinases 1δ and 1ε and their *Drosophila* orthologue Doubletime (DBT) are crucial for normal circadian clock function (Lee et al. 2009) Their best-documented function is Period phosphorylation, which serves to enhance Period degradation in both systems (Kloss et al. 1998; Price et al. 1998; Gallego and Virshup 2007; Meng et al. 2008). PF-670462 and other CK1δ/ε inhibitors severely affect the circadian period in mammalian cells (Vanselow et al. 2006; Eide et al. 2005; Walton et al. 2009, Fig.8.6b).

Transcript oscillations of circadian clock genes were abolished in worms treated with the inhibitor PF-670462 (Fig. 8.6c). and animals displayed arrhythmic daily locomotor activity (Fig. 8.6d) even under LD conditions. Astonishingly, the worms maintained their normal circalunar spawning periodicity with spawning maxima around new moon and minima around full moon (Fig. 8.6e, f) even when their circadian clock was severely affected by the drug treatment. This finding strongly suggests that the circalunar clock-controlled reproductive timing rhythm of *Platynereis* is insensitive to the disruption of circadian transcript oscillations (Zantke et al. 2013). It is noteworthy that PF-670462 also induces a faster maturation process in the worms (i.e., animals mature even when they are younger), but even these faster maturing worms obey the circalunar timing (Zantke et al., unpublished).

The conclusion that the circalunar clock is independent of circadian clock transcript oscillations is further supported by our aforementioned findings that the circadian expression dynamics of most core circadian clock genes are very different between FM/LM and FR-FM/LD (see 8.5), while the observed monthly spawning peaks are indistinguishable (Fig. 8.1d–f).

8.8 Summary

Here we discuss findings on the interactions of circadian and circalunar clocks and the effects of nocturnal light exposure on core circadian clock transcript levels and locomotor activity patterns in *Platynereis dumerilii*. Our results strongly suggest the presence of two molecularly independent, yet interconnected, clocks in the worm: a conventional circadian clock, which is entrained by sunlight (or an artificial diurnal light cycle), and a circalunar clock, which is entrained by moonlight (or a monthly artificial nocturnal light cycle; Fig. 8.7). Both clocks interact on the level of circadian clock gene transcript regulation and behavioral patterns (Fig. 8.7). As is the case in other animals, the circadian system of *Platynereis* is also sensitive to nocturnal light exposure at the level of clock gene expression and behavior.

It is clear that the interplay of both circadian and circalunar rhythms is important to precisely time worm maturation and reproduction. However, the biological importance of the impact of the circalunar clock and of dim nocturnal light on the circadian clock of immature and premature worms remains to be unraveled. The circalunar clock might for instance affect the impact that light at night has on the circadian clock, if the circalunar clock, for example, impacted the circadian phase-response curve.

This is just one of many further open questions. It appears that circalunar clocks have just started to get back into the scientific spotlight. What are their molecular and cellular mechanisms? How do animals sense nocturnal light versus daylight and compute the information? With the advent of functional molecular tools, it appears that there are exciting times ahead for chronobiological research on *Platynereis dumerilii*

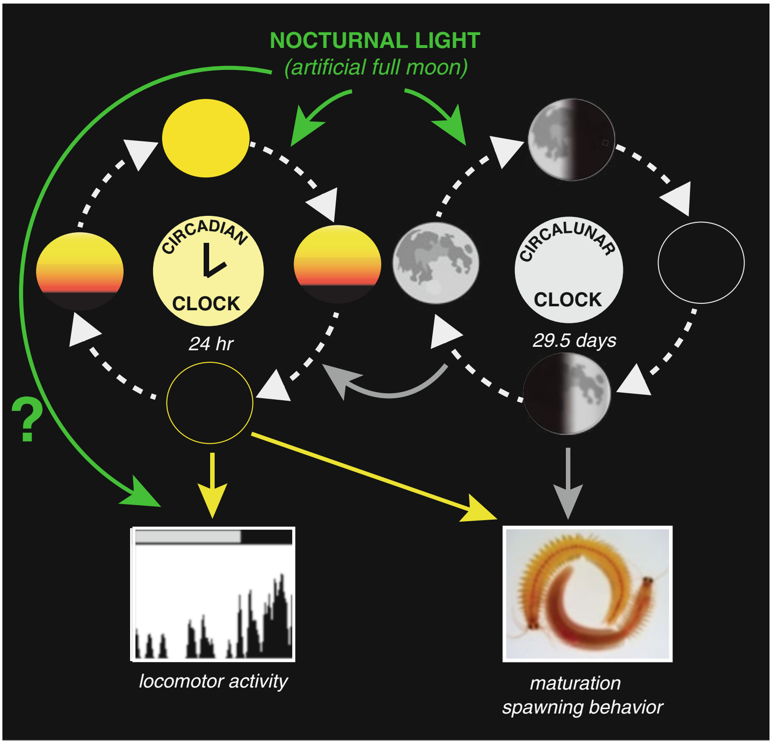


Fig. 8.7 Circadian and circalunar clocks and the impact of light in *Platynereis dumerilii*. As the oscillations of the circadian transcriptional-translational clock are not required for circalunar rhythm generation, it is most plausible that *Platynereis* possesses two distinct molecular clocks that generate circadian and circalunar rhythms, respectively. Although the circadian clock, entrained by sunlight, drives daily nocturnal locomotor behavior and nocturnal spawning (*yellow arrows*), a circalunar clock, entrained by moonlight/dim nocturnal light, governs the worms' monthly spawning rhythm (*grey arrow*). The circalunar clock modulates circadian locomotor activity and circadian clock gene transcript levels. This modulation can be direct or indirect and affect one or more genes (*grey horizontal arrow*). Nocturnal light simulating full moon (*green arrows*) affects circadian clock transcript levels and circadian locomotor activity rhythms

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Chapter 9
Lunar Clock in Fish Reproduction

Taro Ikegami, Yuki Takeuchi, and Akihiro Takemura

Abstract Certain fish exhibit lunar and semilunar reproductive activities that are repeated at regular intervals of 1 month and 2 weeks, respectively. Fish with the lunar cycle exhibit spawning around a selective moon phase, whereas those with the semilunar cycle repeat spawnings twice within a month. Environmental changes in moonlight or tides may be involved in the synchronization of fish reproductive activity. Spawning of the goldlined spinefoot *Siganus guttatus*, a lunar spawner around the first quarter moon, is disrupted under constant moonlight conditions. Exposing fish to moonlight around the full moon resulted in suppression of melatonin, suggesting the perception and utilization of changes in moonlight for synchrony. Of the clock genes examined, *Period* (*Per2*) in the pineal organ was higher at midnight around the full moon than the new moon, whereas *Cryptochrome* (*Cry1* and *Cry3*) in the brain peaked around the first quarter moon. Although circalunar-specific genes have not yet been identified in any organism, some elements of the circadian system are likely involved in the exertion of lunar-related reproductive activities.

Keywords *Cryptochrome* • Melatonin • Moonlight • Ovary • Spinefoot

9.1 Introduction

Many organisms on the Earth possess endogenous clocks that respond to rhythmic changes in light and temperature (Pittendrigh 1993; Panda et al. 2002). Endogenous clocks help organisms to anticipate upcoming daily and annual environmental changes in their habitat and to adjust biochemical, physiological, and behavioral processes accordingly (Pittendrigh 1993). The acquisition of these clocks is closely related to long-term evolutionary and adaptation processes under constant repetition of the Earth's rotation and revolution. In addition, the moon is also considered to affect the entrainment of endogenous clocks in organisms. Although information on lunar-related clocks is limited, rhythmic changes in a habitat arising from the

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movement of the moon around the Earth undoubtedly are utilized for synchronization of various biological activities, including locomotion, migration, and reproduction. Some lunar-related activities even oscillate under constant conditions (Hsiao and Meier 1989; Motohashi et al. 2010), implying the involvement of endogenous clocks (e.g., circalunar, circasemilunar, or circatidal clocks).

Several lunar-related cycles exist: the lunar cycle, the semilunar cycle, and the tidal cycle. The lunar cycle involves a periodic change repeated at 1-month intervals, and biological activities of organisms peak around the species-selective lunar phase. The semilunar cycle is periodic activity that seems to appear at a 14.7-day interval. In many examples of this cycle, peaks of biological activity occur twice within a month. The tidal cycle also involves moon-related activity, which is well correlated with a daily tidal cycle and caused by the combined effects of gravitational forces exerted by the moon and the sun and the rotation of the Earth (Leatherland et al. 1992).

Marine organisms inhabiting shallow waters in tropical and subtropical zones often exhibit various types of lunar-related rhythmicity (Harrison et al. 1984; Thresher 1984). One possible reason why such patterns are typical of these regions is that the lower variation in water temperature and photoperiod in these zones is correlated with a relative increase in the importance and reliability of cues from the moon (Takemura et al. 2010). In this chapter, we focus on the lunar and semilunar cycles occurring in the reproductive events of teleost fishes and review physiological processes of these lunar-related reproductive rhythms. Based on recent information regarding the molecular mechanisms of circadian clocks, we propose a possible role of lunar-related clocks in the exertion of reproductive activities in fish.

9.2 Moon-Related Cycles in Fish Reproduction

9.2.1 Lunar Spawning Cycle

This lunar-related periodicity is characterized by a relationship between a trigger point of a reproductive event and a lunar phase and can be observed in various reproductive events of teleost fishes. For example, the downstream migration of the European eel *Anguilla anguilla* toward their spawning area, the Sargasso Sea, is initiated between the last quarter and new moon period (Miyai et al. 2004). In the North Equatorial Current to the west of the Mariana Islands, searches for hatched larvae of the Japanese eel *Anguilla japonica* by research cruises have suggested the possibility of synchronous spawning around the new moon period (Tsukamoto 2006). Cues peaking around the new moon period appear to trigger reproductive activities in eels. Lunar-related spawning migration has also been reported in certain groupers inhabiting coral reefs. Groupers aggregate at species-selective spawning sites and spawn around a peak of the lunar period; the honeycomb grouper *Epinephelus merra* spawns around the full moon period (Lee et al. 2002), whereas the coral trout *Plectropomus leopardus* releases its gametes around the new moon period (Samoilys and Squire 1994). Following a synchronous spawning event, matured groupers return from the spawning site to the inner reef that they most commonly inhabit. This coming-and-going behavior around a specific lunar phase is repeated during the spawning season (Samoilys and Squire 1994), which occurs annually at a fixed period of a year. Therefore, cues peaking toward a specific lunar phase may be transduced as internal stimuli and may consequently trigger lunar-related reproductive activities.

Comparative studies on lunar-related spawning rhythmicity have been conducted in four spinefoot species (formerly known as rabbitfish) inhabiting Okinawan waters, Japan, in the subtropical zone (Takemura et al. 2010). The white-spotted spinefoot *Siganus canaliculatus* spawns around the new moon from April to June, the little spinefoot *Siganus spinus* spawns around the new moon from May to July, the goldlined spinefoot *Siganus guttatus* spawns around the first quarter moon from June to July, and the streamlined spinefoot *Siganus argenteus* spawns around the last quarter moon from May to July (Takemura et al. 2010). The spawning rhythmicity of the same four species distributed in the tropical zone has also been examined. Results of these studies have indicated that the goldlined spinefoot spawns around the first quarter moon both from March to May and from September to November in waters around the Karimunjawa Islands, Indonesia (Susilo et al. 2009), and the little spinefoot spawns around the new moon both from January to March and from July to September in Chuuk Lagoon, Micronesia (Park et al. 2006). Although the spawning season of each species differs among habitats with different backgrounds of seasonal cues, exactly the same lunar phase is utilized for the same species in all regions of the Indo-Pacific Ocean. Notably, differences in the spawning lunar phase among these spinefoot species partially suggest the evolutionary acquisition of chronologically based reproductive isolation (Takemura et al. 2010).

Lunar cyclic ovarian development has been histologically examined in certain spinefoot species. In all cases reported so far, a clutch of oocytes in an ovary synchronously develops from the immature to mature stage toward a species-selective lunar phase. In the case of the goldlined spinefoot, for example, oocytes at vitellogenic stages appear among immature oocytes around the full moon period and develop fully around the new moon period. Following final oocyte maturation, ovulation and release of gametes from the maternal body occur around the first quarter moon (Fig. 9.1a) (Rahman et al. 2000). When weekly changes in plasma steroid hormone levels were measured, we found lunar patterns in estradiol-17β (E_2) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP), which are responsible for induction of vitellogenesis and final oocyte maturation, respectively (Nagahama 1994). Both steroid hormones increased toward the first quarter moon period. More precise experiments using an in vitro culture technique showed that under the presence of human chorionic gonadotropin (hCG) used as a stimulator of steroidogenesis, synthesis of E_2 and DHP in ovarian segments increased around the new moon period (1 week before spawning) and the first quarter moon period (just before spawning), respectively (Fig. 9.2) (Rahman et al. 2002). Therefore, profiles of these steroid hormones are clearly in accordance with sequenced events in the ovary based on lunar phases. Biological characterizations of fish with lunar reproductive cycles

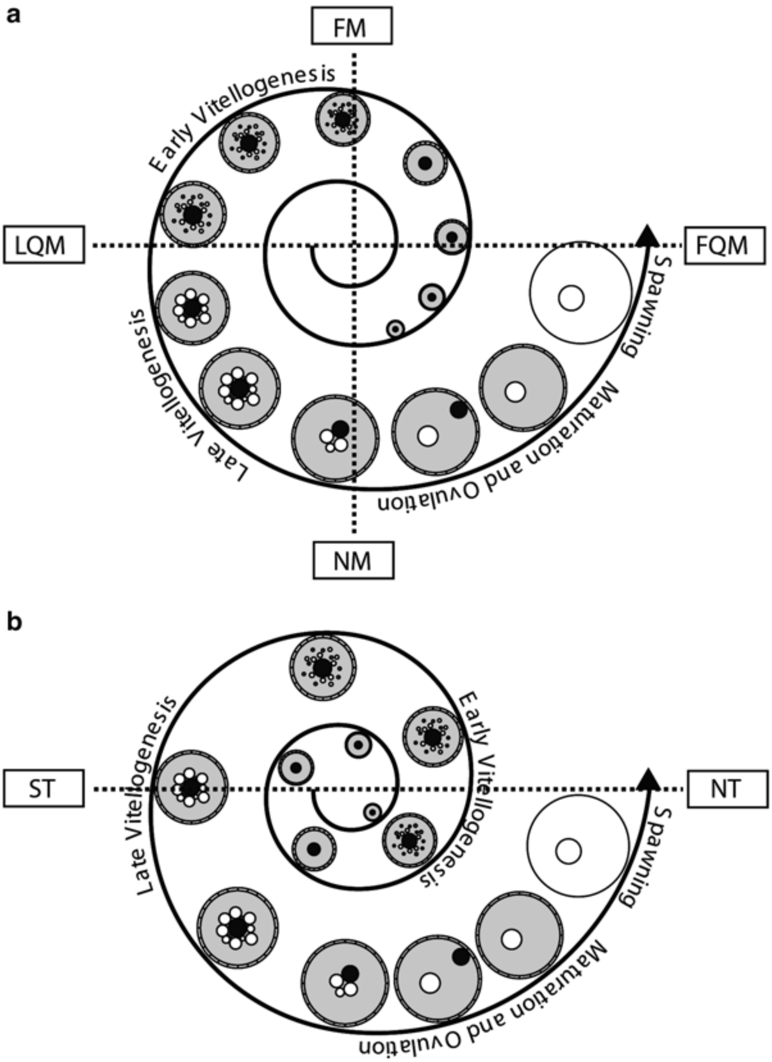


Fig. 9.1 Schema of ovarian development according to lunar spawning cycle in the goldlined spinefoot (**a**) and semilunar spawning cycle in the brackish damsel (**b**). The lunar spawning cycle completes the whole processes of ovarian development within a month and spawning at 1-month intervals. In the semilunar spawning cycle, a clutch of oocytes develops from vitellogenic oocytes and spawning is repeated at intervals of 2 weeks. *FM* full moon, *FQM* first quarter moon, *LQM* last quarter moon, *NM* new moon, *NT* neap tide, *ST* spring tide. (Redrawn from Takemura et al. 2010)

have shown that an entire cycle in relationship to ovarian development and spawning proceeds to a species-selective lunar phase and is completed within 1 lunar month.

How do fish recognize a species-selective moon phase for their spawning rhythmicity? Because the goldlined spinefoot repeats spawning at the selective moon

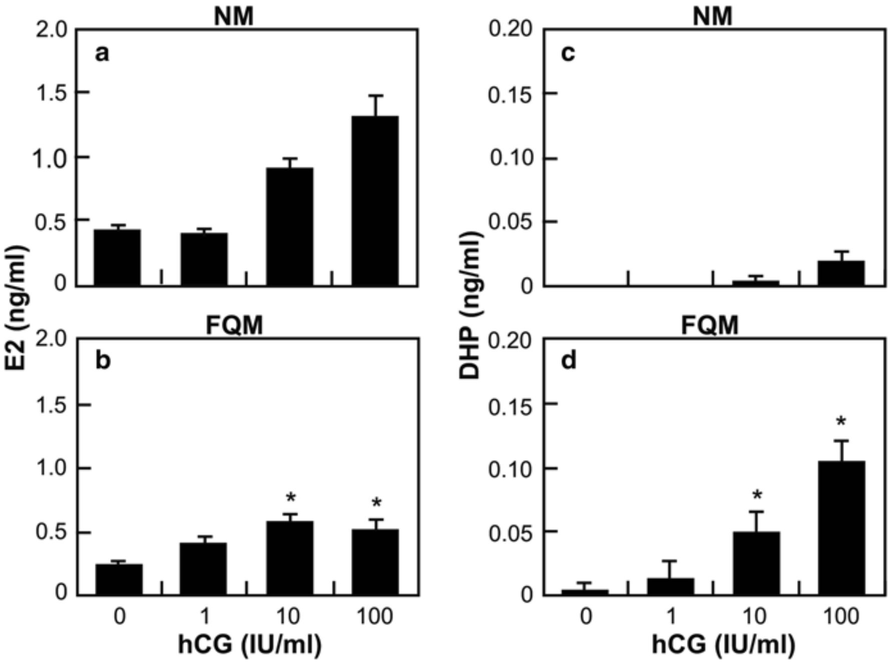
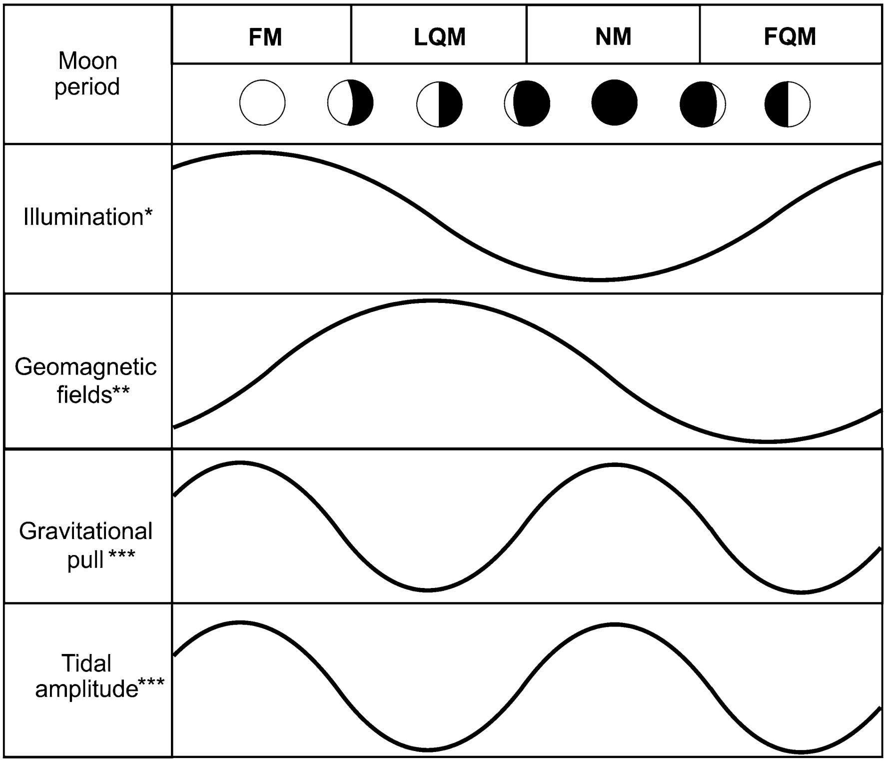


Fig. 9.2 In vitro production of steroid hormones by intact follicles of oocytes of the goldlined spinefoot in response to different concentrations of human chorionic gonadotropin (hCG). Estradiol-17**β** (E_2) production at (**a**) 1 week before spawning (*NM* new moon) and (**b**) just before spawning (*FQM* first quarter moon). 17**α**,20**β**-Dihydroxy-4-pregnen-3-one (DHP) production at (**c**) 1 week before spawning (*NM* new moon) and (**d**) just before spawning (*FQM* first quarter moon). *Asterisks* indicate significant difference ($P < 0.05$) in E_2 and DHP productions between the new moon and the first quarter moon. (Modified from Rahman et al. 2002)

phase (the first quarter moon), even in tanks without tidal changes for several years (Rahman et al. 2000), this species likely does not require tidal stimuli for the continuity of the lunar-related spawning cycle. The fish may be able to perceive and utilize dynamic changes such as moonlight illumination (Horning and Trillmich 1999) and geomagnetic fields (Stolov 1965; Bell and Defouw 1966), which peak around the full moon and the last quarter moon, respectively, at 1-month intervals (Fig. 9.3). When the goldlined spinefoot is reared in tanks under artificial constant full moon and new moon conditions, the expected spawning during the spawning season is disrupted or delayed (Takemura et al. 2004). This observation indicates that cyclic changes in moonlight intensity play at least a partial role in the synchronization of the timing of spawning in this species. In contrast, a 4-year tagging study on the migration behavior of smolts of the chinook salmon *Oncorhynchus tshawytscha* demonstrated that the timing of saltwater entry from a river was closely related to the date of lunar apogee (the farthest distance from the earth to the moon), followed by the date of quarter moons (Fig. 9.3) (DeVries et al. 2004). Salmonid fish may thus perceive changes in lunar gravitation for the start of migration.



* Horning and Trillmich 1999; ** Stolov 1965; Bell and Defouw 1966; *** Leatherland et al. 1992.

Fig. 9.3 Lunar effects on the Earth. Moonlight illumination and geomagnetic field fluctuate at 1-month intervals and peak around the full moon and the last quarter moon, respectively. Gravitational pull and tidal amplitude fluctuate at a 14.7-day interval and peak around the full moon and the new moon. *FM* full moon, *FQM* first quarter moon, *LQM* last quarter moon, *NM* new moon

9.2.2 Semilunar Spawning Cycle

Semilunar reproductive events are explained as apparent spawning cycles at a 14.7-day interval at a selective lunar phase; these cycles have been observed in many fish species that, in most cases, inhabit coastal areas. For example, the grass puffer *Takifugu niphobles*, a common coastal puffer species in Japan, aggregates at certain seashore locations and spawns several hours before the morning or evening high tide, only during the spring tide (Yamahira 1994, 2004). The group spawning of the grass puffer occurs at the same location every year and every 2 weeks during a spawning season (Motohashi et al. 2010). Similar spawning behavior at spring tide has been reported in the California grunion *Leuresthes tenuis* (Clark 1925) and the killifishes (*Fundulus heteroclitus* or *F. grandis*) (Hsiao and Meier 1989). In these cases, the fertilized eggs or hatched larvae are washed out from the coast to the open sea until the high tide of the next spring tide. One advantage of this reproductive strategy is that it appears to reduce predation risk by aquatic predators for offspring (Leatherland et al. 1992).

Details on ovarian development in terms of semilunar spawning cycles have been reported in two species, the brackish damsel *Pomacentrus taeniometopon* and the amboina cardinalfish *Apogon amboinensis*. Both are brackish species exposed to vigorous changes in tidal stimuli. Females of the brackish damsel spawn around the first quarter moon and the last quarter moon (Fig. 9.1b). Because territorial males protect fertilized eggs for 1 week, hatching occurs around the new moon and full moon periods (Pisingan et al. 2006). In the brackish damsel, vitellogenic oocytes can always be found in an ovary during the spawning season. A clutch of vitellogenic oocytes starts to mature and is spawned around both quarter moons. Females of the amboina cardinalfish spawn around the first quarter moon and the last quarter moon period. Because this species is a mouth-brooder, the fertilized eggs are incubated in the mouth of males for 2 weeks until hatching. The release of hatched larvae from the male mouth occurs around the first quarter moon and the last quarter moon period (Pisingan and Takemura 2007). In the amboina cardinalfish, a clutch of yolk-laden oocytes appears among immature oocytes after spawning and then develop synchronously toward the first or last quarter moon. When ovarian segments of the amboina cardinalfish were cultured in medium with hCG, the synthesis of E_2 increased around the new and full moon periods, whereas that of DHP increased around the first and last quarter moon (Fig. 9.4a, b). These studies indicated at least two types of semilunar spawners, which may be categorized as the true or apparent spawner. The former (e.g., brackish damsel) repeats oocyte development and spawning at an interval of 2 weeks; thus, an individual experiences two spawnings within a month during the spawning season (true semilunar spawner). On the other hand, the latter (e.g., amboina cardinalfish) undergoes one-time oocyte development and spawning within a month. In this case, therefore, there may be two populations spawning around the first quarter moon or last quarter moon period (Takemura et al. 2010).

Because the timing of larval dispersal from the male's territory or mouth is correlated with the peak of spring tides (Fig. 9.3), tidal stimuli appear to be perceived by fish species to synchronize gonadal development and spawning. Notably, females of the red-clawed crab *Chiromantes haematocheir* repeat the release of zoea larvae to the sea during high tide around the spring tide. This semilunar spawning rhythm has been suggested to be induced not only by cyclic changes in tides but also by changes in moonlight illumination (Saigusa 1988). Therefore, the semilunar rhythmicity of organisms is likely affected by a conjugated system of cues from the moon. Robertson et al. (1990) hypothesized that the release of hatched larvae with the spring tide is likely related to the rapid dispersal of the offspring from the coast to the open sea, consequently reducing predation pressure and enhancing the survival of the offspring (Robertson et al. 1990).

Hsiao and Meier (1989) collected the gulf killifish *F. grandis* and the mummichog *F. heteroclitus* from the Gulf Coast and the Atlantic Coast, respectively, and reared them under standard laboratory conditions. They found that several spawning

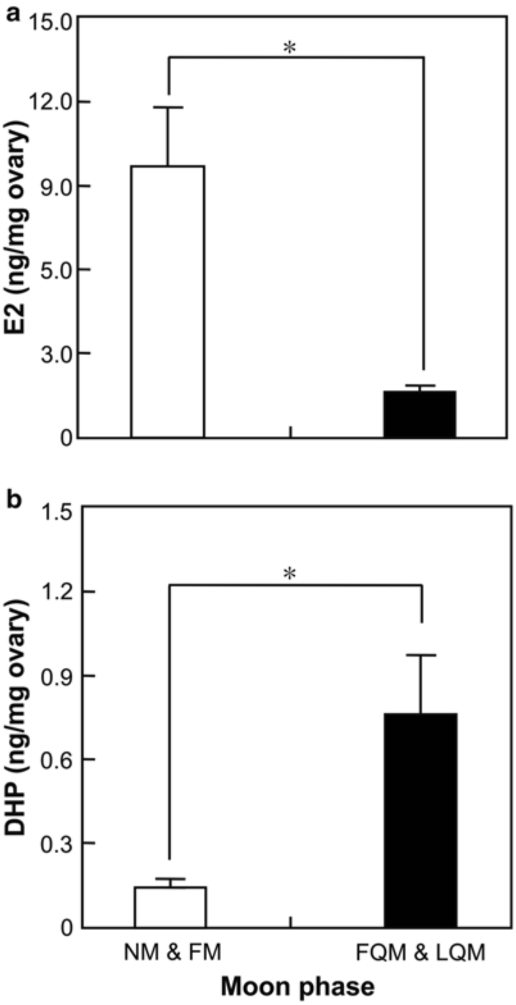


Fig. 9.4 Comparisons of (**a**) estradiol-17**β** (*E2*) and (**b**) 17**α**,20**β**-dihydroxy-4-pregnen-3-one (*DHP*) from the cultured ovarian segments of the amboina cardinalfish in response to human chorionic gonadotropin (hCG) between the new and full moon (NM and FM) and the first and last quarter moon (FQM and LQM). E_2 and DHP were measured using ELISA. *Asterisks* indicate significant difference ($P < 0.05$) between the new and full moon (NM and FM, *open columns*) and the first and last quarter moon (FQM and LQM, *filled columns*). (Modified from Pisingan and Takemura 2007)

cycles were maintained at a mean duration of 13.7 days for the gulf killifish and 14.8 days for the mummichog, and the spawning cycle of these two species was synchronized with the moonlight and tidal cycle in their respective habitats (Hsiao and Meier 1989). Motohashi et al. (2010) observed the aggregating and spawning behaviors of the grass puffer in an aquarium lacking tidal changes. Although spawning was not confirmed in the aquarium mimicking field conditions, the small group of mature fish only aggregated repeatedly in the rising tidal phase during and after the spring tides (Motohashi et al. 2010). Furthermore, in the mudskipper *Boleophthalmus pectinirostris*, the synthesis of plasma steroid hormones (i.e., testosterone, E_2, and 17α-hydroxyprogesterone) was regulated by a biological clock; these steroids peaked twice on the third day after the first quarter moon and on the fourth day after the last quarter moon, although steroid hormone profiles and gonadal development were not positively correlated (Wang et al. 2008). These findings suggest that an endogenous clock controls spawning periodicity and that this clock can oscillate temporarily without external moon-related stimuli. Interestingly, mummichogs (the Arasaki strain) reared for generations under laboratory conditions became daily spawners during the spawning season (Shimizu 1997). Exposing the fish to moon-related stimuli at specific stages of their life cycle may be necessary to exert the semilunar spawning rhythmicity.

9.3 Melatonin as a Possible Transducer in Lunar Spawners

Melatonin is an indoleamine hormone synthesized mainly in the pineal organ and the retina (Zachmann et al. 1992). This hormone is believed to be a transmitter of photoperiodic information to central and peripheral organs, as its level in the blood increases during the nighttime and decreases during the daytime (Bromage et al. 2001). The pineal organ of teleost fishes serves a dual biological function as a neuroendocrine organ and photoreceptive organ. With the exception of salmonid species, the pineal organ of most fish species can release melatonin in a circadian manner under constant dark conditions of in vivo and in vitro experiments, implying that the entire system of the circadian clock functions in the pineal organ (Iigo et al. 1991, 2004; Molina-Borja et al. 1996).

A daytime-low and nighttime-high fluctuation persisted in plasma melatonin levels under light–dark (LD) conditions in the goldlined spinefoot (Takemura et al. 2004), suggesting that melatonin also serves a common role in transducing daily variation in environmental photoperiodic information into the central and peripheral organs in this lunar spawner. Moreover, nocturnal plasma melatonin levels during the new moon period were higher than those during the full moon period (Fig. 9.5a) (Takemura et al. 2004). This result was supported by in vitro experiments; when the pineal organ of the goldlined spinefoot was cultured under natural illumination during the full and new moon periods, secretion of melatonin into the culture medium was higher during the new moon period than during the full moon period (Takemura et al. 2006). In addition, exposing spinefoots to "brightness at the full moon or new moon night" resulted in a rapid suppression of plasma melatonin levels (Rahman et al. 2004; Takemura et al. 2004). Therefore, the melatonin profile in a lunar spawner would likely show a composite pattern at 1-month intervals: the amplitude in melatonin levels between daytime and nighttime during the new moon period would be large, whereas that during the full moon period would be small.

The actions of melatonin are mediated via melatonin receptors belonging to the G protein-coupled receptor superfamily. Melatonin receptors are also expected to be involved in the exertion of lunar rhythmicity in fish. Because three subtypes of melatonin receptors are known in lower vertebrates, the MT1 (formerly known as Mel_{1a}), MT2 (formerly known as Mel_{1b}), and Mel_{1c} (Ikegami et al. 2009a, b), the cDNAs of MT1 and Mel_{1c} have been cloned in the goldlined spinefoot and their expression

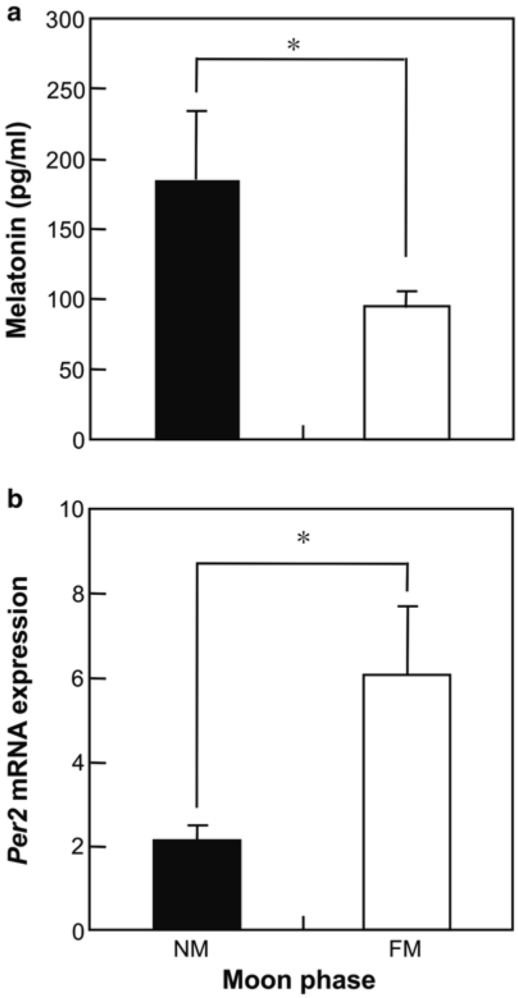


Fig. 9.5 Comparisons of melatonin concentration in the plasma (**a**) and clock gene expression in the pineal organ (**b**) of the goldlined spinefoot between the new moon (NM) and the full moon (FM). The plasma sample and the pineal organ were taken from the fish at midnight during the new moon (*filled columns*) and full moon (*open columns*). Melatonin levels in the plasma were measured by time-resolved immunofluoroassay. Expression of *Period2* (*SgPer2*) mRNA in the pineal organ was determined by quantitative real-time polymerase chain reaction (PCR) and normalized by the amount of *Sgβ-actin* mRNA. Each *bar* represents mean ± SE. *Asterisk* indicates significant difference ($P < 0.05$) between the new moon and the full moon. (Modified from Takemura et al. 2004 and Sugama et al. 2008)

patterns in the pineal organ have been examined using quantitative real-time polymerase chain reaction (qPCR) (Park et al. 2007a, b). The mRNA abundance of MT1 and Mel_{1c} exhibited diurnal variation, with an increase during nighttime and a decrease during daytime under light and dark conditions (Park et al. 2007a, b). An in vitro experiment using the goldlined spinefoot pineal organ revealed that melatonin production is strongly correlated with melatonin receptor mRNA expression (Park et al. 2007a), suggesting that melatonin regulates the expression of these melatonin receptors. These results raise the possibility that melatonin receptors fluctuate according to changes in the intensity of moonlight illumination under regulation of melatonin. In fact, MT1 and Mel_{1c} mRNA abundance was higher at midnight of the new moon than midnight of the full moon (Park et al. 2014). As demonstrated with plasma melatonin levels, exposing fish to “brightness at the full moon night” resulted in downregulation of Mel_{1c} mRNA in the pineal organ (Park et al. 2014). Therefore, the melatonin–melatonin receptor system is potentially involved in generating 1-month periodicity in certain lunar synchronized spawners.

Melatonin likely becomes a useful messenger of various lunar-related environmental changes (Takemura et al. 2010). In fact, a magnetic field generated by Helmholtz coils (maximum flux density 40 mT, frequency 1 Hz, 200 ms on, 800 ms off) resulted in increases in nocturnal pineal and serum melatonin levels of brook trout *Salvelinus fontinalis* (Lerchl et al. 1998). This stimulatory effect of a magnetic field on melatonin production may be attributable to an increased Ca^{2+} influx into pineal photoreceptors (direct effect; Gasser and Gern 1997) or to secondary stress responses (indirect effect; Bullock 1977). Wagner et al. (2007) cultured the pineal organ of two abyssal fish species, *Coryphaenoides armatus* and *Synaphobranchus kaupii*, whose habitats in deep seas experience rhythmic changes in current velocity and direction at intervals of 12.4 h. Melatonin release tended to increase at the beginning of the lunar day and night and was lower during the second half of the lunar day and night and during moonrise and moonset. Thus, tidal currents may act as a zeitgeber at the bottom of the deep sea, and melatonin metabolism with tidal periodicity may be a possible sign of the transduction of tidal periodicity in deep-sea fishes (Wagner et al. 2007).

9.4 Clock Genes in Lunar Rhythmicity

As noted, circumstantial evidence exists that endogenous clocks are involved in the semilunar reproductive cycle in certain teleost fishes. However, how circasemilunar and circalunar cycles are regulated endogenously and what types of biological clocks are involved in oscillating such cycles remain unclear. A pioneer study on the involvement of clock genes in lunar spawning synchrony was performed in the reef-building coral *Acropora millepora*, which spawns around the full moon period (Levy et al. 2007). The mRNA abundance of light-responsible genes, *Cryptochrome* (*Cry1* and *Cry2*), exhibited daily variation with a peak during daytime under light–dark cycles, and *Cry2* mRNA abundance at night was higher during the full moon period than during the new moon. These results suggest that an element of the circadian system is involved in the lunar-related mass spawning in corals.

Initially, *Period* and *Cryptochrome* received the most attention in terms of the genetics of lunar clocks in fish because the family of these elements is believed to include photo-responsible genes in lower vertebrates (Cahill 2002; Yasuo et al. 2004). In the goldlined spinefoot, following the cloning of cDNA, the expression patterns of *Period* (*SgPer2*) and *Cryptochrome* (*SgCry1* and *SgCry3*) mRNAs were examined in the pineal organ and the medial part of the brain (mesencephalon and diencephalon), respectively (Fukushiro et al. 2011; Sugama et al. 2008). The abundance of *SgPer2* mRNA displayed a daily expression pattern with increases during photophase. The abundance of this gene was higher at the culmination of the full moon period than during the new moon period (Fig. 9.5b). Additionally, exposing the fish to moonlight irradiation around the full moon period resulted in upregulation of *SgPer2* mRNA abundance, suggesting that the expression of *SgPer2* mRNA in the pineal organ was affected not only by daylight, but also by moonlight (Sugama et al. 2008). On the other hand, the mRNA abundance of *SgCry1*, but not *SgCry3*, exhibited daily fluctuations, with a peak at dawn and gradual decreases during daytime. Notably, the expression of both genes showed lunar-dependent variation; their abundance increased around the first quarter moon and decreased around the full moon and the last quarter moon period (Fig. 9.6) (Fukushiro et al. 2011). Thereby, an increase in *SgCry* mRNA abundance occurred around the spawning lunar phase of the goldlined spinefoot.

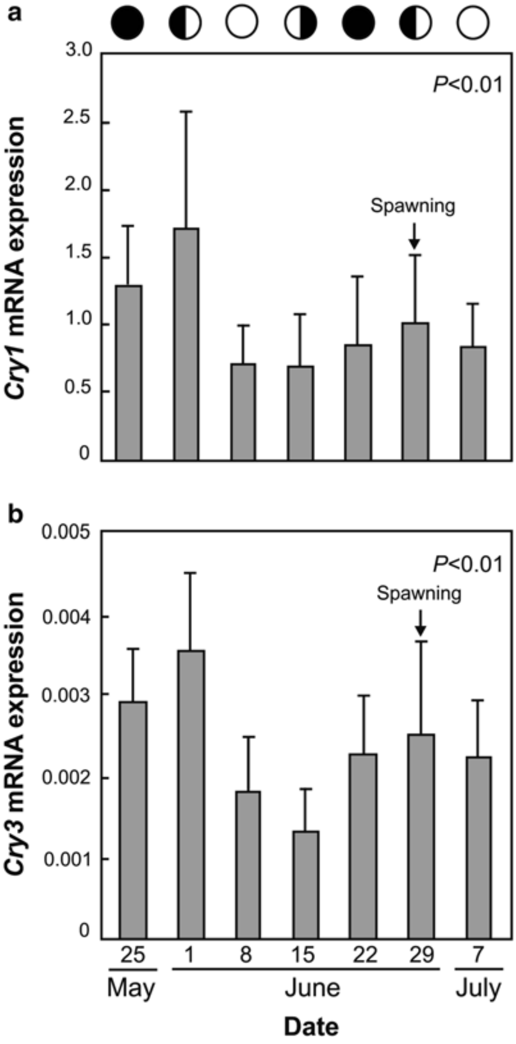


Fig. 9.6 Lunar-dependent variations in the expression of genes encoding *cryptochrome*, *SgCry1* (**a**), and *SgCry3* (**b**) in the brain of the goldlined spinefoot. Each *Cry* mRNA was calculated as a value relative to that of *Sgβ-actin* gene. Each *bar* represents mean ± SD. Lunar phases are indicated above the graph by schematic moon images. Probability values (*P*) of ANOVA for statistically significant difference among dates are indicated. Second peak of mRNA expressions of *SgCry1* and *SgCry3* coincided with spawning day (June 29) whereas the first peak of those existed before spawning season. (Modified from Fukushiro et al. 2011)

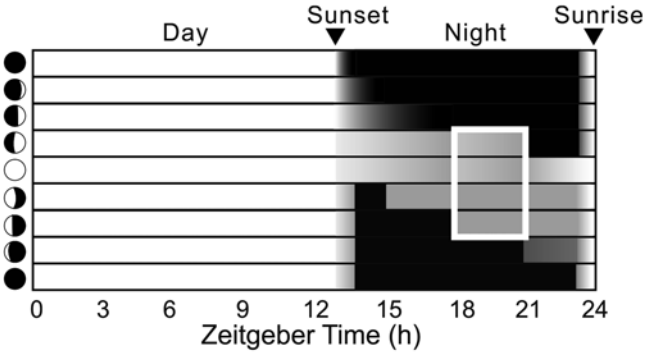


Fig. 9.7 Schematic pattern of solar light phase, moonlight phase, and moonlight intensity in Okinawa, Japan (26°38′N, 127°51′E) during the spawning season (June) of the goldlined spinefoot. A putative photo-responsible phase is indicated by the *white outline. Shading* indicates differences in light intensity. (Modified from Fukushiro et al. 2011)

Despite the lack of information for characterizing the fish lunar clock system in detail, further research should address hypotheses that test whether certain elements of the circadian system are involved in oscillations of lunar-dependent rhythmicity and if periodic changes in moonlight irradiation act as a possible zeitgeber to synchronize this rhythm. Differences in moonlight irradiation between the new moon and full moon period may simply affect the expression patterns of clock genes. Alternatively, lunar phase-dependent gene expression is presumably controlled by moonlight signals around a putative "photo-responsible phase," which is expected to appear at a certain point of the night (ZT18–ZT21). This hypothesis was derived from comparisons between a transient pattern of moonlight irradiation at night (Fig. 9.7) and weekly changes in *SgCry* mRNA expression.

9.5 Closing Remarks

The purpose of this chapter was to provide an outline of the physiological and molecular mechanisms influencing lunar and semilunar rhythmicity in fish reproduction. To date, whether lunar- or semilunar-specific genes are expressed in the central nervous system and are involved in regulating lunar-related rhythms in fish remains unknown. However, the findings detailed in this chapter reveal that the main players, melatonin and clock genes, of the circadian system are associated with the entrainment of fish reproduction to a peak point of cues from the moon. Lunar and semilunar rhythmicity in fish has presumably evolved as an applicable form of the circadian system, particularly if fish share a common transduction system for sensing photic information such as daylight as well as moonlight. Alternatively, the lunar/semilunar rhythmicity is a potentially innate characteristic in most fish but one that oscillates robustly in only certain fish species after adaptation to aquatic environments. If so, it is not necessary for fish to evolve a novel system for lunar/semilunar rhythmicity.

The genome project of the tiger puffer *Takifugu rubripes* was completed in 2002 (Aparicio et al. 2002). Because *Takifugu* species share very high similarity in their genome sequences (Yamanoue et al. 2009), the grass puffer offers a particular advantage for elucidating the molecular mechanisms of the semilunar cycle using the genome resources of the tiger puffer. Despite vigorous accumulation of ecological evidence, physiological and molecular studies on lunar and semilunar cycles in fish have been limited. Advances in genome-wide search might allow us to identify novel clock genes in relationship to the circalunar/circasemilunar cycle or to find new functions of known molecules in oscillating lunar/semilunar cycles. By capitalizing upon new approaches, further studies can help to achieve an overall understanding of lunar and semilunar cycles in fish.

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Chapter 10
The Clock-Work Worms: Diversity and Function of Clock Expression in Marine Polychaete Worms

Kim S. Last and Vicki J. Hendrick

Abstract Polychaetes are marine worms that display a surprising array of reproductive modes that are usually highly synchronized to the complex cycles of the marine environment. From the extremely long metonic (19-year) cycles of reproduction in the Palolo worm to the short ultradian tidal (12.4 h) cycles of feeding in the ragworm, polychaetes demonstrate a great diversity of entrainment to all known environmental cycles in the oceans. They are able to tell the time of the year, month, day, and tide and hence they may be considered the ultimate marine chronometers, a trait that is under strong selection pressure, ultimately increasing fitness. Polychaetes evolved before the great Cambrian explosion 600 million years ago, and hence it seems probable that their clock phenotypes and genotypes constitute the ancestral protoclock that either predates, or at least coevolved with, the circadian time piece we know so much about today.

Keywords Biological clock • Lunar • Polychaete • Synchronous reproduction

10.1 Introduction

At the mention of lunar periodicity, many chronobiologists may think of Carl Hauenschild with his famous work on the polychaete *Platynereis dumerilli* (see chapter by Zantke et al., this volume). However, only some of those scientists will know what a polychaete actually is, and we suspect very few will appreciate that these worms have been the focus of chronobiological study for the past 50 years, because scientists such as Franke, Fong, and Olive, who sought to unravel the mysteries of how polychaetes are able to time their reproductive precision, usually synchronously and often perfectly matched to the annual, lunar, and tidal environmental phases, were invertebrate reproductive biologists. These scientists did not consider themselves chronobiologists and their science lies deep within invertebrate reproductive literature with a readership of mainly marine biologists and ecologists.

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In this chapter we describe the cycles and rhythms expressed by polychaetes, particularly in the orders Eunicida (family Eunicidae) and Phyllodocida (families Nereidae and Polynoidae), from the very long metonic cycling of reproduction to ultradian circatidal emergence patterns associated with foraging and predator evasion (Olive 1995). Some of the evidence of orchestrated reproduction presented in this chapter is manipulatively experimental, much is observational, but only very little is actually chronobiological. There are no free-running actograms depicted in this chapter to show the endogenous nature of the polychaete clock, a graphical language with which we are familiar as chronobiologists. The reason is possibly that there are no commercially available actographs for measuring activity in burrow-dwelling marine organisms, and when bespoke equipment is manufactured (such as by Last 2003; Riisgard et al. 1992), the measured locomotor activities are mostly labile, and free-running rhythms short lived, a characteristic of marine organisms in general. However, we hope that this chapter shows that evidence for clock expression in polychaetes is undeniable and that the range of clocks is as a consequence of their cyclically complex habitat.

10.2 Marine Worms Rooted to the Spot

Polychaetes, oligochaetes, and leeches comprise the phylum Annelida, which are also known as the segmented worms. Most polychaete species are marine, and they can be found from the nearshore to the deep hadal zones of the oceans. They have a distinct head and a number of sensory appendages, including the well-described nuchal organ believed to be associated with chemoreception and food searching (Lewbart and Riser 1996). Their slender bodies are made up of many segments, each of which is adorned with a pair of paddle-like appendages called parapodia. These parapodia enable the worms to crawl along the seabed and burrow into sediment. Crawling and burrowing is facilitated by having elaborate bristles (a characteristic taxonomic feature of polychaetes) that protrude from their parapodia, giving rise to their common name of "bristle worms," as so beautifully illustrated in Fig. 10.1.

Polychaetes that live in burrows, such as the nereids, are not spatially mobile and are analogous to terrestrial plants in that they may be considered "rooted to the spot." They have the advantage however, that their home provides shelter from predators and a harsh environment. Their burrows maybe either permanent, such as those made by the beautiful Christmas tree worms (*Spirobranchus* sp.) that burrow into the calcium skeleton of tropical corals, or temporary, such as those made by the king ragworm (*Nereis virens*) or the lugworm (*Arenicola marina*) that produces the characteristic "casts" with which we are familiar in muddy or sandy temperate estuaries.

There is, however, a distinct disadvantage to being "rooted to the spot." For both hermaphroditic and dioecious polychaete species, successful fertilization of gametes between individuals is required. If individuals live in solitary burrows and want to avoid being eaten they have two options: spawn and release their gametes to the water column or, very dramatically, send out "sexual satellites" that will increase

Fig. 10.1 The beauty of bristle worms is depicted here in an illustration by Ernst Heckel from the 96th plate of "Kunstformen der Natur" (1904), depicting organisms classified as Chaetopoda to which polychaetes and oligochaetes (such as the common earthworm) belong. (From http://en.wikipedia.org/wiki/File:Haeckel_Chaetopoda.jpg)

chances of fertilization considerably (and are elaborated on later). Indeed Olive (1995) also referred to these satellites as "stolons," which are single or multiple fragments of the parent worm detached for the purposes of sexual reproduction, and which effectively enable the worm to be in more than one place at once! Whichever reproductive strategy is used, the majority of polychaetes have a very restricted breeding season usually punctuated by very discrete spawning events; indeed, year-round breeding is actually relatively rare (Schroeder and Hermans 1975), although Olive (1995) makes a distinction between year-round breeding by individuals or cohorts and year-round breeding in a population where the latter is symptomatic of a lack of synchrony between individuals in a population and also rare (but observed by him in the first species that he studied, *Cirratulus cirratus*; see Olive 1971 and Olive 1973). Spawning events are usually synchronous, either for the whole population or cohorts of the population, and critical to this process is timing. Mistimed gamete release results in wasted sexual resources and, for many polychaetes species, death of the parent stock after spawning (semelparity) without successful recruitment.

Because most polychaetes live for a number of years and gamete development usually takes many months, the environmental signal transduction required to reproduce synchronously involves hierarchical input from many environmental oscillators; this requires that they are able to tell the time of year, month, day, and tide. Because orchestrated synchronous reproduction is so critical for survival of the population, it follows that there will be strong selection pressure to possess an endogenous clock(s) that is able to entrain to the prevailing zeitgebers found in these environments (Olive et al. 2005).

10.3 Cyclic Complexity in the Marine Environment

The marine environment in which polychaetes thrive is cyclically complex, and it is perhaps appropriate to briefly introduce this complexity, which may be unfamiliar to uninitiated terrestrial chronobiologists.

Although the moon's gravitational pull exerts its influence in the open ocean as a very gentle rise and fall of the sea surface, its effects are far more dramatic in the shallow waters near coastlines. Here strong surface currents flood and ebb the coastal shelves in an oscillatory manner, and the amplitude of these tides is constantly modified by the relative positions of the moon, the sun, and the earth, ranging from spring (higher-amplitude) to neap (lower-amplitude) tides. Where the sea meets the land we find an extreme environment with well-described demarcation of species (zonation) influenced by individual physiological tolerances and competitive niches. This area is known as the "intertidal zone" and is characterized by a marked alternation of favorable and less favorable living conditions. Because the combination of tidal and day–night cycles results in a large number of regular habitat changes, it has been postulated that marine organisms are probably entrained by a variety of zeitgebers. Indeed, cycles of salinity, temperature, pressure, and wave action have all been shown to synchronize and entrain endogenous rhythms (Naylor and Atkinson 1976; Reid and Naylor 1988).

The majority of the world's tides are semidiurnal: approximately two high and two low tides occur every day with a mean period of 12.4 h per cycle. In some areas, however, such as the Caribbean Sea, we only see one diurnal tide per day where the mean period between successive high or low waters is 24.8 h. Although there will clearly be a match between the amplitudes of the semidiurnal or diurnal tides and the lunar cycle (semilunar spring/neap cycle), there is a mismatch between the tidal period and the period of the solar day (24 h). As a consequence, tidal cycles progressively "march" through the days so that for any given day the high or low water the following day will be approximately 50 min later in areas exposed to semidiurnal tides (although this is highly dependent on local topography and meteorological conditions). The relationship between such tides and the solar cycle will typically come into phase approximately every 15 days (the 15-day cycle being called the semilunar cycle), meaning that if it is low tide today at midday then it will be low tide again at midday in 15 days.

It is well known that most terrestrial organisms have evolved to be active at a specific time of the daily cycle. Marine organisms are not different and may be also diurnal, nocturnal, or crepuscular, usually dictated by predatory avoidance or prey abundance strategies. If, for example, an animal is nocturnal but can only feed on the high tide, then the period between successive peaks of foraging activity will be 24.8 h, that is, the time between every other high tide at night. We refer to such cycles of activity as lunidian or lunar day (Palmer and Williams 1986; Palmer 1995a), and these become circalunidian under free-running conditions. Of particular interest is that 24.8-h lunidian tidal cycles are periodically less variable than the period between successive 12.4-h tidal cycles that show great deviation from tide to tide, depending on the phase of the moon (and semidiurnal inequality). Palmer (1997) has therefore argued that a clock entrained by a lunidian zeitgeber would be more precise than one entrained by the tidal zeitgeber; however, this is not universally accepted (Naylor 1996; Naylor and Rejeki 1996).

10.4 Tidal Periodicity in Polychaetes

Nereid polychaetes are common in the intertidal, particularly in muddy or sandy temperate coastal habitats. Of particular interest are the cyclic behaviors of both *Nereis virens* and *Nereis diversicolor*. These polychaetes live in U-shaped burrows and emerge, usually at night, to forage on the muddy seabed. They are predated on by birds, waders, and pleurenectenoid fish, which are all known diurnal, visual feeders (Carter et al. 1991; Thijssen et al. 1974; Wilson 1991a, b). To minimize predation they rarely leave their burrows completely (at least in the laboratory from personal observations) and retain their tails in their burrow entrances when foraging. (This precaution allows the dorsally located giant axon to rapidly retract their elongated bodies back into their burrows in response to any predator signals such as shadowing or vibration.) Because they do not leave their burrows they are effectively "tethered" and can only scavenge on food within reach of their outstretched bodies.

Measuring activity cycles in such tube-dwelling polychaetes is challenging. However, both Last (2003) and Riisgard et al. (1992) have used bespoke actographs to house polychaetes and do just that. Both actographs are based on a similar design of an artificial burrow, the openings of which are monitored by infrared opto-couplers. Animals readily take up residence in the glass or plastic tubes and instances of any out-of-burrow activity are captured on computer. Actographs developed by Last (2003) have recently been improved to include out-of-burrow activity video capture coupled with motion detection software (Schaum et al. 2013). Here the authors showed that the predominantly nocturnal polychaete *N. diversicolor* is able to detect chemical cues associated with the presence of juvenile flounder (*Platichthys flesus*) with number of emergences, emergence duration, and distance from burrow entrance significantly reduced during exposure to flounder-conditioned seawater and flounder mucus-spiked seawater. The data implied that *N. diversicolor* must have well-developed chemosensory mechanisms for predator detection and is consequently able to effectively minimize risk with a marked modification of its nocturnal foraging cycle.

Last et al. (2009) found that the dominant activities in *N. virens* maintained under both a light–dark (LD) and simulated tidal cycle (STC) in the laboratory were either tidal (12.4 h) or, more frequently, lunar day (24.8 h). The latter cycle of activity coincided with the rising tides at night, the safest time to forage and when food would be both supplied and redistributed on the shore. When animals were maintained under free-running conditions (very dim light) only a quarter showed significant rhythms, but these were in the circadian range (which includes the lunar day of 24.8 h) without any evidence of tidal rhythmicity. It appears therefore that in this organism the clock, at the behavioral level at least, may be governed by a "circadian" -type oscillator.

Of particular interest in the experiment by Last et al. (2009) was that in one animal that had been maintained under only tidal cycles and constant light for 6 weeks, only a circadian expression of activity was ever recorded. Astonishingly, the phase relationship of locomotor activity was maintained with the subjective night but not the tidal cycle, which suggested that there may be "crosstalk" between the signal transduction pathway activated by the tidal stimuli (in this case vibration and water-level change) of the tidal signal and the light (circadian) entrainment pathways. We suggested that either the tidal signal is interpreted as a circadian Zeitgeber because of circadian control on the input pathways (Zeitnehmer) or that the entrainment of the tidal clock directly results in the entrainment of the circadian clock. Further evidence of this type of "crosstalk" has been witnessed more recently in the sympatric *Nereis diversicolor* where LD entrainment resulted in a tidal rhythm of activity (Last, unpublished data). This finding is of interest because it is generally assumed by chronobiologists that LD cycles can only entrain circadian rhythms and are not zeitgebers for circatidal rhythms (Palmer and Round 1967; Palmer 1974, 1995b; Webb 1971). However, Barnwell (1966) showed that under a LD photo-regime without imposed tides, the fiddler crab *Uca minax* would be tidally active for many weeks and that critically, under free-running conditions of LL, the tidal cycles become circatidal. Further evidence from Saigusa (1992) showed that the tidal timing of larval release from the crab *Sesarma pictum* was entrained by a LD cycle but, when the photoperiod was advanced or delayed, the tidal larval release rhythm was shifted accordingly. It has been suggested that the observed tidal rhythm in this species is only a variation of the day–night rhythm (Saigusa and Oishi 2000), a hypothesis that is supported in the marine oyster *Crassostrea gigas* (Mat et al. 2012; Tran et al. 2011). It may be that "cross-modal" entrainment is not that uncommon in marine organisms and that, with the advent of more modern gene silencing techniques, further work can now address the relationship between the putative different clock pathways in polychaetes, as recently demonstrated by the work of Zantke et al. (2013).

10.5 Semilunar and Lunar Periodicity in Polychaetes

Possibly the most famous and certainly the oldest recorded example of the phenomenon of lunar periodicity of reproduction in a marine organism is that of the Pacific palolo worm, *Eunice viridis* (Caspers 1961; Gray 1847; Hauenschild et al. 1968). It is well documented that every year during the moon's third quarter in either October or November these polychaetes undergo mass, synchronous spawning on the reefs of some Indo-Pacific islands, most notably Samoa. Adult worms have very large jaws that are used to dig burrows into the limestone skeleton of coral and feed off algae and small crustaceans. As the spawning date approaches, the animals undergo an extraordinary transformation in which the anterior segments degenerate and the posterior ones fill with either eggs or sperm and become heavily muscled. On the spawning night itself the anterior segments, or stolons, detach and "swim" in the water column as "sexual satellites." This poetically termed "nuptial dance" only lasts about an hour, after which the stolons rupture lengthwise, releasing their gametes. Fertilized zygotes stay in the water column for a few days before the worm larvae settle out on the reef. The adults do not necessarily die, but remain within their burrows with anterior regeneration of somatic tissue and wound healing taking about a week. This annual breeding occurrence is so predictive that it has allowed locals to harvest large quantities of the worms very effectively in an orchestrated, well-timed fashion with canoes and hand nets (Naylor 2010).

The accuracy and synchrony of spawning times in the palolo worm have fascinated various chronobiologists over the years (principally Hauenschild, Caspers, and most recently Naylor). Spawning records date back over a century, and it is possible to plot the "Palolo Calendar" (as depicted in Fig. 10.2), showing the coincidence of spawning with the metonic, that is, 19-year, cycle (Naylor 2010). This cycle arises from the progression of the lunar calendar relative to our Julian calendar such that a particular phase of the moon occurs approximately 10–11 days earlier every year. Consequently, a particular phase of the moon will only occur again on a particular calendar date every 19 years.

For the palolo worm there is a spawning "window" of 6–8 weeks each year. If there is a lunar third quarter during that window at the end of October, spawning will occur, but if there are two lunar third quarters during this window, with one occurring in early October, then the next third quarter will be in early November, which is also then a potential spawning date. Unfortunately, the breeding cycle of *E. viridis*

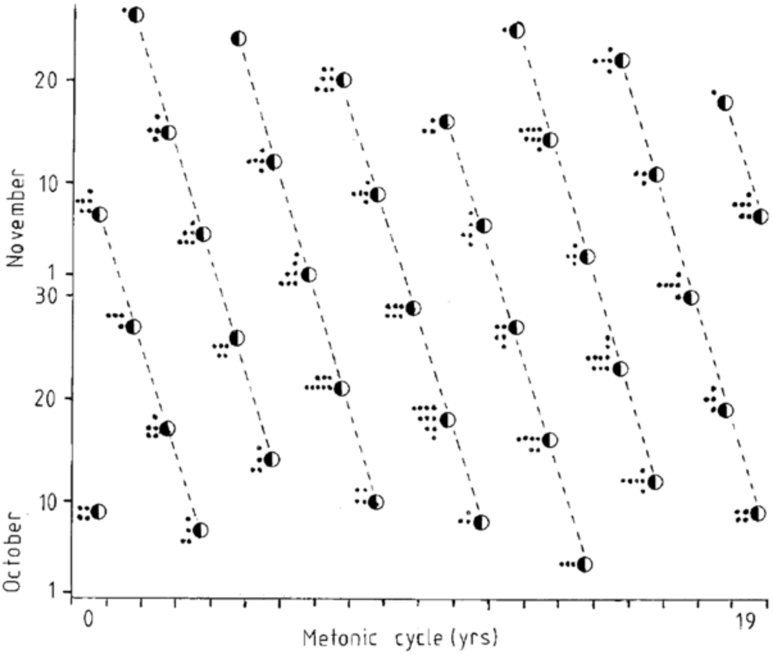

Fig. 10.2 Spawning dates of the Pacific palolo worm (*Eunice viridis*) based on observations from 1843 to 1999. The spawning dates (*dots*) are plotted on the 19-year metonic cycle with times of the moon's third quarter indicated. (Data provided by the Fisheries Division, Samoa.) (From Naylor 2001)

has not been free run under laboratory conditions and hence it is not known whether the spawning window in October/November is synchronized through a circannual clock or indeed whether the spawning time is synchronized by a circalunar clock.

In contrast to the palolo worm, there are other polychaete species with semilunar reproductive cycles but with an extended breeding season. *Nereis succinea*, *Platynereis dumerillii*, and *Platynereis bicanaliculata*, for example, all spawn with semilunar periodicity but do so for most of the summer when seawater temperatures are above a critical temperature. Male and females leave their burrows and come into close proximity, usually at or near the sea surface after dark, where they engage in a swimming behavior known as a nuptial dance originally described in *Nereis limbata* by Lillie and Just (1913). This nuptial dance and consequent release of eggs and sperm has been shown in some nereids to occur in response to sex pheromones from both sexes. Two studies (Fong 1993; Hardege et al. 1998) exposed *P. bicanaliculata* to a series of LD and lunar cycles and showed that the gradual decline in illumination from full moon to the last quarter moon is the cue that synchronizes swarming.

Although the literature provides many examples of lunar reproductive timing, with some examples of lunar periodicity under free-running conditions, there is only one example in which the timing mechanism has been proven to exhibit all the characteristics of a true biological clock. A series of experiments conducted by

Franke (1985, 1986a) demonstrated at the behavioral level the existence of a lunar clock in the syllid polychaete *Typosyllis prolifera*. Franke (1986b) fully characterized the reproductive rhythm in this species with the first lunar phase-response curve shown in Fig. 10.3. Similar to the palolo worm *T. prolifera* undergoes stolonization, which occurs approximately every 31 days during the second quarter of the moon where artificial light at night in the laboratory acts as the entraining zeitgeber. He showed that a critical number of nights with light at night ($2<N<4$) result in the same synchronization response of stolonization as is witnessed in wild populations post full moon. As is the circadian clock, the lunar timepiece in *T. prolifera* was found to be temperature compensated, at least between 15 ° and 25 °C, which is representative of the natural thermal range of this polychaete during the breeding season (March to October). Of further interest is that the temperature-compensated lunar clock is limited to the rhythmic initiation of the stolonization phase, not sexual development which, once initiated, proceeds autonomously as though controlled by an interval timer that may be influenced by temperature. Changes in temperature can phase shift the time of sexual development and are believed to account for differing phase relationships between the lunidian cycles and the cycle of stolon abundance in the wild where seasonal temperature cycles will phase shift the time of stolon production.

Franke (1986b) subsequently detailed a remarkable set of experiments using *T. prolifera* in which some animals were maintained under LD 16:8 with constant dark nights followed by 4 days of “moonlit” nights applied over the lunar calendar (type 1) while others had constant “moonlit” nights with a 4-day dark or “moon-off” period also applied at different points over the lunar calendar (type 2). The resulting response curves shown in Fig. 10.3 are identical. Franke showed that the resetting zeitgeber is the transition between light at night and darkness at night, and he consequently suggested that the lunar clock is probably much more rudimentary than the circadian one, but he did not elaborate why.

It is clear from the literature that moonlight plays an important role in the synchronization of reproductive events in polychaetes. Further, this is not restricted to tropical latitudes where the moonlight signal is usually overt. Indeed the cosmopolitan *Platynereis dumerillii* will swarm on a semilunar basis during the summer months off the west coast of Scotland (Last, personal observations) where the sky is only infrequently clear. Thus, we can postulate that either moonlight transduction pathways are very sensitive in these animals or the endogenous lunar/semilunar clock provides the necessary information to spawn in the absence of clear lunar signals. Clearly when moonlight is lacking there will still be additional environmental signals that may provide lunar zeitgebers for swarming, such as the semilunar spring–neap tidal cycle and associated cyclic changes in hydrostatic pressure, temperature, and salinity that are prevalent in such a fjordic environment.

In all the examples of reproductive strategies described herein it is necessary to make clear that the expression of cycle or rhythm is a population-level phenomenon because any one individual worm will only leave the burrow and spawn once, after which it dies without reentering the burrow. The worm will only have one chance to “get the timing right,” and if it is not successful in fertilizing its gametes then it will be taken out of the gene pool.

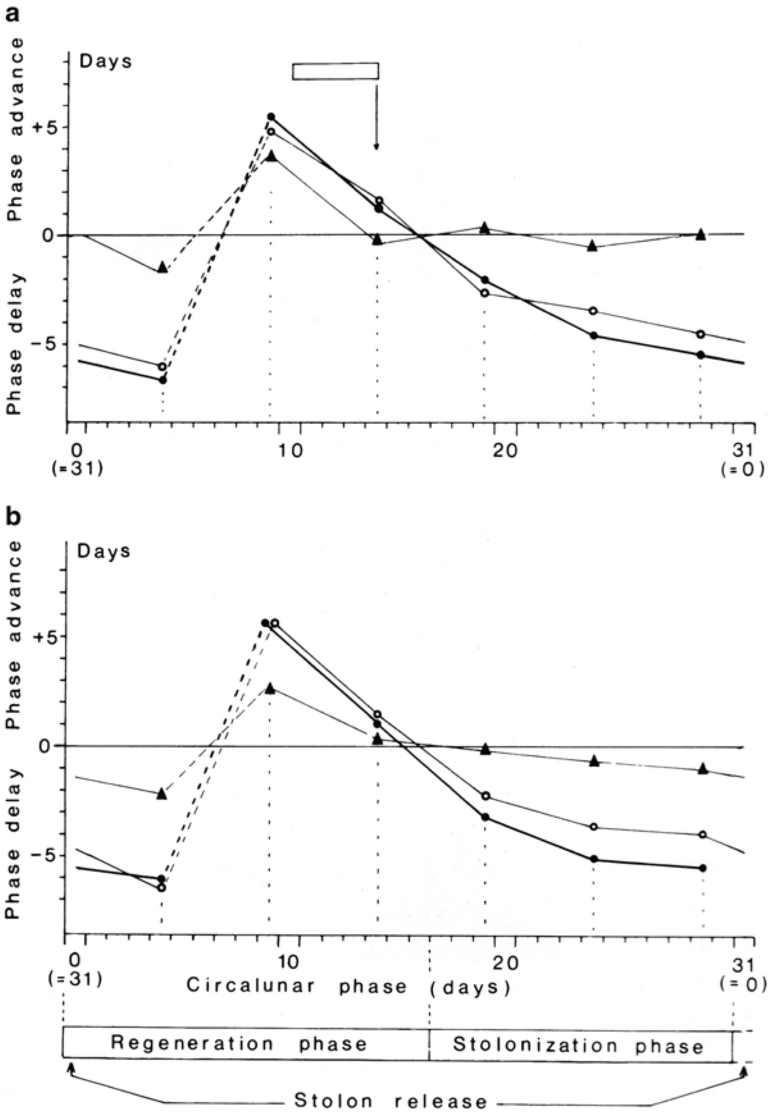

Fig. 10.3 Phase–response curves for the circalunar reproduction rhythm of *Typosyllis prolifera*. The *points* of the curves represent the mean phase shifts (relative to unstimulated controls) in the otherwise free-running rhythms of about 50 worms each, effected by single type 1 pulses (*upper panel*) and type 2 pulses (*lower panel*), respectively, at different phases of the worms' circalunar cycle. Different curves show the shifts measured at the times of the first (*triangles*) and second (*open circles*) stolon release after perturbation. On the *upper margin* of the graph, the approximate position of the curve relative to the "moonlight" Zeitgeber program (four successive "moonlit" nights recurring every 30 days) is indicated (stolon release about 17 days after "moon-off"). (From Franke 1986b)

10.6 Circadian Periodicity in Polychaetes

In contrast to lunar and semilunar periodicity, evidence from the literature of circadian rhythms in polychaetes is surprisingly sparse. In one of the limited examples, Scott et al. (1976) found an alternating aerobic and anaerobic metabolic circadian rhythm in the polychaete *Nereis virens* under constant illumination. Data show long ventilation rest periods (~1–9 h) superimposed on short-period ventilation bouts. Anaerobic respiration produces lactic and other organic acids, and it is believed that the spontaneous switch from anaerobic to aerobic respiration serves to reduce dangerous levels of organic acid accumulation.

Similarly, the capitellid polychaete *Mediomastus ambiseta*, which is widely distributed around the central Americas, has a circadian periodicity in fecal pellet production (Fuller et al. 1988). Even after a month under constant conditions in the laboratory *M. ambiseta* were defecating the most during the subjective night and least often during the evening. It was assumed that this rhythm may either have a diurnal predator avoidance strategy or be caused by physiological constraints.

A more colorful example can be seen in the polychaete *P. dumerillii*, which displays a daily cycle of camouflage. These worms have white reflecting chromatophores speckled across their dorsal surface that undergo a circadian cycle of pigment concentration, the mechanism of which is unknown (Fischer 1965; Fischer and Dorresteijn 2004).

Although these examples document circadian periodicity in polychaetes, several studies suggest that diel activity, and modification thereof, may be an adaptive trait in an environment that is highly labile. A recent study by Schaum et al. (2013), for example, has shown that the polychaete *Nereis diversicolor* has acute chemosensory abilities that allow it to detect very small quantities of predatory fish mucus in the water. However, its diel behavior is highly modified in response to mucus. Nocturnal out-of-burrow foraging activity is virtually completely suppressed and shows no habituations. Similar behavioral modifications to predator cues occur in *N. virens* (Watson et al. 2005), although it is not known whether this actually disrupts the circadian rhythm previously found to be expressed in about 30 % of *N. virens* populations under free-running conditions (Last et al. 2009). It is suggested, therefore, that the clock of *N. virens* is probably weakly coupled, labile, and readily modulated by external stimuli or the dominant environment cycle (Last et al. 2009).

10.7 Photoperiodism in Polychaeta

It should be apparent from the preceding sections that some polychaetes time their reproductive activities to the seasons, and the measurement of daylength or "photoperiodism" is central to this. Although the subject of photoperiodism strays slightly from the topic of enigmatic clocks, biological clocks are believed to be central to underpinning photoperiodic responses (Bünning 1936; Saunders and Bertossa 2011) and so are worthy of discussion.

The most conclusive evidence for overt photoperiodism is the attainment of a photoperiodic response curve. One of the first such curves drawn for a non-arthropod invertebrate was for the polychaete *Harmothoe imbricata* (Garwood and Olive 1982). Animals were maintained under constant summer conditions (LD 16:8 and 16 °C), and subsamples of the populations were transferred to various shorter daylengths and cooler temperatures. It was found that in this scaleworm both temperature and daylength affect the rates of various processes in the gametogenic cycles of both sexes. It was later found that females must experience at least 40 days with the light period less than the critical daylength between LD 10:14 and LD 11:13 if the eggs are to complete their development (Clark 1988). Olive (1995) subsequently produced a general model demonstrating the environmental control of reproduction focusing on the Polychaeta, postulating that an endogenous oscillator, which is entrained by the environmental zeitgeber, defines the time during which a proportion of the population makes a transition to the sexual maturation program.

Another polychaete for which a photoperiodic response curve has been generated is *N. virens*. Last et al. (1999) demonstrated that there is a critical photoperiod of 11 h in which mean out-of-burrow activity (foraging excursions) is inversely related to the duration of the scotophase, that is, as the nights become shorter the foraging excursions become longer even though the available time for foraging is less. Working in the northeast of England, where the worm is locally enshrined in folklore (see Fig. 10.4), it was found that photoperiod further influenced other physiological functions such as segmentation, regeneration of truncated caudal segments, and natural growth rates. These findings were interpreted by Last and Olive (1999) as a modification of the diel (and probably circadian) activity providing a mechanism by which the transduction of the changing duration of photophase and scotophase can generate a photoperiodic, that is, annually cyclic, physiological response.

Similarly, Fong and Pearse (1992a) found, in the polychaete *Neanthes limnicola*, that the timing of parturition, fecundity, and lifespan were all affected by photoperiod. Parturition was synchronized within the populations of individuals maintained under LD 12:12, becoming asynchronous under any other regime. Reproduction in this species occurred consistently under spring light regimes even when regimes were 6 months out of phase. It was suggested that the worm must experience either one or more critical daylengths, or increasing daylengths, that mimic spring light regimes before initiating sexual development. They concluded that in this polychaete seasonally changing photoperiods determined the optimal time of parturition.

In certain polychaetes photoperiod acts synergistically with temperature to modulate gametogenesis. In populations of *Autolytus prolifera* and *Autolytus brachycephalus*, long days and warm waters induced stolonization whereas short days and low temperatures prevented it. Under long days with cold water, however, stolonization was drastically reduced, suggesting that temperature is the dominant environmental cue in these species (Schieges 1979). Conversely, photoperiod and temperature also influence oocyte growth in the polychaete *Kefersteinia cirrata*, but in this case the

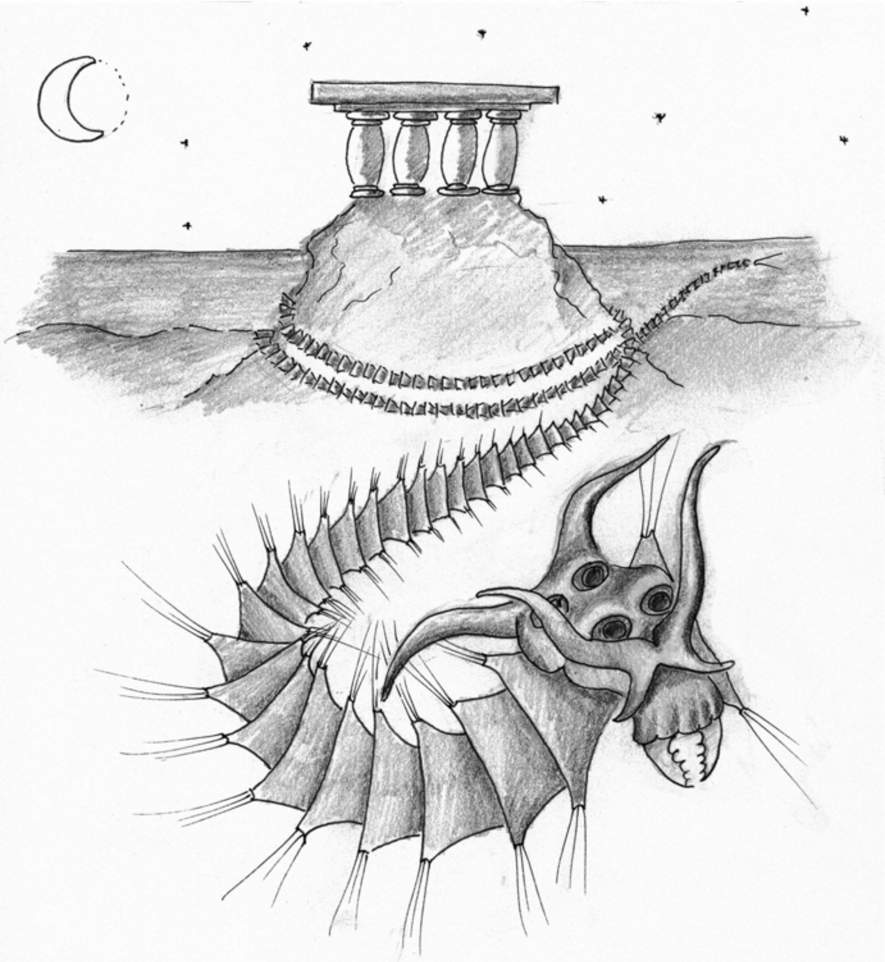

Fig. 10.4 The story of the "Worme of Lambton" is a popular traditions of the northeast of England, UK. This story has been handed down by oral tradition through many centuries and involves a giant worm, not unlike a nereid polychaete, causing much trouble and seen here coiling seven times round Penshaw Hill. (From Last 2000)

photoperiodic effect is dominant (Olive and Pillai 1983). Similarly, Olive (1980) showed that the apparent control of oogenesis in *Eulalia viridis* lies in the interaction between temperature and an endogenous reproductive cycle in which the most important zeitgeber is temperature, not photoperiod. Obviously, the effects of temperature and photoperiod are largely dependent on the particular species investigated.

Although the photoperiodic control of reproduction in polychaetes has received the most coverage in the literature, a few workers have shown photoperiodic effects on growth and behavior. Chu and Levin (1989), for example, found that in the polychaete *Streblospio benedicti* overall body size and setiger (segment) number, brooding activity, and brood size were increased with lengthening days or long fixed daylengths. However, survivorship and maturation were unaffected by daylength. The animals showed cessation of reproduction and decrease in growth rate in autumn and winter, with increased growth rate and initiation of reproduction in spring and summer. These findings are similar to those of Last and Olive (1999) who showed that short-day treatments result in significantly reduced growth, segment proliferation, regeneration rate, and feeding in the polychaete *N. virens*. In this species the critical photoperiod was found to be LD 12:12, suggesting that key events (growth and reproduction) will occur at the same time at all points in the geographic range of that organism, regardless of local temperature and feeding conditions (Olive et al. 1997). In summary, the role of the photoperiod in polychaete life history is probably to coordinate an ongoing inevitable process with the appropriate, and possibly most adaptive, season.

10.8 Annual Periodicity in Polychaetes

We have already shown that in many polychaetes reproductive events occur at specific times of the year. Changes in photoperiod, moonlight, and temperature have all been implicated in structuring this annual calendar of breeding, yet there are times when gamete development cannot be manipulated. A good example of this is in the scaleworm *Harmothoe imbricata*, where overt refractory periods in the animals' calendar year (in this case in the spring when spawning occurs naturally) make it impossible to induce gametogenesis even with photoperiod/temperature manipulation (Clark 1987; Garwood 1981; Garwood and Olive 1982). In explanation, Olive, Garwood, and coworkers proposed the "gating" model that effectively prevents breeding at inappropriate seasons (Garwood 1980; Olive and Garwood 1983). Later, Olive (1984) and then Clark (1987) working with the polychaete *N. diversicolor*, suggested an 'endogenous oscillator/external variable interaction model' to account for the observed cyclic development of mature gametes. This concept suggests that there must be an interaction between an external environmental zeitgeber and an endogenous timing mechanism believed to take the form of a circannual rhythm. The timer was described as having the following properties: an exogenous cycle that determines the birthdates; an endogenous circannual cycle of the "gated" type which determines when the final stages of gametogenesis may be initiated; and an interval timer that maintains a constant phase relationship between initiation and completion of gametogenesis. This model was later refined (Olive 1995) to include the input of late-acting spawning triggers during the period of "readiness to spawn" and photoperiodic resetting of the endogenous oscillator, resulting in phase advance of spawning "readiness" (see Fig. 10.5).

In contrast to those studies investigating circadian rhythms, circannual free-running protocols rarely use constant dark or light photo-regimes but instead use static light–dark cycles to unmask seasonal rhythmic changes. When the polychaete

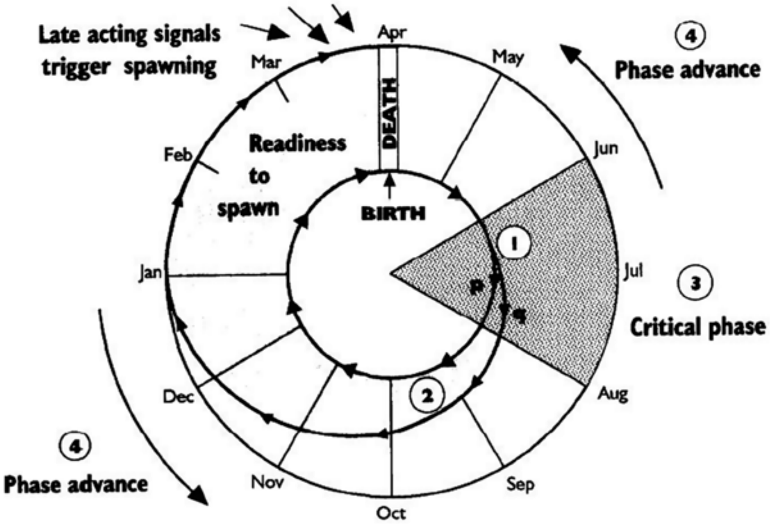

Fig. 10.5 A model to describe the observed environmental control of reproduction in the Nereidae. A putative endogenous oscillator defines the time or "critical phase" at which a proportion of animals switch from somatic growth to sexual maturation. The oscillator, however, may be phase advanced to allow the animals to take advantage of "late-acting" environmental signals such as the lunar cycle and chemical signals (see Bentley and Pacey 1992 for review) that will illicitly reproduce at an earlier time and maximize reproductive fitness. (From Olive 1995)

Neanthes limnicola was reared under LD 12:12, all animals reached parturition within 9–11 months whereas those under different static photo-regimes did not (Fong and Pearse 1992b). Although the study was not carried out for two full cycles (the minimum duration required to demonstrate a circannual clock), it did at least suggest the presence of a "seasonal" cycle of reproduction that was effectively free running under LD 12:12.

The polychaete *N. diversicolor* has shown a similar circannual reproductive response. When maintained under constant temperature and static photoperiods from birth, animals became sexually mature at the same time as worms in the field, suggesting that external zeitgebers were not essential in determining the timing of reproduction (Garwood and Olive 1978; Olive and Garwood 1983). In another dramatically colored bright green polychaete, *Eulalia viridis*, the gametogenic cycle is characterized by an annual period of rapid vitellogenesis in the spring, which is hypothesized to be caused by the interaction between two out-of-phase cycles, both with a periodicity of one year (Garwood and Olive 1978; Olive 1980). Differing from the scaleworm *H. imbricate*, which requires the existence of a circannual rhythm entrained by photoperiod to control the reproductive cycle, *E. viridis* is suggested to have one exogenous cycle that is entrained by temperature (considered the most important zeitgeber for this species) and a second, endogenous cycle which modulates the oocyte growth rate over the course of the year (Garwood and Olive 1978; Olive 1980).

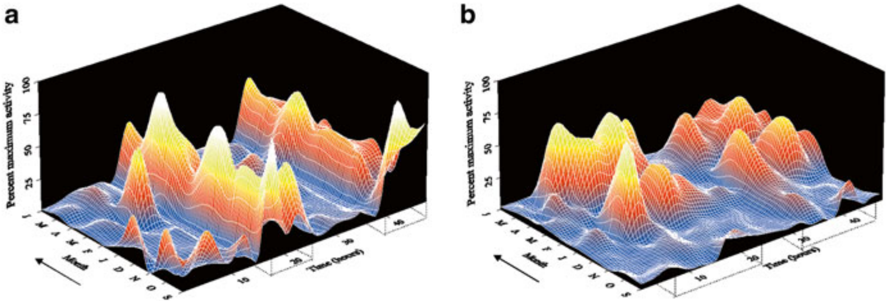

Fig. 10.6 Mean monthly burrow emergence activity (September to June) in *Nereis virens* under two static photoperiods representative of summer (LD 16:8) and winter (LD 8:16) conditions (*top* and *bottom*, respectively). Under constant summer daylengths, nearly all activity is confined to the night except for perturbations in September, October, and March ($n = 4$). However, under constant winter daylengths there is a cessation of activity between September and the middle of December. In January there is a sudden spontaneous increase in activity during the night, which is continuous for the rest of the experiment, except for a decline in activity in March ($n = 4$). (From Last and Olive 2004)

A long-term behavioral experiment conducted by Last and Olive (2004) using an actograph showed that the influence of an endogenous seasonal or circannual timer is not restricted to gametogenic development. A single population cohort of *N. virens* was maintained either under constant LD 16:8 or LD 8:16 for 9 months (September to May) and their activity recorded continuously (Fig. 10.6). Those animals maintained under LD 16:8 were strongly nocturnal during the whole trial whereas those under LD 8:16 showed almost complete cessation of activity between September and December, followed by a spontaneous resurgence of activity thereafter. This cessation of activity during the subjective autumn was postulated to minimize risk at a time when the worms were growing their gametes. In contrast, while under constant LD 16:8 conditions gamete development was inhibited and the worms continued to forage to maximize their energy intake. The spontaneous resurgence of activity under constant LD 8:16 after 6 months in the laboratory coincident with springtime outside the laboratory remains unexplained. However, one explanation may be to accrue enough metabolic reserves to reproduce, and therefore the response could be considered adaptive to maximize fitness. It is unknown whether this activity is caused by a seasonal, possibly annual endogenous timer in this polychaete.

10.9 Polychaete Proto-Clocks

It is apparent that biological clocks (annual, lunar, daily, and tidal) are well manifested throughout the class Polychaeta. What then, does this tell us about the evolutionary history of these clocks? Polychaetes belong to the stem group of the Bilataria, the Urbilataria, which are the hypothetical last common ancestor of the bilaterian clade believed to have existed from the fossil record about 600 million years ago (Peterson 2001; Peterson et al. 2004). These marine worms would have inhabited shallow seas and lagoons around the supercontinent of Pangaea and the Central Tethys Sea at least 200 million years ago until the great Cambrian explosion saw our arthropod ancestors crawl onto land. Such lagoons probably provided a hostile environment, particularly from DNA-damaging ultraviolet radiation as a result of the rather sparse atmosphere at this time (Benn 2001). Migrating away from dangerous surface waters during the day and back again at night may have been critical to prevent excessive DNA damage (Boeing et al. 2004), although diel vertical migration (DVM) in modern zooplankton is attributed to mostly predator avoidance (Bollens et al. 1992). Lagoons would also have been subject to extreme tidal cycling (because the moon was closer to the Earth at the time), and therefore it is not hard to imagine a prehistoric marine environment influenced by extreme solar and lunar signals where a clock would have been adaptive.

It is also of note that any ancestral proto-clocks evolving at this time must have entrained to cycles that do not match the present-day ones. The rotation of the Earth about its axis has slowed over evolutionary time because of tidal friction (and the resulting retreat of the moon from Earth), and hence the period of the solar day would have been much less than it is now, around 20 h during the Cambrian (Krasinsky 2002). During evolutionary history, therefore, clock periods will have lengthened quite considerably, although such musings are probably outside the scope of this chapter but detailed elsewhere (Tauber et al. 2004). What is noteworthy, however, is that the proto-clock to all terrestrial arthropods and vertebrates would have stemmed from a polychaete-like animal. Furthermore, the clock behaviors we see now in polychaetes are probably core to the circadian pacemaker because we have clear evidence of tidal clock timing in terrestrial dipterans (Neumann and Heimbach 1985). It seems improbable that the proto-clock from the polychaete-like animal would have reinvented itself in this fly after the great radiation of the animal clades, but until molecular evidence becomes available we only have an incomplete picture of this story.

10.10 Marine Clock Hypothesis and a Look into the Future

This chapter would be incomplete without mention of the three hypotheses proposed to explain tidal clock behavior in marine organisms, even though they are not based on the behaviors of polychaetes. Naylor (1996), who worked on the common shore crab (*Carcinus maenas*), proposed that there are two oscillators, one that measures the day (circadian) and the other which measures the tide (circatidal); Palmer and Williams (1986) and Palmer (1995a), who worked with tropical fiddler crabs, proposed a dual oscillator with a periodicity of 24.8 h but locked in antiphase, hence producing tidal (12.4 h) and near-circadian (24.8 h) rhythms; and finally Enright (1976), who worked on isopods, proposed that a single bimodal clock can produce both circadian and circatidal rhythms. It is clear that we cannot decipher the clock mechanisms in marine organisms based on behavior alone (Aldrich 1997); we need molecular tools, and we probably should be looking at organisms with a sequenced genome. One such candidate is *Platynereis dumerillii*, and it has been suggested (Tessmar-Raible et al. 2011) that, with its remarkable lunar clock, it will make a particularly fitting model with which to find the molecular basis of the marine clock. Indeed, only recently a significant step forward has been made at deciphering the workings of the circalunar clock in this animal (Zantke et al. 2013). Zantke et al. (2013) found that when circadian clock function (at the behavioral and molecular levels) was inhibited using casein kinase 1d/ε, the expression of circalunar spawning activity remained uninhibited. The conclusion was that the circalunar clock in *P. dumerillii* is independent of the oscillations of the circadian transcriptional clock. The molecular workings of other marine examples, although not polychaetes, have come under the spotlight just recently. For example, the Pacific oyster *Crassostrea gigas* has been shown to display a suite of behavioral clock phenotypes with circadian clock gene analogues sufficient to generate a circatidal rhythm (Mat et al. 2013; Tran et al. 2011), and the marine woodlouse (*Eurydice pulchra*) has revealed the coexistence of molecularly independent circatidal and circadian clocks (Zhang et al. 2013). We certainly live in exciting times with regard to better understanding marine clocks, aided now by modern molecular techniques that will ultimately help us to better understand the workings of these remarkable timekeepers.

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Part II
Circannual Rhythms

Chapter 11
Circannual Rhythms: History, Present Challenges, Future Directions

Barbara Helm and Tyler J. Stevenson

Abstract Circannual rhythms are endogenous biological oscillations that underlie a wide range of seasonal processes. Without knowledge of these underlying mechanisms, it is difficult to fully understand what drives the ways organisms change over the course of a year and to predict how they will respond to environmental conditions. The study of circannual rhythms is particularly timely given the substantial interest in seasonal processes in relationship to environmental change, and further given insights that seasonal change in human physiology and behavior is greater than previously thought. The present chapter outlines basic definitions and ideas on circannual rhythms, summarizes ground-laying work, and highlights some current developments. It then addresses challenges in this field and the opportunities that arise from the rapid development of new technologies.

Keywords Endogenous • Migration • Photoperiod • Reproduction • Seasonal

11.1 Introduction

Among the "Enigmatic Clocks" that are the subject of this book, perhaps the most enigmatic in terms of their mechanistic basis are circannual clocks. Circannual rhythms oscillate with daunting period lengths of roughly 365 days and provide the endogenous basis of long-term timing. Even today, nearly 30 years after Gwinner's (1986) authoritative assessment of the field, it is difficult to understand the biological processes that could create rhythms with such extremely long time constants. Furthermore, organisms differ greatly in the extent to which circannual rhythms are displayed in the absence of environmental cues and in the conditions under which

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these rhythms persist. Nonetheless, rhythms on this timescale are taxonomically widespread, and in some species circannual processes recur with admirable temporal precision. Circannual rhythms are typically measured as recurring biological processes over the lifetime of individuals (e.g., reproduction, migration, metabolic change). In short-lived species, rhythms may emerge on a population level, for example, by periodic recurrence of phases in which population members undergo individual developmental transitions (e.g., pupation of insects; see also the chapter by Miyazaki et al., this volume).

Increased research efforts to better understand circannual rhythms are important and timely. Interest in the seasonality and annual cycles of organisms has grown enormously over the past years. In particular, phenology (i.e., the seasonal timing of recurring biological processes; Foster and Kreitzman 2009; Visser et al. 2010) has attracted substantial scientific and public attention in view of the rapid changes in global environments. The study of phenology has benefited from methodological and analytical advances, which in turn yielded data that indicate great differences in the ways organisms modified their annual cycles in view of changing conditions. Simultaneously, evidence is increasing that seasonal changes in a range of physiological processes are more pervasive in many species, including our own species *Homo sapiens*, than previously thought (Hazlerigg et al. 2013; Helm et al. 2013; Ebling 2014; Martinez-Bakker et al. 2014; Schwartz and Andrews 2013). We are convinced that an improved understanding of the mechanisms that underlie seasonal biology, and in particular those of circannual rhythms, will contribute considerably to addressing urgent concerns in ecology, health, and global change biology.

Research during the past decades has consolidated the evidence for circannual rhythms and is inching toward tractable approaches to resolve their mechanistic basis. Several of these advances are represented by the chapters presented in this book. This introduction gives general background on circannual rhythms, discusses some difficulties, and ends with an outlook on innovative methods to resolve them.

11.2 What Are Circannual Rhythms?

The study of biological rhythms is concerned with behavioral, physiological, morphological, developmental, or molecular processes that recur with periodicities related to those of geophysical cycles (Dunlap et al. 2004). It centers on distinguishing cycles that are endogenous and persist even under constant conditions from those that are solely driven by external (environmental) factors. Formally, the definition of circannual rhythms develops along the following steps: rhythms in general are defined as regular spatial or temporal repetitions of patterns. Endogenous rhythms are temporal repetitions under conditions that provide no external information about the period they normally assume. An endogenous rhythm with a period length of approximately 1 year is called *circannual* (from *circa*, about, and *annus*, year; Gwinner 1986). Convincing evidence for an endogenous basis is provided if the periodicities of circannual rhythms are close, but not identical, to 1 year. Because under constant conditions they then progressively drift away from the calendar year, they are termed "free running." Jointly these features specify the necessary and sufficient conditions for phenomena to be regarded as "circannual rhythms." In addition, the following features further characterize circannual rhythms as being based on *bona fide* endogenous clocks (Gwinner 1986; Dunlap et al. 2004):

1. Entrainment of the free-running rhythm to environmental cues (i.e., *Zeitgeber*). Under real life conditions, circannual rhythms are usually not free running but instead are entrained to the annual cycle. Based on experimental evidence, the most important zeitgeber is the annual change in daylength ("photoperiod"). Entrainment by other *Zeitgeber* has been shown to be possible, but evidence for their effectiveness is currently limited to proof-of-principle studies (see the chapters by Heideman, and by Goymann and Helm, this volume; Immelmann 1971; Heideman and Bronson 1994; Helm et al. 2013). Entrainment has also involved transient cycles, at least in some species (e.g., Concannon et al. 1997) (Fig. 11.1).
2. At least in some species, the free-running rhythm is temperature compensated (Gwinner 1986, p. 42ff). In contrast to the usual increase in the rate of physiological processes with increasing temperature (so-called Q_{10} properties; Rensing and Ruoff 2002), circannual period length was barely affected by experimental differences in constant ambient temperature. Nonetheless, similar to its effects on circadian rhythms, temperature had clear, and often phase-dependent, modifying effects on aspects of circannual cycles, in particular in some hibernating species (reviewed in Gwinner 1986, p. 43; Wikelski et al. 2008; Helm et al. 2013; also see the chapter by Miyazaki et al., this volume).

Although these features unambiguously characterize endogenous circannual rhythms, they have been established for only a limited number of organisms. Annual cycles are ubiquitous in most environments on earth, but such rhythmic patterns can be based on various mechanisms, including instantaneous responses to environmental fluctuations. Thus, without study under constant conditions the contribution of circannual rhythms to these cycles is unclear. Regrettably, the attribute "circannual" is nonetheless sometimes applied in the general sense of "annual cycles." To avoid confusion and to properly identify pertinent mechanisms, the use of the term "circannual" should be restricted to processes that persist under constant conditions, whereas in all other cases, the correct term is "annual" or "seasonal" cycle.

11.3 Historical Perspective on Circannual Rhythms

The expression of circannual rhythms differs between organisms and often requires specific, "permissive" conditions (Gwinner 1986). For example, in birds, rodents, and dinoflagellates, closely related taxa may differ substantially in the degree to which circannual rhythms persist under constant conditions (Anderson and Keafer 1987; Gwinner 1996; Prendergast et al. 2002). Differences in circannual

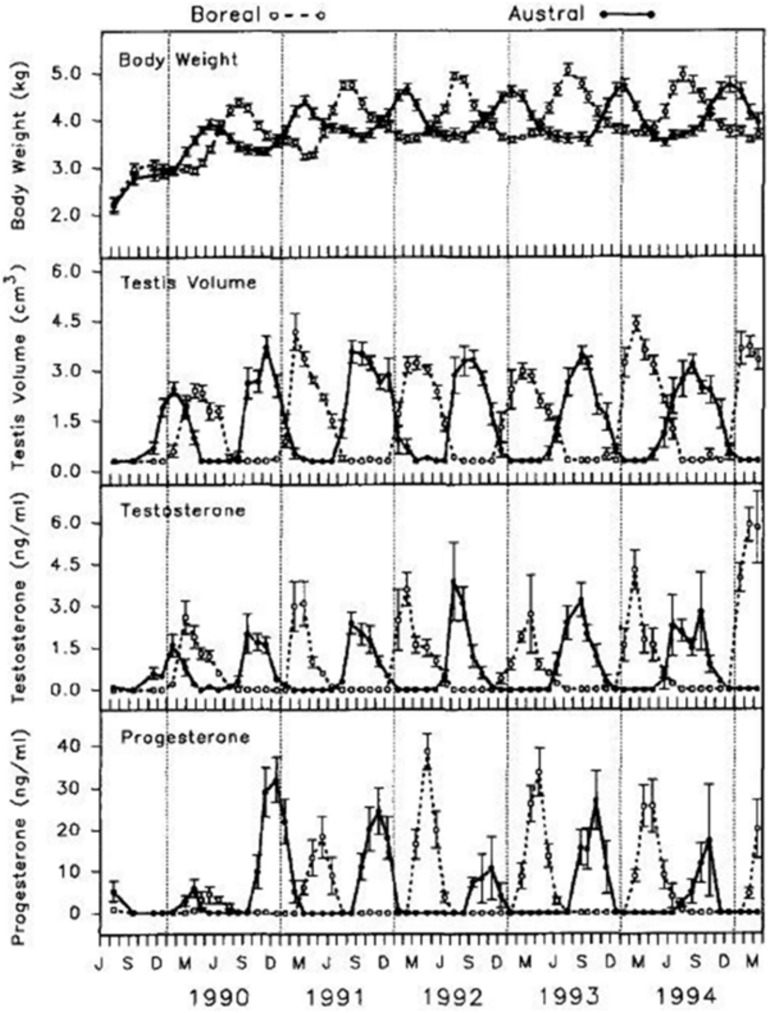

Fig. 11.1 Photoperiodic entrainment of circannual rhythms: The figure shows cycles of body mass, testis volume, testosterone, and progesterone of woodchucks *Marmota monax* in the process of phase-shifting from photoperiodic conditions of the Northern (boreal) to the Southern (austral) Hemisphere. Entrainment involved transients for at least two cycles. *Curves* show means and standard error. Animals that did not re-entrain were removed from the study. (Reprinted from Concannon et al. (1997) with friendly permission of *Biology of Reproduction*; see there for more information)

organization had been predicted from the outset of the field (Aschoff 1955, 1958) and were thought to relate to environmental seasonality and to an organism's life cycle along the following considerations. Although in most habitats on earth environmental conditions change over the course of a year, these changes differ geographically in amplitude and year-to-year predictability (see the chapters by Heid elevated eman, and by Goymann and Helm, this volume). A high amplitude in seasonality commonly implies alternations in the availability of conditions that are conducive to growth and reproduction. Accordingly, organisms in these environments are expected to alternate between phases of growth and reproduction on the one hand, and phases of avoidance or endurance of unconducive conditions, for example, by migration or dormancy, on the other. Within a given seasonal environment, the pressure to align with the seasons will be elevated for organisms that depend on resources that are highly seasonal (e.g., in birds feeding on aerial insects compared to those feeding on seed) (Foster and Kreitzman 2009; Bradshaw and Holzapfel 2007).

If seasonal changes are predictable, organisms can anticipate them and prepare in advance, instead of simply responding to their occurrence. Classical examples for highly precise, anticipatory actions include avian long-distance migrations, in which departure dates of populations and individuals are often highly repeatable between years and occur well in advance or even in the absence of environmental deterioration (Gwinner and Helm 2003; Helm et al. 2012; Altshuler et al. 2013). In their attempt to predict in which species circannual rhythms would be the most clear cut, researchers were aware that photoperiod (annual change in daylength) usually provide information for anticipating seasonal changes (Rowan 1925, 1926; reviewed in Foster and Kreitzman 2009; Nelson et al. 2010). Therefore, they expected endogenous information to be most useful for organisms that had limited access to reliable photoperiodic or other predictive information about seasonal change. For example, Rowan (1926) pointed out that photoperiodic information alone was insufficient to explain the timely return of equatorial or transhemispheric migrants, which in their winter quarters experienced either constant or long daylengths. The plant physiologist Erwin Bünning (Bünning 1949) conducted extensive experiments with seeds that pointed in the direction of long-term rhythms but remained inconclusive because environmental influences (especially humidity) were not strictly controlled and period lengths were very close to that of the solar year.

Following the more rigorous formalization of endogenous rhythms on a circadian time scale, Jürgen Aschoff (Aschoff 1955, 1958) wrote two influential papers in which he suggested that these concepts be applied on an annual timescale. Aschoff (1955) proposed that rewarding biological systems for the study of endogenous circannual rhythms would be mammalian hibernation and avian long-distance migration. Furthermore, near the equator photoperiodic information is available, but because its annual changes are minute the information content was thought to be low. Therefore, equatorial species were also predicted to profit from clear circannual rhythmicity. Aschoff (1958) discussed possible experimental approaches to investigation and specifically recommended to keep organisms under constant light–dark (LD) cycles, which he knew were more likely to retain robust rhythmic organization than unchanging light intensities. Almost simultaneously, the first rigorous data on circannual rhythms were published. Below we introduce main study systems and also refer the reader to other sources for more complete coverage (Gwinner 1986).

11.4 Main Circannual Model Systems

Mammalian Hibernation The first unequivocal evidence that endogenous circannual rhythms were indeed involved in annual-cycle timing came from a study of hibernation in the ground squirrel *Citellus lateralis* by Pengelley and Fisher (1957). This seminal publication inspired decades of work on the regulation of seasonal processes in hibernators. Next to their formative role for research on circannual rhythms and photoperiodic entrainment (Fig. 11.1), studies of hibernating mammals were also highly relevant for understanding seasonally regulated physiology. These studies continue to reveal important information on a wide spectrum of features, including further characterization of circannual rhythms and physiological and molecular regulation of metabolism (Ebling 2014; Schwartz and Andrews 2013). For example, Kondo et al. (2006) studied circannual cycles over as long as 13 years and reported evidence for a novel protein that may be involved in the regulation of hibernation, and Olson et al. (2013) characterized circannual fluctuations in sensitivity to an agonist that induces torpor. Monecke et al. (2009) have provided detailed information on interactions of circannual rhythms and photic input in the European hamster *Cricetus cricetus*, including phase-response curves to long and short days (Fig. 11.2). Recently, they also demonstrated for this species photoperiodic entrainment independently of systemic melatonin production, which they suggest represents a specific, circannual pathway (see also Monecke et al. 2014). The chapter by Monecke and colleagues in this volume reports on these and other advances.

Pupation in Carpet Beetles Blake (1958) published a first documentation of circannual rhythms of pupation in the varied carpet beetle *Anthrenus verbasci* and

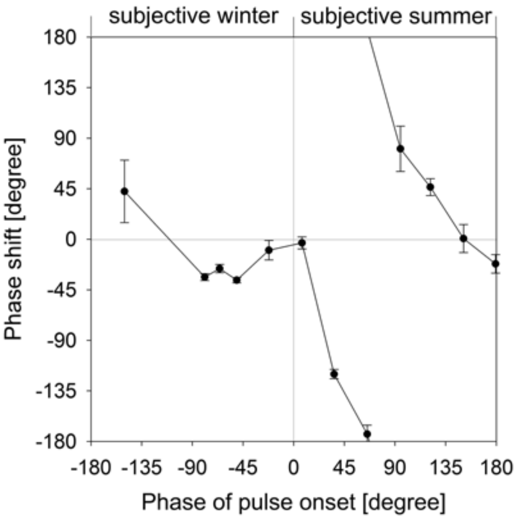

Fig. 11.2 Circannual phase-response curve to short-day pulses: European hamsters *Cricetus cricetus* were kept under constant light-dark (LD) 16:8 h conditions, and during different circannual phases, were exposed to 1 month of LD 10:14. *Curves* show shifts in the timing of reproductive cycles. (Reprinted from Monecke et al. (2009) with friendly permission of *Journal of Biological Rhythms*; see there for more information)

thereby initiated a tractable study system that has been used for circannual research up through the present day (see the chapter by Miyazaki et al., this volume). Carpet beetle larvae live in secluded and often light-sheltered places, from where they pupate after spending one or several years in larval stages. Under constant conditions pupation of a cohort occurs with circannual rhythmicity, so that most pupate after one, and smaller numbers after subsequent, circannual intervals. Circannual rhythms are thus measured on a population level as the number of larvae pupating at any given time. Research on this model system has contributed the most in-depth characterization of formal properties of circannual rhythms so far (Miyazaki et al. 2005, 2006, 2007), and is summarized in the chapter by Miyazaki et al. in this volume.

Avian Migration As predicted by Aschoff (1955) and earlier by Rowan (1926), long-distance migration of birds has been a rewarding system for circannual studies. The central question was what triggered the departure of equatorial and transequatorial migrants from the winter quarters in early spring to return to higher latitudes for breeding (Newton 2008). Daylengths are nearly uniform at the equator and are longer in Southern Hemisphere winter quarters than on Northern Hemisphere breeding grounds. The winter habitat clearly sustains the migrants and allows many local birds to breed at this time. Despite the absence of any obvious environmental drivers or cues, migrants nonetheless leave on time to return to the breeding grounds in spring. The groundwork for addressing this question was laid by Eberhard Gwinner, who spearheaded circannual research and was a passionate ornithologist. Since his first circannual publications in 1967 and 1968, his trademark studies involved both free-living and captive birds. Gwinner spent many months in the Republic of Congo, where he observed *Phylloscopus* warblers in the wild and in local captivity. Simultaneously, conspecifics were kept in aviaries in Southern Germany under circannual conditions (Gwinner 1967, 1968). Gwinner found that the birds developed migration-related behavior (nocturnal restlessness or *Zugunruhe*) in African and German captivity in time for their actual departure in the wild. Under constant conditions, *Zugunruhe* drifted rhythmically with circannual period lengths. These and many ensuing studies (Hamner and Stocking 1970; Gwinner 1986; Holberton and Able 1992; Berthold 2001; Newton 2008) provided rigorous evidence that in many species the signal for migratory restlessness and related processes (e.g., fat deposition) came from a circannual clock. In most songbirds, species of the order Passeriformes, periodicities were shorter than 1 year whereas in a different avian class, waders (species of the order Charadriiformes), period lengths were considerably longer (Piersma et al. 2008).

A main insight from avian studies was the wide range of processes that were under circannual regulation. For example, using laboratory testing devices for directional preference (“Emlen funnels”), researchers were able to show that birds shifted their orientation behavior based on annual and circannual programming (Emlen and Emlen 1966). With a “clock and compass” navigational system, unexperienced migrants could direct their behavior toward the winter quarters on their first journey. Another insight was that features of circannual rhythms differed greatly between

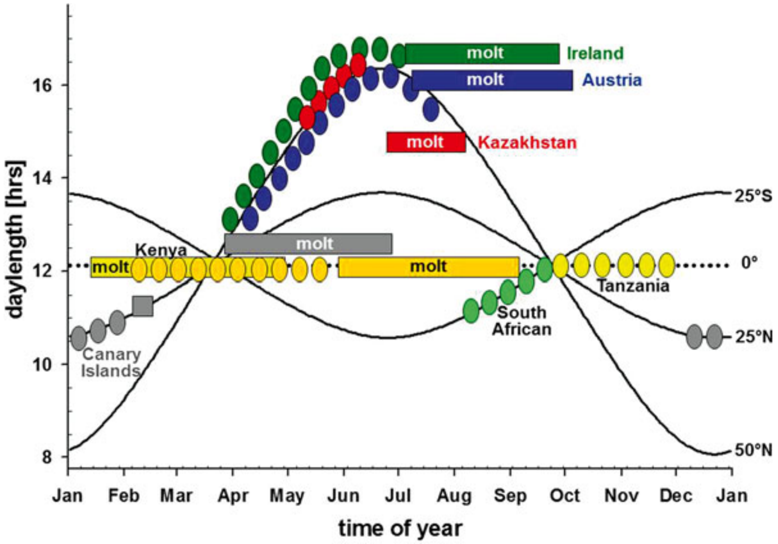

Fig. 11.3 Photoperiodic conditions under which stonechats *Saxicola torquata* breed and molt: overview of information on the timing of reproduction (*oval shapes*) and molt (*boxes*) of stonechat populations from different latitudes. Reproduction and molt are plotted on the respective daylength curves of the birds' breeding ranges; the different populations are indicated by *color-coding*. (Reproduced from Helm (2009) with friendly permission by *Integrative and Comparative Biology*; see there for details)

species and, in some cases, even local populations (Gwinner 1996; Helm et al. 2009). In flycatchers and sylvid warblers, the robustness of the rhythm and the permissive conditions under which it was expressed differed with migratory distance and daylengths normally experienced across the annual cycle (Gwinner 1988). To investigate differences between closely related taxa, Gwinner established a circannual model system using a species with an extensive north–south breeding range, the stonechat *Saxicola torquata*. This species became paradigmatic for sustained rhythms and for geographic differentiation of annual and circannual properties (Helm et al. 2009; Fig. 11.3). A succinct overview of circannual rhythms in migratory birds is given by Gwinner (1996).

Equatorial Species As suggested by Aschoff (1955), equatorial species living under nearly invariable daylengths are primary candidates for showing distinct circannual rhythms. Two chapters in this volume (Heideman, and Goymann and Helm) are centrally concerned with equatorial, and more generally, tropical species. These chapters detail that many equatorial species show robust circannual rhythms in captivity, but that only a few, notably island-breeding seabirds (Chapin 1954; Reynolds et al. 2014), show free-running cycles in the wild. A main question, therefore, is the nature of the *Zeitgeber* that enable most tropical species to synchronize with the external year. The chapter by Heideman (this volume) points out bats with circannual rhythms that do not entrain to photoperiod, and the chapter by Goymann and Helm (this volume) reports on entrainment of birds to subtle, photic cues at the equator. Both chapters also place circannual and photoperiodic timing in ecological and evolutionary context.

Algae Another environment that may be nearly constant, and thereby favor robust circannual rhythms, is the deep-sea region of oceans. Research examining different species of algae indeed established circannual rhythms in various processes, including germination, growth, and cell division (Costas and López Rodas 1991; Lüning and Kadel 1993; Matrai et al. 2005). A landmark paper by Anderson and Keafer (1987) documented the presence of circannual cycles even in the unicellular dinoflagellate *Gonyaulax tamarensis*. The mechanisms of the long biological time constants are difficult to conceive of even in multicellular organisms, let alone in unicellular organisms (see also the chapter by Lincoln and Hazlerigg, this volume). However, the relative tractability of these organisms and their unicellular organization could provide key insights into regulatory mechanisms. It would be highly desirable that work on this promising, and probably relatively tractable, circannual model system were continued.

Sheep The species introduced here have in common that their environments or life styles make photoperiodic information hard to obtain or potentially misleading, so that they would have been expected to have evolved robust circannual rhythms. However, persistent circannual rhythms can also be found in some species that live year around in temperate areas with unhindered access to photoperiodic change. Among those are breeds of sheep, for example, Soay sheep (see chapter by Lincoln and Hazlerigg, this volume). These animals have provided significant advances in our understanding of the neuroendocrine mechanisms that underlie circannual rhythms, including evidence for a circannual pacemaker located in the pituitary gland in the brain (Lincoln et al. 2006). Even after disconnecting the pituitary from neuronal input, which brought reproductive cycles to a halt, these animals showed persistent circannual cycles of the hormone prolactin secreted by lactotrophic cells in the anterior pituitary gland. These data are tantalizing and indicate that circannual timing of specific seasonal processes may reside within specific tissues in more complex species. For further background of these studies, and of the mechanistic outlook that has developed from this work, see the chapter by Lincoln and Hazlerigg (this volume).

11.5 Challenges in the Study of Circannual Rhythms

Although circannual rhythms on a whole meet the full set of criteria expected of biological oscillators, there is still some confusion in the literature about their endogenous nature and biological role (Mrosovsky 1970; Menaker 1974; Dawson et al. 2001; Dawson 2007; Newton 2008; Wikelski et al. 2008). We believe that many perceived difficulties have originated from differences in research traditions, terminologies, and choice of model species, which can ultimately be harmonized.

Circannual rhythms have sometimes been regarded as irrelevant or artefacts because under natural conditions powerful *Zeitgeber* are commonly available. This criticism is a shared legacy of endogenous rhythms in general. Good arguments for the relevance of endogenous rhythms under natural conditions include specific entrainment properties (Johnson et al. 2003) and benefits of internal representation of time and temporal coordination of physiology (Dunlap et al. 2004). For circadian rhythms, the major difficulties arising from their disruption certainly support their relevance (Fonken et al. 2013). However, for a broader appreciation of endogenous rhythms, identification of the mechanistic basis is essential. Circadian rhythms are ubiquitous in a world where photic *Zeitgeber* are available, but critics were only convinced of their existence when the formerly intractable mechanistic basis was elucidated. Today it seems hard to believe that pioneers such as Jürgen Aschoff were long accused of reporting on mere artifacts, and that the endogenous character of circadian clocks was scientifically debated at least until 1970 (Brown et al. 1970). Rhythms on other timescales, including circannual but also circatidal and circalunar rhythms, are still faced with criticism, although researchers now begin to reveal their molecular basis (see the chapter by Zantke and Tessmar, this volume; and Zhang et al. 2013).

Of the endogenous clocks, circannual rhythms are obviously the slowest to be tested (Menaker 1974). Data are still available only for some species and for relatively few circannual cycles (Gwinner 1986). Furthermore, the outcomes of these investigations were sometimes inconsistent, leading to further skepticism. For example, when several processes were studied in parallel (for example, reproductive state, body mass, and a hormone), they sometimes differed in rhythmic behavior, so that molt rhythms may have persisted although reproductive cycles became arrested (Gwinner 1986; Newton 2008). Similarly, in several but not all systems these long-term rhythms were relatively variable (Mrosovky, personal communication), and the conditions under which these rhythms persisted varied among organisms. In some species, circannual rhythms were maintained under a wide range of constant daylengths (e.g., sylvid warblers; Berthold 2001; Helm et al. 2013), including continuous light (Fig. 11.4; Rani and Kumar 2013; Holberton and Able 1992), indicating that cycling requires no alternation between light and darkness. However, in other species rhythms continued only under a limited daylength range or were not expressed at all (Dawson 2007). These observations provide challenges for circannual research, but they also point to understudied and underappreciated features of seasonal biology and photoperiodism.

Dissociation of Processes The observation that, under circannual conditions, physiological processes can dissociate, is a powerful indication that their fixed sequence results from synchronization by light. Thus, although hormones mediate the transitions between life history stages (Jacobs and Wingfield 2000), the underlying processes are physiologically sufficiently independent to become uncoupled (see also the chapter by Lincoln and Hazlerigg, this volume). Under natural conditions, the wide range of variation in the sequence of life history stages gives further testimony to a principally modular, flexible composition of the annual cycle.

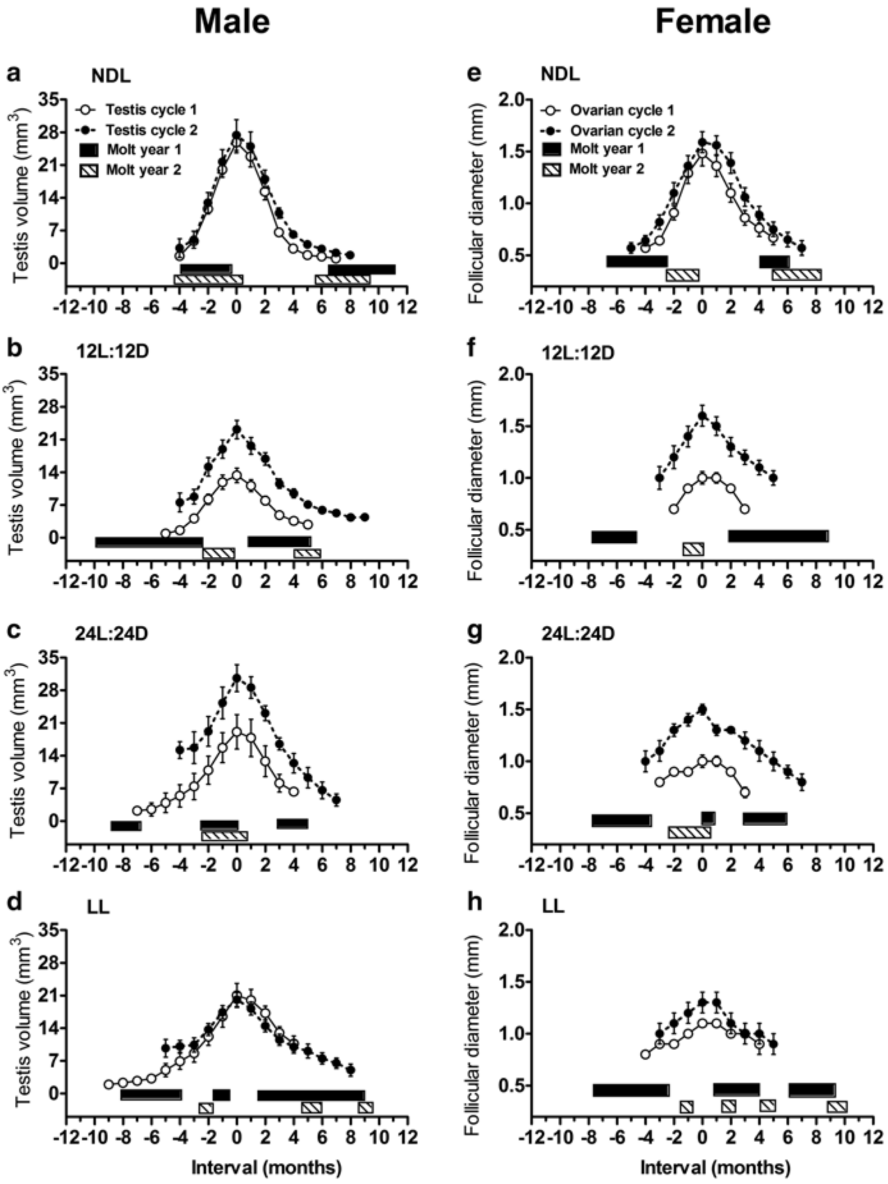

Fig. 11.4 Persistence of avian circannual rhythms under different light conditions: reproductive (*curves*) and molt (*bars*) cycles of male and female spotted munia (*Lonchura punctulata*) kept under different light conditions. In all cases, data are shown for two cycles (first year, *white circles*; second year, *black circles*). Male (**a**–**d**) and female (**e**–**h**) birds were kept under natural (*NDL*) and artificial lighting conditions (12 L:12D, 24 L:24D and LL; L = 22 ± 2 lux, D = 0 lux). On the *x*-axis, zero indicates the timing of maximal gonadal size in a given year. The preceding and following months are plotted from −12 to +12. (Reproduced from Rani and Kumar (2013), with friendly permission by *General and Comparative Endocrinology*; see there for details)

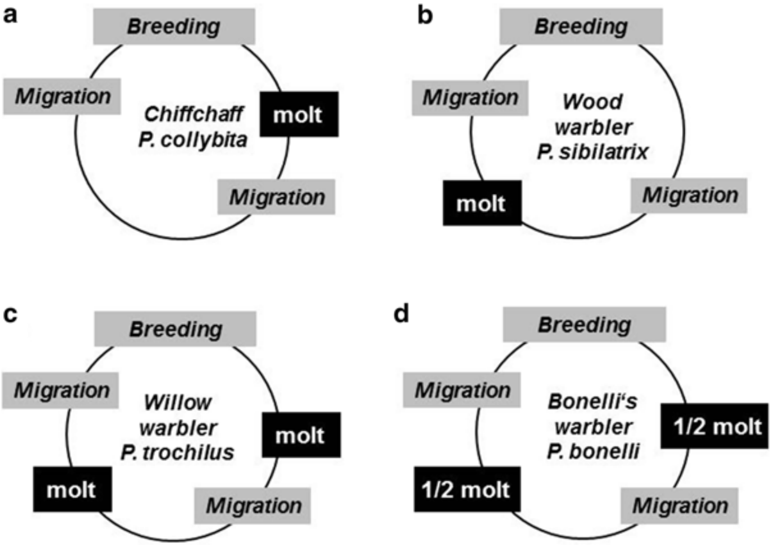

Fig. 11.5 Variable position of molt within the annual cycle: schematic shows timing of molt in four sympatrically breeding species of *Phylloscopus* warblers. In these migratory songbirds, molt occurs either (**a**) solely after breeding, or (**b**) solely in the winter quarters, or (**c**) is fully completed twice per year, or (**d**) is split between the winter and breeding quarters. These patterns are thought to relate to migration strategy. (Based on summary in Newton 2008)

For example, warblers of the genus *Phylloscopus* are similar in appearance but differ in the extent of their migrations, as well as in their annual cycles (Newton 2008). Figure 11.5 shows schematically the position of molt with respect to breeding and migration. Four closely related species molt either directly after reproduction, or in winter after autumn migration, or partially at both times, or undergo two complete molts (Newton 2008). This plasticity in nature and in the experimental studies should inspire fresh mechanistic thinking. Circannual rhythms were sometimes seen to arise as sequences of connected stages with sequential negative feedback, adding up to roughly 1 year (Mrosovsky 1970; Wikelski et al. 2008). For example, reproductive activation could activate pelage or molt, which would inhibit reproduction. After molt completion, inhibition could be lifted, and reproductive activation could recapitulate. The continuation of circannual cycles in some but not all traits, and the plasticity of sequences in wild organisms, makes this explanation unlikely and calls for other ideas. Menaker (1974) correctly considered the discussion of whether circannual rhythms were really rhythms or merely a series of sequential steps to be unproductive, and explained: "Of course, both circannual and circadian rhythms must consist of sequences of interdependent steps, the productive question in both fields (but perhaps more easily studied in circannual rhythms) is at what level of organization do the crucial steps occur and can they be identified and their interactions analyzed." The chapter by Lincoln and Hazlerigg in this volume outlines how we are beginning, 40 years later, to distinguish different levels of organization on which sequential steps can occur. The annual cycle may be composed of modular, circannual processes, such as reproductive state, molt, metabolic state, and migration, which may be integrated through photoperiodic synchronization and be mediated by endocrine control (see the chapters by Monecke et al., and by Heideman, this volume).

Circannual Rhythms and Photoperiodism Research on photoperiodism and circannual rhythms, respectively, has not always been sufficiently integrated, which has sometimes led to the false perception of noncompatible mechanisms. From a circannual perspective, it is entirely clear that in the real world, circannual rhythms normally are entrained by photoperiod as its main *Zeitgeber*, much as circadian rhythms are normally entrained by the daily light cycle. Recent findings from several circannual study systems have underpinned this basic insight by elaborating phase-response curves, which closely resemble those of circadian rhythms (Fig. 11.2, Monecke et al. 2009; see also the chapter by Miyazaki et al., this volume). These curves detail how the response of the organism to a given daylength depends on the phase of the circannual cycle, underpinning the realization that photoperiodic sensing is mechanistically distinct from the underlying long-term timer (Johnston et al. 2003; Lincoln et al. 2005). Correspondingly, from a photoperiodic perspective there is ample evidence that responses to daylength depend on the phase of the annual cycle (Bradshaw and Holzapfel 2007). In view of these convergent findings, the remaining difference is in an emphasis on endogenous cycles and an associated *Zeitgeber* role of photoperiod, or on photoperiod as the driver of the cycle via long-term timing mechanisms, respectively.

Harmonizing these perspectives is made complicated by terminology used by many researchers to describe photoperiodic regulation and circannual rhythmicity, respectively, and by the choice of study organisms and photoperiodic conditions (Butler et al. 2010). Circannual studies have mainly focused on systems on the extreme end of sustained rhythmicity, selecting species with robust cycles and constant daylengths that optimized rhythmicity. Photoperiodic studies, in turn, have focused on species whose cycles are highly responsive to or completely dependent on photoperiod, and have chosen daylength conditions that efficiently switch between life history stages or arrest rhythmicity (Gwinner and Wozniak 1982). The definitions that are central to the two approaches reflect these respective focal interests. For photoperiodism, the main interest was in robust reproductive responses to relatively short-term changes in photoperiod. Birds such as the European starling *Sturnus vulgaris* maintained on short daylengths (e.g., <12 h) showed a prepubertal-like state and were then often referred to as being *photosensitive* (Nicholls et al. 1988; Dawson et al. 2001). Exposure of these birds to long daylengths initiates a cascade of events that results in a state referred to as *photostimulated*, characterized by gonadal development and reproductive competence. However, long days also lead to the generation of a state referred to as *photorefractory*, in which these birds experience complete involution in reproductive physiology. Recent comparative work has highlighted that the term “photorefractoriness” stands for a host of forms of temporary or permanent inhibition of the advance of the annual cycle under particular photoperiodic conditions

(Nicholls et al. 1988; Hahn et al. 1997; Hahn and MacDougall-Shackleton 2008; MacDougall-Shackleton et al. 2009). Detailing these forms and relating them to the progress or dampening of circannual rhythms is beyond the scope of this introduction but is certainly worth further exploration. The experimental data and the associated characterization of the neuroendocrine light input pathway hold considerable potential for identifying circannual mechanisms. Unfortunately, the polarization between photoperiodic and circannual perspectives has slowed the much-needed progress in understanding long-term timing processes.

Highly flexible interactions between circannual rhythms and photoperiodism are supported by the wide range of avian breeding and molt cycles under natural conditions. For example, many species or even local populations breed on short and even declining photoperiods. Figure 11.3 shows that breeding cycles of closely related stonechats from different geographic regions are spread out across the year (Helm 2009). The respective daylengths under which these processes occur underpin their photoperiodic flexibility. For avian populations inhabiting arid and hot environments, breeding in winter is common, even if conspecifics in different environments breed in summer. We consider it unlikely that such populations would use a markedly different mechanism than their summer-breeding conspecifics to time reproduction and molt. Correspondingly, migratory species show highly diverse responses to the daylength conditions they experience in winter. For example, from a photoperiodic point of view, transequatorial migrants such as bobolinks (*Dolichonyx oryzivorus*) would be expected to breed in winter when austral daylengths are long (Hamner and Stocking 1970; Gwinner 1988). Instead, these species display greatly extended relative photorefractoriness, which however eventually dissipates under these long days, allowing migrants to reactivate their reproductive system in preparation of breeding. When tested under constant circannual photoperiods, species such as the garden warbler *Sylvia borin* (Berthold et al. 1972), and also the white-crowned sparrow *Zonotrichia leucophrys* (Wingfield 1993), reactivated their reproductive system under long days (16–18 h of light). Clearly, therefore, birds appear to be relatively flexible in the adjustment of their response to components of the photoperiodic cycle. Evolutionary shifts seem to be compatible with the idea of an underlying circannual rhythm (Helm et al. 2009). Changes in the timing of events, such as winter breeding of stonechats or winter molt of *Phylloscopus* warblers (Fig. 11.5; Newton 2008), would thereby arise from adjustments of the phase relationship of components of the circannual cycle to components of the photoperiodic cycle. Such adjustments could be effected by shifts of the molecular mechanisms that regulate the neuroendocrine control of reproduction (e.g., neuropeptides), permitting reproduction under daylengths that better predict local environmental conditions favorable to breeding (Perfito et al. 2012).

The chapters in this book provide valuable information for linking the diversity of annual cycles to differences in photoperiodic responses and underlying mechanisms. Phase-response curves (Miyazaki et al., and Monecke et al., this volume) pinpoint phase-specific responses of the circannual system, and the physiological underpinnings of variation in photoperiodic responses are discussed in depth for mammals (see Heideman, this volume).

11.6 Opportunities and Perspectives for Unraveling Circannual Mechanisms

Despite the many challenges facing the circannual biologist, these are exciting times. Pioneering nucleic acid sequencing conducted by Edward Sanger had been considered the gold standard for molecular biology, especially DNA sequencing, for the latter part of the twentieth century (Sanger et al. 1977). His advances were essential for the direct sequencing of target genes, such as insulin, and his work resulted in being awarded the Nobel Prize in Chemistry in 1980. Recent developments of second-generation Sanger sequencing methods, also known as next-generation sequencing (NGS), have permitted massive high-throughput sequencing that has allowed an entire genome to be sequenced in less than 1 day. These new methods are quite ingenious: instead of sequencing a single nucleic acid region, NGS technologies simultaneously sequence millions of nucleic acids at a time (Metzker 2010). Truly, our ability to provide whole genomes has provided immense molecular information at an unprecedented rate. The availability of NGS technologies will undoubtedly provide significant advances for our understanding of the molecular mechanisms that generate the immense range of weird and wonderful rhythms described in this book.

The ability to sequence entire genomes will be an extremely valuable tool for the identification of evolutionarily conserved sequences that facilitate or dampen oscillations in physiology and behavior. Functional genome comparisons are currently hampered by the lack of genomes from nontraditional species. With the advent of NGS methods, it is now possible to obtain large bits of genomic information from simple organisms such as the dinoflagellates (see the chapter by Lincoln and Hazlerigg, this volume), to more complex organisms such as beetles, stonechats, European hamsters, and ground squirrels (see the chapters by Myazaki et al., Heideman, Monecke et al. and Goymann and Helm, this volume). Full genome-wide comparisons of circannual species with those that exhibit less pronounced oscillations (e.g., humans, mice) will yield valuable fundamental knowledge that will in turn allow for a better understanding of naturally occurring annual or possibly circannual rhythms in humans (Foster and Roenneberg 2008; Martinez-Bakker et al. 2014).

The application of NGS has also permitted the broad analyses of other molecular levels, such as RNA expression and genome-wide profiling of epigenetic markers and chromatin structure. Emerging techniques that permit these large-scale analyses of molecular processes include RNA-sequencing (RNA-seq); methylation-sequencing (methyl-seq), and chromatin precipitation assays (ChIP). RNA-seq consists of sequencing all RNA pieces within a cell or tissue and ultimately provides a snapshot of the RNA that is expressed at a single point (Metzker 2010). Using this technique an ambitious chronobiologist could unravel the seasonal “transcriptome” and ultimately map all genes that are transcribed during the circannual cycle. Furthermore, RNA-seq will also result in the discovery of substantial changes in noncoding (nc)RNA. ncRNAs range in size from small or microRNA (<50 bp) to longer ncRNA (>200 bp). It is widely believed that ncRNA serve to permit genetic flexibility, and we suggest that the phenotypic plasticity associated with circannual rhythms may, in part, be regulated by these ncRNA (Cech and Steitz 2014). Our understanding of the role these ncRNAs play for regulating circannual rhythms is in its infancy. During the next few decades, delineating how ncRNA participate in the generation and regulation of circannual rhythms will undoubtedly identify novel agents for orchestrating the large changes in seasonal genomic, physiological, and behavioral plasticity.

Epigenetic modifications, including DNA methylation and histone acetylation and their enzymatic effectors, are becoming major players in the molecular regulation of biological timing (Fig. 11.6). Methyl-seq involves the sequencing of the genome with a specific focus on the location of DNA methylation, resulting in the creation of a "methylome." DNA methylation is an important molecular biological event, consisting of the addition of a methyl (CH_3) group to cytosine nucleotides in the genome sequence. Increased levels of methylation serve to prevent the ability of transcription factors from binding to the DNA template, effectively inhibiting gene transcription and reducing RNA expression. Given the recent identification of seasonal changes in enzyme expression involved in methylation (Stevenson and Prendergast 2013; Fig. 11.6b), it is likely that several genomic regions will exhibit seasonal variation in methylation levels (Fig. 11.6c); these epigenetic modifications most likely contribute to the extensive genetic plasticity identified in various microarray studies (Ross et al. 2004; Nakao et al. 2008; Mukai et al. 2009; Stevenson et al. 2012a). Furthermore, given the observation of heritable variation in neuroendocrine traits associated with circannual timing of reproduction, epigenetic modifications such as DNA methylation provide a strong candidate mechanism involved in the selection underlying this phenotypic plasticity (Avigdor et al. 2005; see the chapter by Heideman, this volume). For example, DNA methylation of key genomic regions in parents provides a molecular level at which selection can act to regulate the probability of gene expression, either by permitting or inhibiting, and thereby regulate circannual or annual timing that is subsequently passed on to the next generation. By employing methyl-seq approaches, it now has become possible to identify specific genomic targets that may facilitate the divergence in circannual physiological and behavioral processes despite the lack of genomic changes.

Advances in proteomics are another exciting technological advancement that will have significant impacts for the circannual biologist. The proteome consists of the entire set of proteins present in a cell, tissue, or organism. Our understanding of annual or seasonal changes in protein expression has primarily focused on select

Fig. 11.6 (continued) and circannual rhythmicity, exhibit light-dependent changes in expression. When hamsters are in the summer breeding condition (*green bars*), there is an increase in enzyme expression compared to the winter nonbreeding condition (*red bars*). **c** *dnmt3b* expression in immune cells (i.e., peripheral leukocytes) also changes in a seasonally dependent manner. (Data from Stevenson and Prendergast 2013; Stevenson et al. 2014, with friendly permission by the publishers)

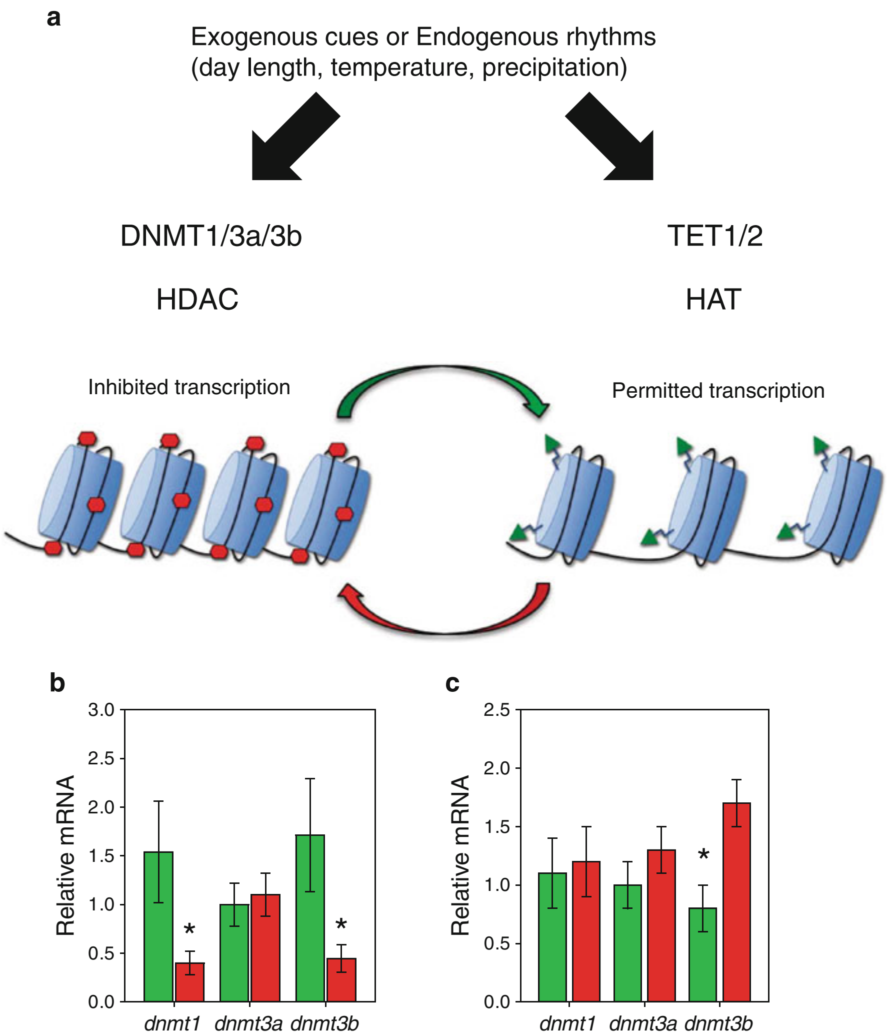

Fig. 11.6 Epigenetic modifications and the regulation of circannual or seasonal rhythms. **a** Endogenous circannual rhythms or exogenous environmental cues act to regulate gene transcription by regulating enzymes involved in epigenetic modifications. Two epigenetic events, DNA methylation and histone acetylation, can act independently or together, leading to the inhibition or enhancement of gene transcription. Epigenetic enzymes can be divided into those involved in DNA methylation or histone acetylation. DNA methyltransferase enzymes (isoforms 1, 3a and 3b; DNMT) are critical for adding methyl groups (indicated by *red hexagons*) onto the DNA. Another family of enzymes referred to as ten–eleven translocator (TET1 and TET2) are important for initiating the removal of methyl from the DNA, permitting the ability of genes to be transcribed. Another class of enzymes has been identified to increase or decrease the levels of acetyl molecules (indicated by *green arrows*) on histone proteins. Histone deacetylase (HDAC) and histone acetylase (HAT) enzymes are the primary effectors that remove or attach, respectively, acetyl to histones. **b** Recent work in the seasonally breeding Siberian hamster has found that *dnmt1* and *dnmt3b* enzyme expression in the hypothalamus, a key brain region implicated in the control of seasonal

neuropeptides, for example, gonadotropin-releasing hormone (GnRH; Stevenson et al. 2012b), neuropeptide VF precursor [NPVF; also known as gonadotropin inhibitory hormone (GnIH) Tsutsui et al. 2013], and KiSS metastasis suppressor (KISS; Revel et al. 2007). Annual or seasonal changes in these neuropeptides are well documented and provide a strong indicator of different seasonal reproductive states in a wide range of vertebrate species. Indeed, there are several other proteins that also exhibit changes over circannual timescales. Given that protein activity is the ultimate biological effector, a comprehensive understanding of genomic and proteomic plasticity will be important for future research in circannual rhythmicity at molecular and cellular levels.

Finally, there is growing evidence that circannual changes in histogenesis occur in many key brain and peripheral tissues (Hazlerigg and Lincoln 2011; see the chapter by Lincoln and Hazlerigg, this volume). Studies that have examined the neural distribution of bromodeoxyuridine (BrdU), a marker of newborn neurons, indicated that cortical, hypothalamic, and pituitary tissues are targets for neurogenesis. The origin of these new neurons appears to derive from stem cell niches located in the subventricular zone along the third ventricle (Hazlerigg and Lincoln 2011). Cyclical changes in neurogenesis are well established in seasonal species (Tramontin and Brenowitz 2000), however, the challenge is linking the variation in new cells with a functional outcome. Recent work that attempted to resolve this issue employed double-labeled histochemistry and revealed that approximately 10 % of BrdU cells coexpress CD-45, a pan-leukocytic marker (Hazlerigg et al. 2013). These data suggest that seasonal changes in neurogenesis may be involved in neuroimmune mechanisms. Given the large number of other BrdU cells expressed in the hypothalamus, it is likely that annual changes in histogenesis will have pleiotropic effects. Unraveling the relative contribution of cyclical histogenesis and epigenetic modifications for the generation of circannual rhythmicity will be one hurdle facing the next generation of circannual biologists. The next several years will undoubtedly provide exciting and innovative findings.

Conclusions

In the field of circannual rhythms, the decades invested in laying the foundations, by collecting hard-won whole-organism data over many years, may soon provide the basis for understanding the underlying mechanisms. Model systems are principally in place, and the major recent advancements in circannual biology are intriguing and highly innovative. The benefits of understanding seasonal processes are becoming evident for many applications, including ecology, climate change biology, health, and well-being. Given the timeliness, it should be feasible to master perhaps the most difficult of all hurdles: convincing funding agencies to support long-term research on circannual clocks.

For those daring enough to tackle the challenges and rewards in circannual research, they might find solace in an old advertisement posted by the great British explorer Sir Ernest Shackleton: “Men wanted for hazardous journey, small wages, bitter cold, long months of complete darkness, constant danger, safe return doubtful, honor and recognition in case of success.” For today’s explorers, men and women alike, the reward may lie in a much deeper understanding of the genuinely rhythmic life on earth. The wide taxonomic spread of circannual rhythms, down to the level of unicells, and the findings of their phase-specific interaction with photoperiod, suggest the possibility of a fundamental, endogenous basis to organismal seasonality. The appreciation of such a mechanism may have been obscured in the past by conceptual problems (Paul et al. 2008: “The rigid classification of timing mechanisms as either timers or clocks may be physiologically misleading despite its heuristic value”) and by particular photoperiodic experimental settings (Butler et al. 2010: “Distinctions between photoperiodic and circannual seasonal organization erode with the incorporation in the laboratory of ecologically relevant day length conditions”). Moving beyond these difficulties will clear our vision for the relevance of circannual rhythms. The circannual system drives seasonal transitions, provides temporal coordination of physiology, and, importantly, regulates a seasonally changing suite of flexible, species-specific responses to environmental input. The potential importance of this function of circannual rhythms was outlined in an unpublished note left to us by the late Eberhard Gwinner: “Idea: To study only the photoperiodic part of photoperiodism is like studying hormones without considering hormone receptors”.

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Chapter 12
Stem Cell Regulation of Circannual Rhythms

Gerald Lincoln and David Hazlerigg

Abstract Circannual rhythms depend on intrinsic timing mechanisms that operate over months and years. In this chapter, we briefly review the evidence that cyclical histogenesis, regulated by stem cells, underlies the generation of circannual rhythms. Three levels of circannual organization are considered: the concept of a "circannual clock shop" of multiple integrated timers; the concept of tissue-autonomous circannual oscillators dependent on cyclical regeneration; and, last, the remarkable possibility that circannual timing may actually be a cell-autonomous characteristic that first evolved in protists. We conjecture that all three mechanisms can contribute to the generation of the seasonal phenotype in complex organisms, different for each species, and that an evolutionally conserved, cell-autonomous mechanism may be expressed in stem cells to act as a pacemaker for circannual timing.

Keywords Canonical circannual genes • Clock shop • Endogenous clocks • Photoperiod • Seasonal rhythms • Stem cell niche • Tau

12.1 Introduction

The Earth's rotation about its axis and its orbit around the Sun give rise to two fundamental periodicities, daily and annual, to which life has evolved. Across taxa, this has led to the appearance of internal timing mechanisms, which permit physiological and behavioral changes to anticipate forthcoming changes in environmental conditions. That circadian clocks evolved to resonate with the daily periodicity generated by the Earth's rotation is now widely accepted, and much is now known about the structure and mechanism of circadian pacemakers (Reppert and Weaver 2002). Contrastingly, the genetic and cellular mechanisms underlying circannual clocks have received much less attention, and their fundamental nature remains enigmatic (Hazlerigg and Loudon 2008).

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The way the Earth's rotation and orbit have favored the evolution of endogenous circadian and circannual clocks is summarized in Fig. 12.1. In addition, the ability of the circadian system to measure daylength allows the precise synchronization of seasonal biology to the seasons. This synchronization permits organisms to optimize

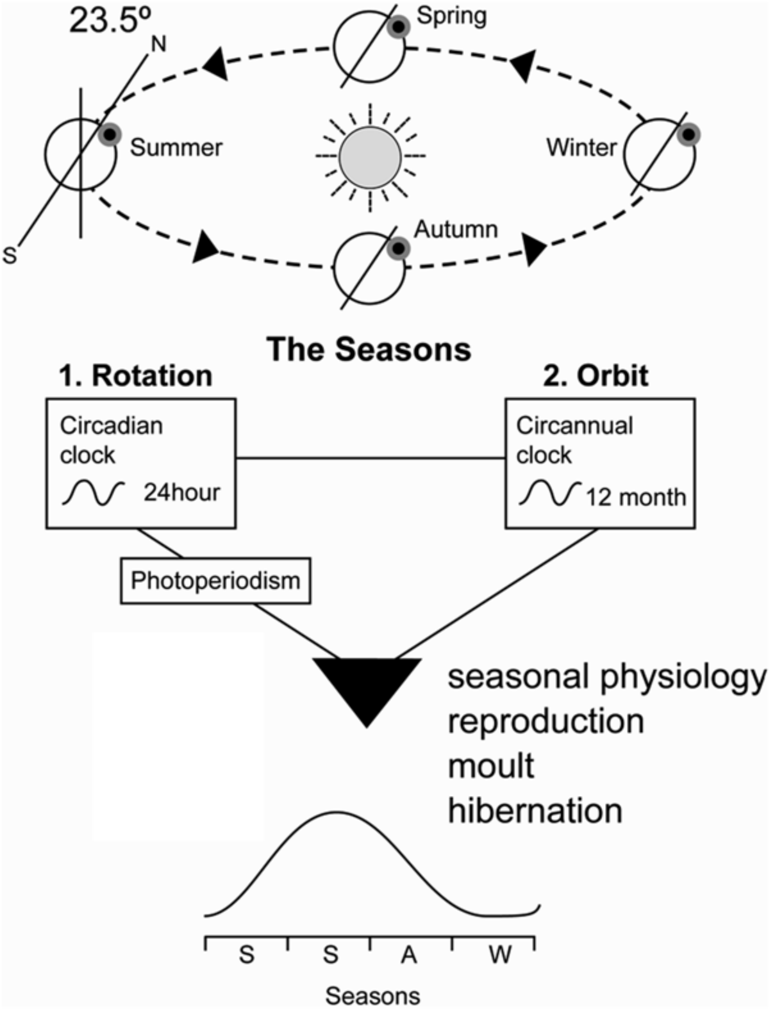

Fig. 12.1 Endogenous timers anticipate the Earth's periodicities. Schematic of the way the Earth's rotation on its axis every 24 h (*top panel*; line s/n across the earth indicates the rotational axis and the *filled circle* represent a UK northerly point of reference) and orbit around the sun every 12 months have favored the evolution of endogenous circadian clocks (based on about a dozen clock genes) and circannual clocks (based on stem cells and tissue regeneration: current chapter). The ability to respond to the annual cycle in daylength that varies with latitude (photoperiodic time measurement or photoperiodism) depends on the circadian system. Most long-lived organisms utilize such entrainment of a circannual pacemaker to precisely regulate long-term rhythms in reproduction, molt, hibernation, and other seasonal characteristics. (After Hazlerigg and Lincoln 2011)

their survival and reproduction, and is thus fixed by natural selection as an adaptive trait, accounting for the remarkable conservation of timing principles across taxa, from protists to vertebrates (Reppert and Weaver 2002; Bell-Pedersen et al. 2005).

Recently we have written two articles proposing that cyclical histogenesis is the basis of circannual rhythm generation (Lincoln and Hazlerigg 2010; Hazlerigg and Lincoln 2011). The long-time domains are the result of the protracted processes of cell differentiation, tissue remodeling, and feedback signaling from within and outside the tissue, as seen in developmental biology. These cyclical regeneration processes occur in multiple seasonally regulated tissues in the brain, pituitary gland, and peripheral tissues with variable phasing to produce each circannual rhythmic phenotype. In making this proposal, we were mindful of the cyclical regenerative biology seen in vertebrate hair follicles, testis tissue, blood-forming tissues, antlers, feathers, and pituitary gland. All these processes are dependent on stem cells that govern the period of the cycle and the morphology and phenotype of the regenerated cells and tissues.

Tissue-specific stem cell niches are viewed as reservoirs fueling cycles of tissue regeneration throughout the life of the organism. The property of circannual rhythmicity emerges from long-term feedback cycles that control stem cell reactivation after the phases of growth and quiescence and other potentially important innate timing processes. Because genes that regulate the development of an organ may also control cycles in the same tissue in the adult (e.g., hair follicle, Schneider et al. 2009; pituitary gland, Vankelecom 2009), it is evident that the adult's stem cells act to recapitulate events of ontogeny conferring cyclicity as a common feature of the adult phase of the life history sequence (Fig. 12.2). We propose that the seasonal transition is similar to a metamorphic event. It affects most tissues, radically changing physiology, but in contrast to the familiar metamorphosis of an amphibian, it is reversible and repeatable. The parallel role of thyroid hormone deiodinases in regulating seasonal transitions (Hanon et al. 2008) and in amphibian metamorphosis (Brown 2005) provides good evidence for the fundamental similarity of these processes.

In this chapter, we consider the basic question of where the long-time constants of circannual timing are generated. We summarize evidence that the adult stem cells residing in their tissue-specific stem cell niche may play a critically important role in circannual timing (Fig. 12.2). Three levels of circannual organisation are considered: (1) network, the concept of a "circannual clock shop" of multiple integrated timers (Hazlerigg and Lincoln 2011); (2) oscillator, the concept of tissue-autonomous circannual oscillators dependent on cyclical regeneration; and (3) basic, the remarkable possibility that circannual timing may actually be a cell-autonomous characteristic that first evolved in protists. It will be evident that there is a major overlap between categories 1 and 2 because a circannual clock shop depends on the presence of multiple circannual oscillators based on cyclical histogenesis, and these have tissue-autonomous characteristics (thus the overlap with level 2). However, we prefer to separate these categories because the special feature of a circannual clock shop is the network of histogenic sites hierarchically organized and networked together by hormonal mechanisms, whereas the key feature of tissue-autonomous control is the local regulation of long-term timing by stem cells potentially using autocrine/paracrine signaling.

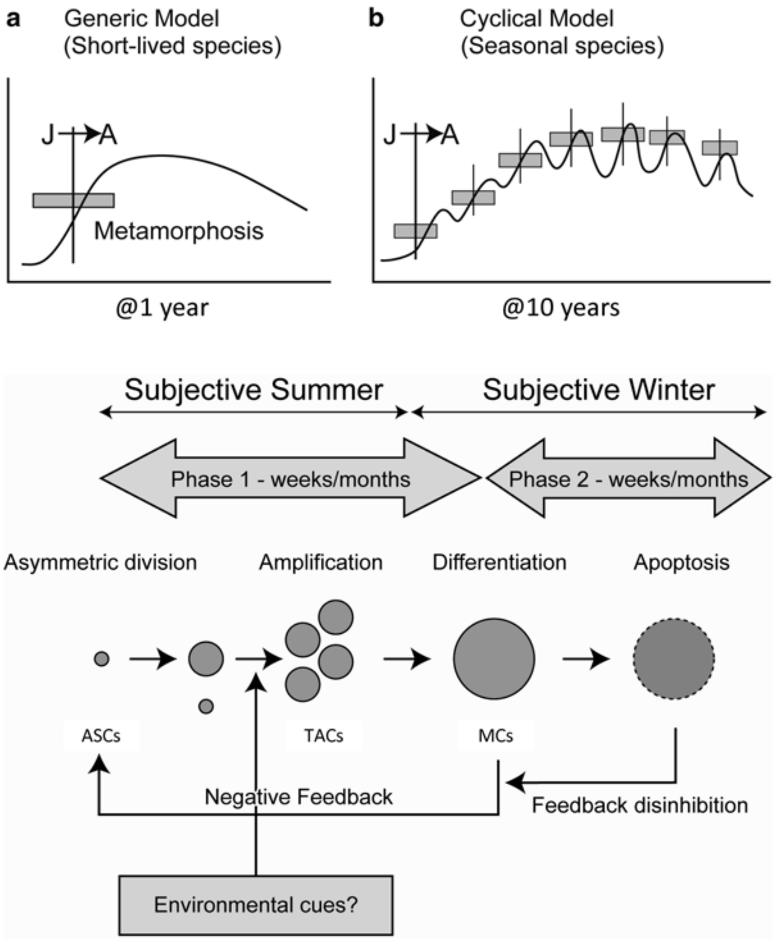

Fig. 12.2 Cyclical life histories. *Top panel*: Vertebrate life history models illustrating changes in body weight or testis size. (**a**) Generic model depicts a smooth change across the life history within a single juvenile–adult (J-A) transition (or metamorphosis). This model represents a short-lived species. (**b**) Cyclical model with a juvenile–adult transition (J-A) followed by repeated transitions throughout the life history each representing an endogenous circannual cycle: circannual cycles are an integral part of the life history program (Lincoln and Hazlerigg 2010). *Bottom panel*: Original generic model for circannual rhythm generation by cyclical histogenesis (Hazlerigg and Lincoln 2011). We proposed that circannual cycles oscillate between phases of subjective summer (*horizontal arrows*) with growth and maturation of tissues by histogenesis, and subjective winter (*dark bar*), during which tissues become quiescent. This is driven by pluripotent stem cells located in a tissue-specific stem cell niche. Stem cells undergo asymmetrical division as a rare event giving rise to populations of transient amplifying cells (TACs), which undergo repeated rounds of cell division to form a new cell population. Migration and differentiation then lead to the formation of new functional tissue, as well as providing feedback signals that arrest cell division in the stem cell niche. Withdrawal of negative feedback, possibly related to apoptosis, initiates a new circannual cycle. *ASC* adult stem cell, *MC* mature cell

12.2 Circannual Timing Based on a "Clock Shop"

Previously we presented a model for circannual organization in metazoan animals based on a coordinated "clock shop" of circannual oscillators residing in many tissues. We proposed that the oscillators are based on cyclical histogenesis controlled by resident stem cells, with coordination of the multiple oscillators achieved by peripheral hormonal signals (Fig. 12.3). The model proposes a hierarchical organization with oscillators in the hypothalamus and pituitary acting as primary pacemakers that respond to photoperiod and other environmental cues to ensure

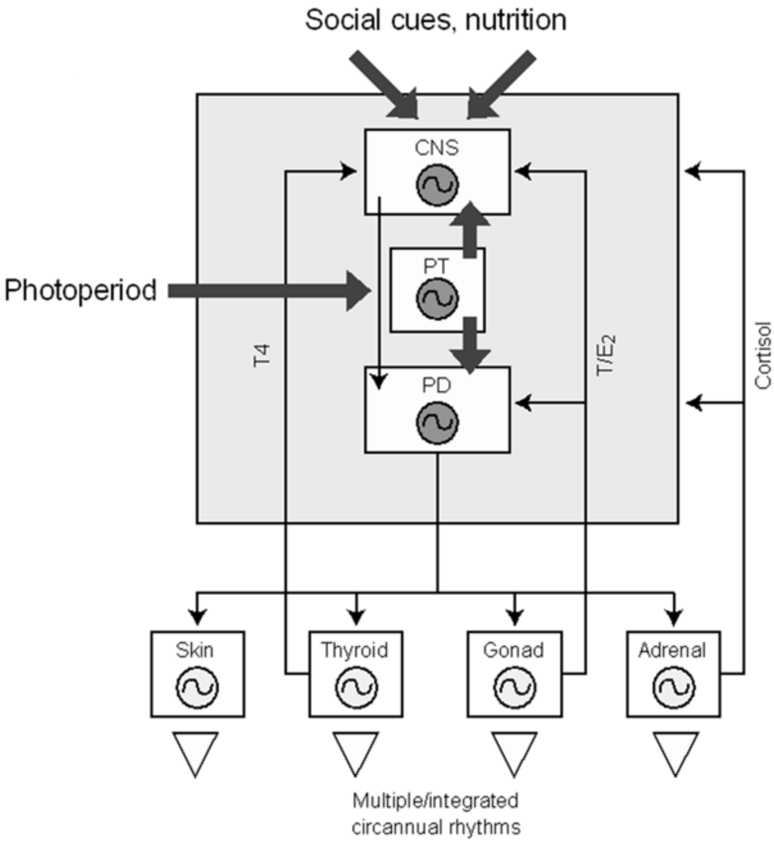

Fig. 12.3 Circannual clock shop. Organism-level circannual coordination is seen as the product of circannual cycles in multiple tissues (Hazlerigg and Lincoln 2011). The analogy to circadian organization can be drawn (Herzog and Tosini 2001). Multiple coordinating signals between pacemaker structures in the hypothalamus, pituitary, and peripheral organs including thyroxine (T_4), glucocorticoids (cortisol), and gonadal steroids (testosterone, *T*; estradiol, E_2) provide for feedback control. Circannual rhythms may be entrained by photoperiod acting at the level of the pars tuberalis (*PT*), as in mammals, or by other cues (social, nutritional, etc.) acting via the central nervous system (*CNS*). *PD* pars distalis, *circle/oscillator symbol* histogenic cycle

seasonal entrainment. Parallels can be drawn with the circadian system where the suprachiasmatic nucleus (SCN) acts as a central pacemaker for peripheral clocks, and also acts to relay effects of daylight for entrainment to the daily light–dark cycle.

Several lines of evidence suggest that multiple circannual rhythms can run in parallel in an organism, even in the absence of changes in feedback signals or effects of photoperiod. Sheep pinealectomized to remove melatonin as a mediator of photoperiodic input, castrated, and given constant-release implants of estradiol, still express circannual rhythms of gonadotropin secretion (Woodfill et al. 1994). These rhythms can be synchronized by seasonally appropriate programmed daily infusions of melatonin, but continue asynchronously in the absence of melatonin. Gonadectomized ground squirrels also express robust circannual rhythms, whose characteristics are modulated by gonadal steroid implants (Hiebert et al. 1998). In circannual rodent species there is also evidence that ablating major endocrine feedback pathways by thyroidectomy or castration fails to interfere with innate rhythms in hibernation, feed intake, and fat accumulation (Henderson and Demeneix 1981). Moreover, in hypothalamo-pituitary-disconnected (HPD) Soay rams, the surgical destruction of the descending hypothalamic control of the pituitary gland that blocks most seasonal rhythms (gonadal activity, food intake, body weight) fails to abolish circannual prolactin secretion (Lincoln and Clarke 1994; Lincoln et al. 2006). Pharmacological suppression of prolactin secretion in this model does not affect the phase of the circannual prolactin cycle (Lincoln et al. 2003), indicating that circannual rhythm generation is tissue localizable and independent of the output response.

Experimental manipulation of hormones known to control cell proliferation, differentiation, and organogenesis also has profound effects on the expression of seasonal rhythms, indicating the key role of histogenesis. Altering thyroid hormone (TH) status is particularly disruptive. Thyroidectomy blocks seasonal breeding cycles in birds and mammals (Follett and Potts 1990; Moenter et al. 1991; Webster et al. 1991; Nakao et al. 2008). In sheep, these effects represent a temporally specific and anatomically localized action of TH on the generation of the circannual reproductive rhythm. Detailed studies using thyroxine (T_4) replacement in thyroidectomized ewes have revealed a window of sensitivity to T_4, which opens in the spring and closes some 6 months later, as part of the circannual cycle (Thrun et al. 1997; Billings et al. 2002). Strikingly, micro-implants of T_4 placed in the histogenic regions of the mediobasal hypothalamus are sufficient to mimic the effects of systemic T_4 replacement (Anderson et al. 2003). Furthermore, central treatments with biologically active TH (tri-iodothryonine, T_3) promote a permanent summer breeding phenotype in quail and Siberian hamster (Follett and Potts 1990; Barrett et al. 2007).

Most recently, an endogenous control pathway regulating seasonal transitions has been characterized in the hypothalamus of mammals and birds (Hanon et al. 2008; Nakao et al. 2008; Ono et al. 2008). This pathway depends on the regulation of the expression of two enzymes, DIO2 and DIO3, which convert thyroid hormones between active and inactive forms, located within tanycytes in the ependymal cell layer lining the third ventricle in the mediobasal hypothalamus. Increased Dio2 gene expression (thus activating thyroid hormone) is seen following exposure to long days, with the converse sequence under short days, and spontaneous reversion occurs under prolonged photoperiod, indicating an innate timing mechanism.

Photoperiodic control of Dio3 (which inactivates thyroid hormone) has been less widely reported, but Dio3 expression increases markedly under a short photoperiod in the Siberian hamster, suggesting a key role in driving the summer-to-winter transition in physiology (Barrett et al. 2007). Studies in sheep, mice, and birds also demonstrate that it is thyroid-stimulating hormone (TSH) produced in the pars tuberalis (PT) of the pituitary stalk that acts locally in the adjacent hypothalamus to regulate Dio2 expression in the ependymal tanycytes. Thus, both inductive and entraining effects of photoperiod on seasonal physiology are regulated at the level of the PT through the control of thyroid hormone-dependent mechanisms in the brain (Dardente et al. 2010). These data are all consistent with a role for TH in supporting cyclical histogenesis in the hypothalamus as part of the long-term timing mechanism; tanycytes are the candidate stem cells for this oscillator (Lee et al. 2012).

The adrenal glucocorticoids also have far-reaching effects on histogenesis. Recently, we have investigated the impact of the glucocorticoid axis on circannual prolactin secretion in the HPD Soay sheep model by giving the long-acting ACTH secretagogue, synacthen-D (Syn-D), to chronically activate the adrenal axis (Hazlerigg and Lincoln 2011). The effect on the circannual prolactin rhythm was phase dependent, detectable immediately if the drug was given during the "subjective spring" phase of the circannual cycle when prolactin levels were increasing, but not detectable until some 3 months later (well after the end of adrenal activation) if Syn-D treatment was delayed until the peak of the prolactin cycle. Full recovery from the later Syn-D treatment was not seen until after 1 to 2 years.

Our interpretation of these results is that SynD causes a glucocorticoid-dependent suppression of cell division within the anterior pituitary (evidenced by the HPD procedure), possibly through induction of cell differentiation, and that the effect depends on the phase of the long-term cycle in pituitary histogenesis. Treatment in the proliferative phase (subjective spring) immediately delays the circannual cycle, whereas treatment later leads to markedly delayed responses. Again, this is strong evidence that histogenesis underlies the generation of circannual rhythms and that the major peripheral hormones act to coordinate the cycles in different physiological systems signals (Fig. 12.3).

12.3 Tissue-Autonomous Circannual Oscillators

Under this second scenario, circannual cycles are generated by a tissue-autonomous mechanism for each different physiological system rather than via a more global network as in a "clock shop." Adult stem cells residing in their tissue-specific niche are regulated locally by feed-forward and feedback signals from the progeny tissues, to produce a histogenic cycle of renewal. One or more such cycles may be required to generate the circannual rhythm. In developing this concept we have used the hair follicle as the prototype wherein the period of the tissue cycle, and the morphology of the new tissues, is regulated by a local population of stem cells. Other cyclical tissues including testis, blood-forming tissue, antler, and pituitary gland are also described.

12.3.1 Hair Follicle

The mammalian hair follicle acts as a semiautonomous miniorgan, capable of iterated cycles of growth and regeneration with timescales of months to years. The intrinsic cellular and molecular control of the hair follicle cycle is well understood (Schneider et al. 2009). The cycle is initiated by stem cell proliferation in the bulge region of the follicle (Fig. 12.4a). Progeny cells migrate to the dermal papilla (DP) and activate regrowth of a complete new hair fiber (anagen phase) and shedding of the old. During the cycle, paracrine signals are generated that exert autonomous feedback control as well acting on neighboring follicles, leading to synchronous cycles of hair growth (Plikus et al. 2008). The wnt/β-catenin signaling pathway, coupled with low levels of bone morphogenic proteins (BMPs), promote anagen whereas rising BMP levels cause growth arrest (catagen) and transition to the quiescent telogen phase. Return to anagen requires diminution of BMP levels, and so the hair follicle cycle can be thought of as a limit-cycle oscillation between the opposing forces of wnt/β-catenin and BMP signaling (Plikus et al. 2008). The stem cells of the individual follicles and tissue paracrine signaling govern both the follicle periodicity and the waves of synchronized hair growth within the skin (Plikus et al. 2008). The autonomy of the follicular cycles is demonstrated by their persistence in in vitro culture systems (Philpott and Kealey 2000).

In seasonal species, the hair follicle can produce more than one hair phenotype, generating distinctive summer and winter coats (Dicks et al. 1994). These phenotypes emerge from follicle growth cycles with different periodicities, leading to different fiber characteristics (Fig. 12.3b). Short cycles typically produce short summer hair fibers, and long cycles produce winter coats, which persist because all follicles enter an extended period of telogen over winter. The annual pelage cycle is thus the composite of more than one tissue cycle in the individual follicle,

Fig. 12.4 (continued) (Schneider et al. 2009). Once the growth of the new hair is complete, the germinal epithelial cells at the base of the hair shaft regress isolating the dermal papilla (DP) to produce the mature phase (catagen) and then the quiescent phase (telogen). The total period of the hair follicle cycle is 2 months to less than 2 years, and the relative time in anagen, catagen, and telogen varies markedly among species and between skin regions and seasons within species. (**b**) Model for seasonal changes in the hair follicle cycle in the sheep or red deer. Hair follicles (illustrated as *horizontal bars 1–8*) show limited local synchronization and cycle repeatedly during the summer; high prolactin levels are permissive to this state. Declining prolactin in the autumn synchronizes follicles throughout the pelage and leads to an extended anagen (*A*), producing a long winter coat. Follicles then remain quiescent in telogen (*T*) during the winter. Synchronous reactivation of the follicles produces the conspicuous spring body molt. The overall circannual period is a composite of multiple histogenic cycles in the hair follicle. (**c**) Experiment in which female red deer were given a small osmotic minipump implanted under the skin of the flank delivering prolactin for 28 days. Treatment in spring induced the local activation of anagen and the development of a skin patch with red summer coat (*S*), contrasting with the longer grey winter coat on the rest of the body. This skin patch developed a winter coat (*W*) some 5 months later, long after the end of prolactin treatment, demonstrating tissue-autonomous control of the cycling between the two pelage phenotypes. (After Loudon and Jabbour 1994)

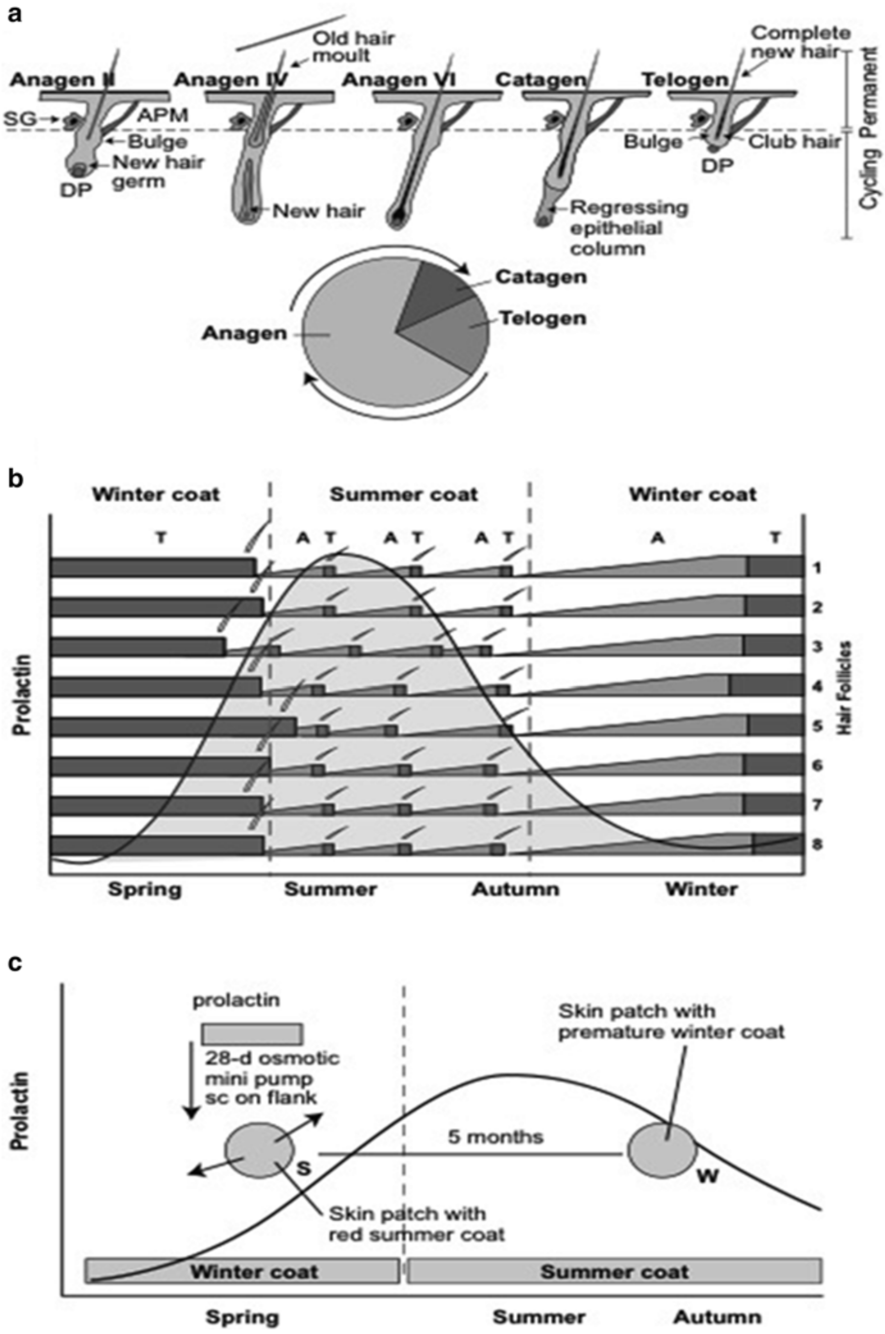

Fig. 12.4 Hair follicle as a tissue-autonomous rhythm generator governed by stem cells. (**a**) The growth phase of the follicular cycle (anagen) is initiated by stem cell activation in the bulge region at the base of the hair as a result of changes in the microenvironment of the bulge niche (*arrow*)

alternating between summer and winter programs. The transition between these summer and winter phases of follicle cycling can be synchronized by the hormone prolactin, in which secretion rises dramatically in spring, causing the conspicuous spring molt, and declines in the autumn, allowing growth of the winter coat (Dicks et al. 1994). Prolactin appears to trigger hair follicle reactivation and the seasonal molt by acting to curtail telogen and promoting a return to anagen across all hair follicles.

Importantly, the seasonal alternation of hair phenotype appears to be, in part, autonomously regulated within the skin, as has been elegantly demonstrated in red deer by the short-term administration of prolactin in the skin using osmotic minipumps to locally alter the hair follicle cycle (Loudon and Jabbour 1994; Fig. 12.4c). The treatment produced localized, premature growth of the red summer coat in winter animals with grey coats. The affected skin patch then remained out of phase with the remainder of the body's molt pattern for at least 5 months, long after the local hormonal manipulation, and in late summer prematurely developed a grey winter phenotype on animals with a natural red summer coat. This observation indicates that the skin tissue has intrinsic control of both hair follicle cycle and the switch between summer and winter phenotypes, striking evidence for a tissue-autonomous circannual clock. We are not aware of studies of these seasonal rhythms using in vitro organ culture: this is a major technical challenge because of the very long time frame required for such experiments.

In birds, the semiautonomous control of feather follicles, based upon intrinsic feedback cycles and communication between neighboring follicles in skin patches, accounts for the complexity in plumage (Mou et al. 2011). Seasonal control of plumage change appears to depend on the capacity of feather follicles for independent cyclicity, with wing flight feather and body feather molts often running asynchronously. In the migratory great knot, phase separation of these molts persists under constant photoperiod, reflecting intrinsic circannual control (Piersma et al. 2008).

12.3.2 Testis Tissue

One of the best examples of stem cell genetics controlling tissue cyclicity can be found in the mammalian testis. Xenotransplantation of spermatogonia (male germ stem cells) between laboratory strains of rats or mice have demonstrated that the period of the spermatogenic cycle (time taken from the differentiation division of spermatogonia until full maturation of spermatozoa within the seminiferous tubule) is regulated by the spermatogonia and not by the supporting somatic tissue of the host testis (Brinster and Zimmermann 1987). The full spermatogenic cycle involves multiple mitotic divisions of primary spermatocytes in the basal compartment of the tubules, differentiation of secondary spermatocytes through a meiotic reduction division, and final differentiation of spermatids into spermatozoa in the center of the tubules. The whole sequence takes 45–70 days, with the duration being species specific. These cellular events are tightly synchronized and locally coordinated into defined cell associations in the seminiferous tubule, which allows division of tubules into defined stages and detailed quantitative analysis of the histogenic process (Hochereau de Reviers et al. 1985).

Seasonally breeding species express robust cycles in testis size and function with marked variation in the degree of involution during the nonsexual phase, and the pattern is circannually regulated under constant conditions (Lincoln 1989). This seasonal gonadal rhythm is primarily regulated through changes in gonadotropin secretion, governed by the circannual timer mechanisms of the hypothalamus (see Sect. 12.2). Interestingly, seasonal involution of the testis results from reduced efficiency in progeny cell production and survival caused by the action of reduced gonadotropins on the somatic cells of the testis (Sertoli and Leydig cells), with no fundamental change in the period of the spermatogenic cycle (Courot et al. 1979; Hochereau de Reviers and Lincoln 1978; Hochereau de Reviers et al. 1985). This finding indicates that the stem spermatogonia determine the period of the tissue cycle, despite major changes in any feedback signaling from the progeny cell population. There is also preliminary evidence that autonomous control of the long-term cycle in the efficiency of spermatogenesis is in part locally regulated. Thus, there can be a mismatch between the seasonal regression of the testis and the decline in gonadotropin secretion, variation in the efficiency of spermatogenesis in different parts of the testis, especially during the nonsexual phase, and a dissociation between cycles in the spermatogenic and androgenic functions of the testis in some seasonal species (Lincoln 1989). The overall story for the testis nicely illustrates autonomous control of tissue cyclicity, along with a global regulation by central circannual pacemakers that allows for the optimal timing of the different aspects of the seasonal sexual cycle.

12.3.3 Hematopoietic Tissues

Another compelling case of long-term cycles with stem cell origins comes from a family of hematological diseases (leukemias) in which long-term cyclical variations in the titers of different blood cell types are observed (Haurie et al. 1998). Periodic oscillations in numbers of granulocytes with period lengths of up to 100 days have been recorded in patients with periodic myelogenous leukemia, and of pancytopenia in which titers of all blood cell types are affected. Importantly for our concept of tissue autonomy, these diseases originate from abnormal bone stem cell function in lineages high in the hematopoietic cascade, thus producing life-threatening effects with periodic anemia, blood-clotting defects, and immunodeficiency. These effects are caused by specific gene defects that have been characterized by genetic analysis in human families with the rare disease; as well as in a breed of greyhound dogs that has been used as an animal model (Haurie et al. 1998). Experimental and modeling evidence indicate that the period length of these blood cell oscillations depends on the properties of bone marrow stem cells, progeny cells, and local signaling between them, rather than feedback cycles involving systemic hormones (Haurie et al. 1998). We are unaware of any corresponding studies of the blood cell histogenesis in seasonal species to assess possible regulation on a circannual time scale.

12.3.4 Antler Tissue

The deer antler is a complete organ composed of multiple tissues that is regenerated annually, or circannually, under specific constant photoperiod regimens (Goss 1977, 1983, 1984). Antler regeneration is a wound-healing response within the permanent antler pedicles on the frontal bones of the skull that can only be triggered during the "summer phase" of the antler cycle (Lincoln 1984), similar to the seasonal window for limb regeneration in newts and other cold-blooded vertebrates in which there is intrinsic seasonal variation in the sensitivity of the wound site to neurotrophic signals (Singer 1974). The periodic antler growth persists in castrated deer where the dominant effect of sex hormones is removed (Goss 1983; Lincoln 1984). Moreover, double antler cycles with a large antler grown in summer and a small one in winter can occur notably in Pere David's deer where the seasonal reproductive cycle is phase advanced relative to the permissive window compared to the closely related red deer (Pocock 1923; Loudon and Brinklow 1992; Lincoln 1992). In male reindeer, the casting of the hard antlers occurs soon after the rut in winter, well in advance of the next cycle of antler regeneration, whereas in many deer species the initiation of antler growth in spring precedes casting of the old antlers (Goss 1983; Lincoln 1992). Thus, the mechanical events of antler casting (wound formation) are not an essential trigger for the start of next antler cycle. These observations are consistent with the idea of dual control of the antler: one mechanism governs the autonomous regulation of antler growth and maturation, and by the other the seasonal sex hormone cycle, itself governed by a brain pacemaker, "gates" the antler cycle to a particular phase (see Sect. 12.2).

Recently, the stem cells of the pedicle periosteum that regenerate the antler have been characterized in more detail using immunocytochemistry and tissue transplantation (Li et al. 2009; Li 2012) and the stem cells have been isolated (Seo et al. 2013). These cells express CD9 antigen, Oct4, and Nanog, and elevated levels of telomerase enzyme activity and nucleostemin, all biochemical markers of pluripotent stem cells (Li et al. 2009). In culture these cells can be triggered to differentiate into cartilage, bone, and fat cell lineages. The inner tissues of the antler (cartilage and bone) are derived from the pedicle periosteum stem cells, whereas the outer tissues (skin, nerves, and blood vessels) are derived from pedicle skin under the inductive influence of unknown factors released by the pedicle periosteum (Li 2012). Interestingly, numerous hair follicles form de novo in the antler velvet-like skin and express repeated hair follicle cycles and waves of synchronous molting as seen in normal skin (Lincoln 1984). Antlers developed in summer (e.g., red deer) produce short velvet hairs, whereas antlers developed in winter (e.g., roe deer) produce long velvet hair that may reduce heat loss and protect the growing tissue. Based on the situation in normal skin, the prediction is that these features of the new antler hair are endogenously controlled both within the antler and by systemic seasonal hormones such as prolactin.

12.3.5 Pituitary Gland

The pituitary gland is one of the putative central circannual pacemakers (Hazlerigg and Lincoln 2011; see Sect. 12.2). Evidence that it may act as a tissue-autonomous pacemaker comes from detailed studies using the hypothalamo-pituitary-disconnected (HPD) Soay sheep model (Lincoln et al. 2006). The HPD operation permanently removes the neuroendocrine control of the pituitary but maintains its blood supply and tissue viability. This procedure should not be confused with hypophysectomy, which totally destroys the pituitary; HPD sheep still maintain some functional activities of the secretory cells that produce the classical anterior pituitary hormones, although no longer controlled by the brain.

Remarkably, HPD sheep express photoperiod-induced cycles in prolactin secretion similar to sham-operated controls, and the cyclicity free runs as a circannual rhythm under constant long days (Lincoln et al. 2006). Under such conditions the pattern persists for at least four cycles with a progressive desynchrony between adjacent animals, consistent with endogenous circannual control. The free-running period for the HPD sheep prolactin rhythm is close to 10 months and is significantly different from the Earth's 12-month periodicity, a temporal feature common to circannual rhythm generation in many different organisms (Gwinner 1986). Because the HPD surgery isolates the pituitary gland from the brain and removes most cyclical influences from other physiological systems (reproduction, food intake, body weight) (Lincoln et al. 2003), we infer that the pituitary gland itself acts as a circannual rhythm generator.

The marginal zone (MZ) of the pituitary cleft region in the anterior pituitary is the most likely site for a stem cell niche and cyclical histogenesis (Hazlerigg and Lincoln 2011). In the MZ there are polarized epithelial cells lining the cleft (remnant of the embryonic Rathke's pouch) that have the characteristics of pluripotent stem cells; these give rise to transient amplifying cells (TACs) that differentiate into new pituitary cells of different cell lineages (Vankelecom 2009). The prediction is that the "summer phase" of the circannual prolactin cycle is the time of proliferation of lactotrophs governed by the MZ stem cells. A second possible site for autonomous rhythm generation is the stalk region of the pituitary gland (PT, pars tuberalis) (Fig. 12.2); this is the location where the melatonin signal that relays the effects of photoperiod is transduced through a circadian clock gene-based mechanism (Dardente et al. 2010). Bromodeoxyuridine labeling experiments in sheep demonstrate that histogenesis in the PT is seasonally regulated (Migaud et al. 2010; Hazlerigg and Lincoln 2011; Hazlerigg et al. 2013). Thus, the PT may be a key convergence point between the photoperiodic input pathway and localized circannual rhythm generation.

12.4 Cell-Autonomous Circannual Time Keeping

If circannual oscillations are generated tissue autonomously, then how simple a level of cellular organization is required? Among higher plants, circannual rhythms of water uptake have been reported in bean seeds (Spruyt et al. 1987), and there are several reports of circannual rhythms in the transition between growth and resting phases in sea kelp (Dieck 1991; Schaffelke and Luning 1994) and in filamentous algae (Costa and Varela 1988; Costa and Lopez-Rodas 1991). For example, experimental cultures of *Spirogyra* maintained exponentially by serial transfer of vegetative cells, and held under constant light and temperature, exhibit progressive changes in phenotype (decrease in growth/cell division and increase in zygote formation) with a circannual pattern. There are parallel changes in cell morphology, notably affecting the spiraled chloroplast and loss of cell organization, features associated with cell aging. None of the plants survived without zygote formation (Costa and Lopez-Rodas 1991); thus, the circannual cycle requires the alternation between vegetative and reproductive phases, where both the aging of the photosynthetic cells and the hatching of the zygote are endogenously regulated.

Equally remarkable are the descriptions of circannual rhythms in a wide range of dinoflagellates, best studied in the genus *Alexandrium* (Andersen and Keafer 1987; Matrai et al. 2005). The life cycle of these complex unicellular organisms comprises asexual and sexual phases of propagation in the surface layers of the sea, as well as a diploid quiescent resting phase (hypnozygotes) during which cysts are formed and sink to the sediment layer on the seabed (Fig. 12.5). Reactivation of the cyst (hatching) is light- and temperature dependent, and in some strains is also subject to a strong circannual rhythm of sensitivity to these reactivating signals. There appears to be a strong ecological basis to whether a strain expresses circannual rhythmicity (Andersen and Keafer 1987). For strains in shallow coastal or estuarine waters, the seabed sediment layer is exposed to strong annual cycles of light and temperature, and emergence is environmentally driven, after an interval of 2–6 months. Hence, the encysted hypnozygote behaves as if it carries an hourglass-like interval timer mechanism to prevent winter emergence. By contrast, in strains found in deeper waters, where the seabed sediment layer is uniformly cool and dark, with only subtle temperature cycles, a robust, high-amplitude circannual rhythm of excystment potential is seen: the free-running period for this rhythm is close to 11 months, again less than the sidereal year.

The dinoflagellates provide the best evidence that a circannual rhythm can be generated within a single cell. In the dinoflagellate life history, it is the sexual diploid, encysted stage that expresses the rhythm, in some ways analogous to circannual

Fig. 12.5 (continued) no further temporal control, implying that an hourglass mechanism operates in the hypnozygote. Remarkably, in *A. tamarense* hypnozygotes collected from deep-water sediments, the temporal gate to excystment follows a circannual rhythm with a period length of approximately 11 months. (For further details, see Andersen and Keafer 1987 and Matrai et al. 2005

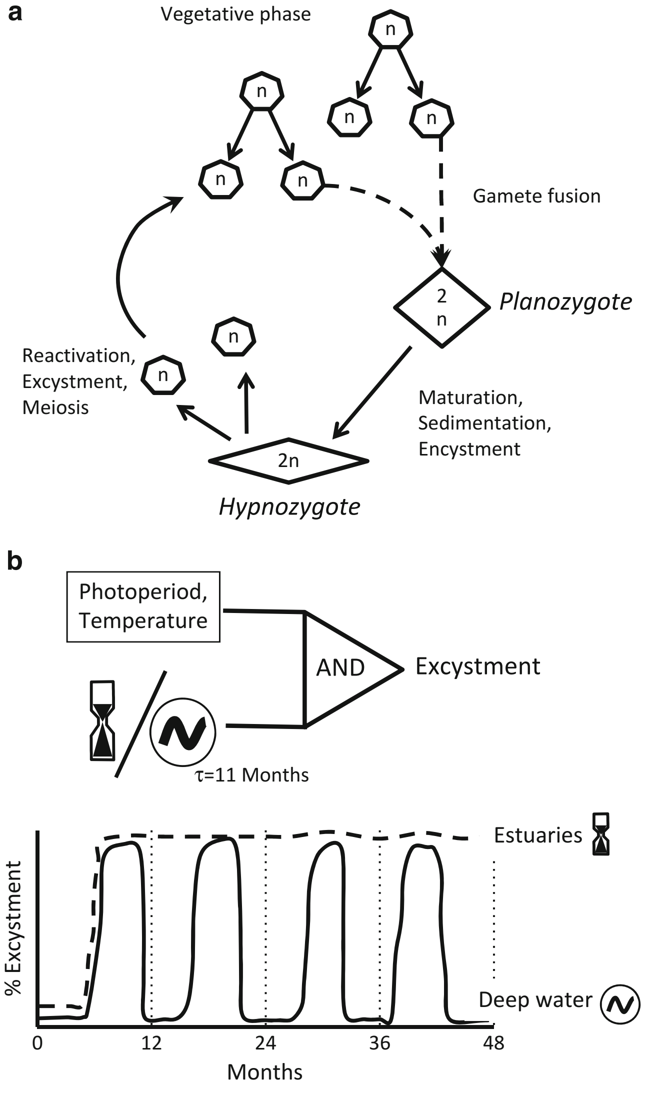

Fig. 12.5 Cell-autonomous circannual rhythms in dinoflagellates. (**a**) The simplified life cycle. Under favorable, typically summer, conditions, haploid ($n=1$) cells propagate vegetatively and are found in the sea surface water layers. A resting phase develops after fusion of two haploid cells, typically in the autumn months; this generates a diploid ($n=2$) "planozygote" that then develops into an encysted "hypnozygote" characterized by accumulation of storage vesicles, a thickened cell wall, and by increased cell density, which causes cysts to sink to the seabed. Reactivation in subsequent year(s) involves meiotic cell division and emergence of new vegetative cells from the cyst, a process known as excystment. (**b**) Long-term timing and circannual rhythms in the hypnozygote. The length of time spent in the hyponozygote phase is controlled by interactions between internal timing mechanisms and reactivating signals, particularly temperature and photoperiod. In *Alexandrium tamarense* strains living in shallow coastal waters and estuaries, excystment will not occur until a certain minimum length of time (2–6 months) has elapsed, but thereafter shows

rhythms in seeds in higher plants. The oscillation occurs without cell division or any overt changes in morphology in a seemingly quiescent encysted cell. It is clearly under genetic control because closely related strains of the same species vary in whether they do, or do not, express the circannual rhythm in cell responsiveness. Ticking in the cyst is a genetic timer. This raises the important question: could the rhythm expressed in single-celled organisms represent the very basic circannual clock that evolved at an early stage of eukaryotic evolution and is the mechanism conserved as a fundamental timer in more complex organisms?

Conclusions

In 1986, Ebo Gwinner published his seminal book on circannual rhythms (Gwinner 1986). He concludes: "Perhaps the most formidable physiological problem in circannual rhythm research arises from the extremely long duration of the processes involved. Some of the changes that occur within or between the various fractions of a circannual cycle have time constants that are way beyond the range of time constants known for any neuroendocrine feedback loop and are in many respects reminiscent of developmental processes occurring during ontogeny."

Decades later we can begin to confirm his vision. Circannual rhythm generation in birds and mammals depends upon reiterated cycles of histogenesis regulated as part of the life history program. Pluripotent, stem cells are set aside during development in defined stem cell niches, different for each tissue, and involve tissue repair and cycle generation over time intervals of months and years, thus accounting for the long-time domains for circannual timing.

The three levels of temporal organization summarized here ("clock-shop," tissue-autonomous, cell-autonomous) may all contribute to the regulation of circannual rhythmicity in complex organisms. It seems logical that the circannual timing mechanisms seen in single-celled organisms evolved first and are fundamental: they may provide the genetic program within the stem cell that regulates rhythmic activity over long time intervals. Tissue-autonomous timing mechanisms, wherein paracrine and autocrine feed-forward and feedback signaling between the stem cell and the progeny cells generate cyclicity, would be the next stage of complexity. And, finally, the "clock shop," with a hierarchy of oscillators from central to peripheral, is the best representation of the complexity of vertebrates.

The new concept that we wish to promote is that the circannual rhythmicity forms part of the life history program and is different for each organism depending on which life history phase requires to be timed predictively. This need may involve periods during the *adult* phase, as in most vertebrates, and affect different aspects of the phenotype (body weight, reproduction, pelage, migration, and hibernation behavior), each potentially timed independently through separate tissue oscillators. Alternatively, circannual rhythmicity may affect just *one part of the life cycle* as in insects (pupation) and dinoflagellates (hatch of the diploid cyst). Here intrinsic control is clearly adaptive because the organism is isolated from the environment.

In addition, circannual rhythmicity may operate *across generations* where the phase of the rhythm is transferred stepwise between generations as in short-lived organisms that alternate between asexual and sexual phenotypes.

Now, the number one challenge is to identify the canonical circannual genes that provide the core circannual timer. These genes are likely to be closely linked to the control of aging because circannual rhythms span long time periods as part of the cyclical life history program. They may also be part of the mechanism that governs cellular energy homeostasis and metabolism because long-term viability depends upon energy stores: thus, a long-term clock must register energy status to be adaptive.

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Chapter 13
Seasonality of Life Histories in Tropical Birds: Circannual Rhythms and *Zeitgeber*

Wolfgang Goymann and Barbara Helm

Abstract In his seminal paper about potential circannual rhythms, Aschoff (*Stud Gen* 8:742–775, 1955) predicted their presence in species that live in relatively constant, tropical environments. Detailed follow-up investigations largely supported this idea, but life-history stages (such as breeding, molt, migration) of wild tropical species can be rhythmic or arrhythmic. We present some classical findings on circannual biology of tropical species and provide ecological and evolutionary context by reviewing the diverse patterns of seasonality in tropical environments. We relate this to the seasonality of life histories in tropical birds and discuss the potential *Zeitgeber* of circannual rhythms, which are still elusive. We emphasize the diversity of tropical environments, roughly covered by the biome concept, which range from extremely erratic and unpredictable to very predictable environments. Predictable environments, which arguably favor evolution of circannual rhythms, are characterized either by a rather constant climate throughout the year or by a pronounced and regular climatic seasonality. Colwells' (*Ecology* 55:1148–1153, 1974) concept of predictability, constancy, and contingency allows a quantification of such diversity of tropical biomes and forms the basis of our discussion of the seasonality of life histories in tropical birds.

Keywords Endogenous rhythm • Environmental cue • Life history • Predictability • Tropical biomes

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13.1 Introduction

From early on, researchers have observed that many tropical species maintained an annual cycle under conditions that appear to be far less seasonal than those at higher latitudes. These observations had led to speculations as to whether tropical animals possessed timers and which aspects of the environment may provide timing cues. In this context, Aschoff (1955) proposed the systematic study of circannual rhythms as the possible basis of such timers. To explain why such rhythms would not be free running in nature, he implemented the concept of *Zeitgeber*[1] (i.e., synchronizing cues) in analogy to circadian biology (see also Immelmann 1972). Accordingly, environmental stimuli act as synchronizing *Zeitgeber* influencing the timing of these internal processes rather than directly inducing or inhibiting internal processes (see also Gwinner 2005 for discussion). Subsequent studies of birds, mammals, insects, and plants demonstrated unambiguously that physiological processes, in the absence of *Zeitgeber*, do show circannual rhythmicity. Furthermore, experimental entrainment to *Zeitgeber* has been successful in most of these species (but see Heideman and Bronson 1994; Chap. 15). For species living at higher latitudes, the most effective *Zeitgeber* is the annual change in daylength (photoperiod). Studies manipulating photoperiod also demonstrated that circannual rhythms generally entrain well to *Zeitgeber* stimuli, but only within certain limits of entrainment, which fits very well with the general oscillator idea (e.g. Daan 1987) elaborated for circadian rhythms. However, near the equator, photoperiod remains almost constant across the year, and the question which *Zeitgeber* entrain circannual rhythms is still not resolved.

As predicted in Aschoff's early review, birds from the tropics show particularly robust circannual rhythmicity. A classic example is the African stonechat (*Saxicola torquatus axillaris*), which has been extensively studied by Eberhard Gwinner and his coworkers. In this species, robust circannual rhythms in molt and reproductive traits continued for more than 10 years under constant 12.25:11.75 h (light:dark) conditions (Gwinner and Dittami 1990; Gwinner 2003; Fig. 13.1). Remarkably, rhythmicity also developed in birds that never experienced other photoperiods (Gwinner 1991, 2003). Hand-raised stonechats reproduced successfully under constant conditions (Gwinner et al. 1995), and their offspring, which had hatched under constant conditions, equally displayed circannual rhythms (Gwinner 1996; Fig. 13.2). A striking example for the circannual basis of annual cycles are blackcaps (*Sylvia atricapilla*) from Cape Verde (Fig. 13.3). In contrast to blackcaps in Europe, these birds breed twice a year during two distinct breeding seasons. When held under constant conditions, males from Cape Verde express two testicular cycles per circannual year (Berthold and Querner 1993) whereas; European blackcaps express a single cycle (Berthold et al. 1972). Moreover, the wide range of conditions under which circannual cycles can be expressed in some tropical species has been

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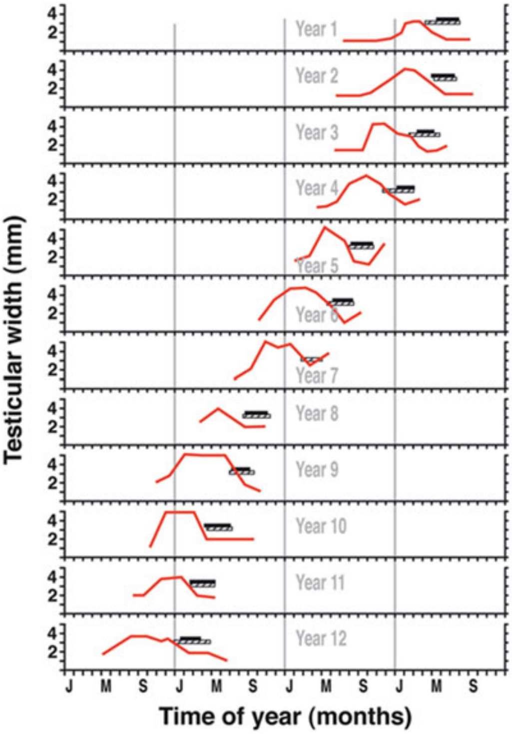
[1] Throughout this chapter, we use the term *Zeitgeber* in its correct German version, in which there is no distinction between singular and plural for this term; hence there can be one *Zeitgeber* or two or more *Zeitgeber*.

Fig. 13.1 Persistence of circannual rhythms. Circannual rhythm of testicular width (*red curves*) and molt (*black bars*, flight feather molt; *hatched bars*, body molt) in an individual African stonechat kept from fledging for more than 10 years in a constant equatorial photoperiod. Successive testicular and molt cycles are plotted underneath each other with the preceding 2 years plotted on the *left* in each case. (Reprinted from Gwinner 2005 with permission of Hokkaido University Press)



documented for the spotted munia (*Lonchura punctulata*), an Asian estrildid finch (Chandola-Saklani et al. 2004). In this species circannual cycles persisted under constant dim light as well as under annual cycles of 24 h of light alternating with 24 h of darkness (Budki et al. 2012). These and other studies (summarized in Gwinner and Dittami 1985 and Rani and Kumar 2013) suggested that tropical environments may indeed favor a robust circannual regulation of life-history stages.

However, next to such rhythmic behavior, life-history patterns of some other tropical birds also gave evidence for divergent mechanisms. According to these studies, some tropical birds prepare to breed whenever environmental conditions become suitable. This flexibility would allow them to take advantage of unpredictable, favorable conditions. For example, rainfall is the most likely cue that initiates gonadal growth in small ground finches (*Geospiza fuliginosa*) from the Galapagos (Hau et al. 2004). Clearly, there is no singular way in which tropical birds time their life cycles. Understanding the diversity of patterns is therefore an important step toward pinpointing the ecological and evolutionary contexts that shape a reliance on endogenous rhythms. The heterogeneity of patterns is presumably best explained by the diversity of biomes in tropical and subtropical regions and by the diversity in life history and ecology of tropical bird species. This extraordinary diversity makes tropical birds an instructive example for the "ecological end" of the study of circannual rhythms and their interaction with the environment.

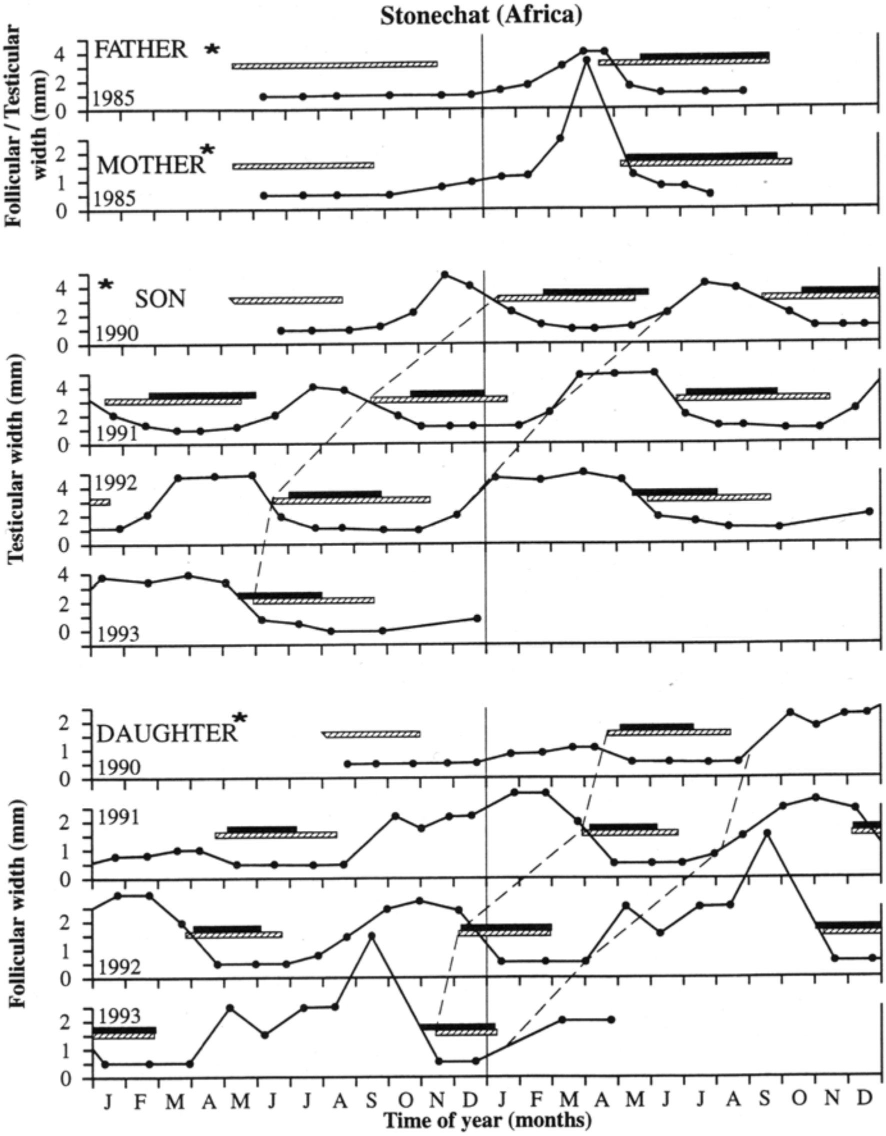


Fig. 13.2 Family patterns. Changes in testicular width, diameter of the largest ovarian follicle, and occurrence of molt (*hatched bars*, molt of body plumage; *black bars*, molt of flight feathers) of four African stonechats kept under a constant 12.25-h photoperiod. *Upper diagram* shows data from two parental birds (father, mother) that had hatched in April 1985 (*asterisks*) in Kenya. These birds were taken from their nests at 5 to 8 days of age and subsequently kept under experimental conditions until November 1986. *Lower two diagrams* show data from a son and a daughter of these parental birds that hatched in January (son) and April 1990 (daughter) from eggs incubated under constant conditions and held until the end of 1993 (son) and mid-1994 (daughter) under the same conditions. In the *lower two diagrams*, data from successive years are plotted from top to bottom with the preceding year replotted on the *left* in each case. For each bird, the *dashed line* on the *left* connects successive onsets of molt of body plumage, and the *dashed line* on the *right*, onsets of gonadal growth. *Onsets* of gonadal growth are defined as the dates at which testicular widths of more than 3 mm and follicular diameters of more than 1 mm were measured for the first time during successive gonadal cycles. (Reproduced from Gwinner 1996 with permission of John Wiley & Sons, Inc.)

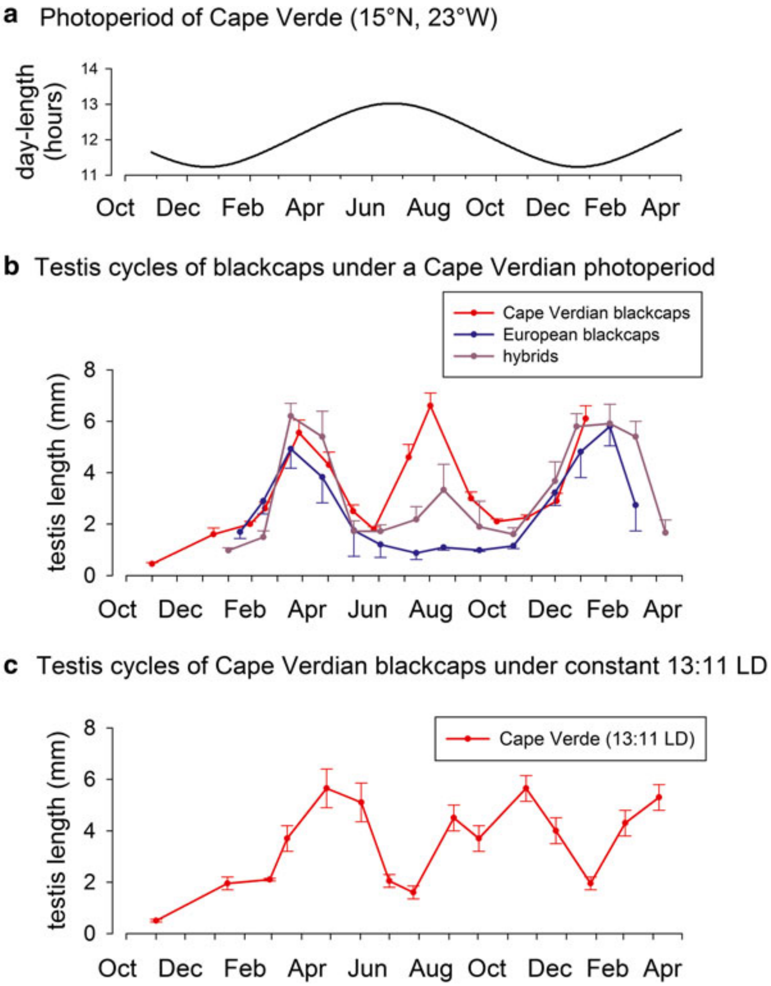


Fig. 13.3 Biannual testis cycles of blackcaps. (**a**) Photoperiod (number of daylight hours) on Cape Verde that has been used for the simulation in (**b. b**) Testicular cycles of 6 male blackcaps from Cape Verde (*red*), 10 male blackcaps of southern Germany (*blue*), and 11 hybrids (*purple*) of the two populations maintained under common garden conditions with a Cape Verdian photoperiod. (**c**) Testicular cycles of seven male Cape Verdian blackcaps maintained under constant conditions (13 h light:11 h dark). (Data from Berthold and Querner 1993)

For addressing the role of ecological context, it is useful to first clarify the ways in which annual cycles are aligned with environmental fluctuations. Following the seminal work by Rowan (1925, 1926) numerous studies have demonstrated that, at higher latitudes, the annual variation in daylength (photoperiod), is the major environmental cue for the timing of life-history stages in most species of birds (e.g. Dawson et al. 2001; Sharp 2005). Baker (1938a, b), distinguished between ultimate and proximate causes to time energetically demanding life-history stages such as breeding or molt, with the ultimate causes being directly related to their successful completion, that is, during periods of favorable weather and high food abundance. Proximate causes, on the other hand, are environmental predictors of such upcoming favorable conditions and have mechanistic functions of triggering or synchronizing events. Proximate causes are not necessarily directly related to fitness (i.e., breeding success or good molting conditions). For example, the photoperiod in temperate and arctic regions is the classical proximate cause, with an increasing photoperiod typically predicting favorable weather conditions and increasing food abundance, although daylength itself probably contributes little to breeding success (Baker 1938a). Other factors, such as temperature or food, have more direct links to fitness and therefore may combine proximate and ultimate aspects.

Proximate causes that do not directly influence fitness, such as photoperiod, are useful in environments with predictable seasonal conditions (see following). In contrast, marked photoperiodic cycles are absent in environments close to the equator, which nonetheless can show considerable seasonal fluctuations in climatic conditions. Based on these features, it has been suggested that tropical breeders rely on an endogenous circannual clock that allows advance preparation for ensuing life-history stages (Gwinner 1992). Long-distance migrants that spend the winter in the tropics face similar challenges, as they may need to rely on their circannual clock to time their journey back to the breeding grounds (for example, discussion in Wikelski et al. 2008). But which environmental cues birds use in the tropics as *Zeitgeber* stimuli to synchronize the circannual clock with the natural year *Zeitgeber*, in particular close to the equator, is still unclear.

In this chapter we present our perspective on the seasonality of life-history stages in tropical birds and discuss potential environmental *Zeitgeber* of circannual rhythms. We endorse an annual-cycle approach but, because of the availability of data, we place particular emphasis on breeding cycles. There are several excellent, more general reviews of avian circannual rhythms (e.g. Rani and Kumar 2013; Aschoff et al. 1980; Farner and Gwinner 1980; Gwinner 1986, 1990, 1996, 2003, 2005; Gwinner and Helm 2003; Helm et al. 2013; Newton 2008; Wikelski et al. 2008), to which readers are referred for more comprehensive coverage. In addition, there is an extraordinary popular science book on seasonal rhythmicity offering a very pleasurable introduction and overview of the field (Foster and Kreitzman 2009).

13.2 Internal Change: Annual and Circannual Life Cycles

Birds as a taxon are, relative to their size, long-lived and commonly undergo well-defined annual cycles of various life-history stages (Jacobs and Wingfield 2000; Wingfield 2008). Usually, in each year of its adult life a bird undergoes at least one bout of reproduction. Most commonly, these breeding events require reactivation (“recrudescence”) of the reproductive system in preparation for breeding and subsequent regression at the end of the breeding season (see Williams and Ames 2004 for an example of a very rapid regression). Reproductive activation proceeds via a cascade of endocrine events, among others via release of hypothalamic gonadotropin-releasing hormone (GnRH), pituitary luteinizing hormone (LH), and follicle-stimulating hormone (FSH), and gonadal steroids such as testosterone and estradiol (Wingfield and Farner 1993; Wingfield and Silverin 2002). In addition to breeding, most species also undergo at least one bout of regeneration of the integument, involving replacement of the plumage and flight feathers (Hahn et al. 1992). In addition, other life-history stages can be added, in particular, migration. Because migration is commonly associated with avoidance of severe winter conditions at high latitudes, its occurrence within the tropics has probably been substantially underestimated (Newton 2008). On closer inspection, many taxa of birds have been found to carry out intratropical migrations, for example, hummingbirds, seabirds, and songbirds (Winker et al. 1997; Rappole and Schuchmann 2003; Styrsky et al. 2004; Pinet et al. 2011).

The phenological patterns of these processes in tropical birds are reviewed below in detail. Overall, there is a tendency for the different life-history stages to occur separately from each other with little overlap. Migration is clearly incompatible with substantial flight feather molt, and also breeding is usually separated from migration (but see Williams 2012, p. 269ff, for instructive exceptions). Breeding is usually also separated from intense molt, but can sometimes overlap, for example, in some tropical species (Foster 1975; Hahn et al. 1992; Komdeur 1996; Astheimer and Buttemer 1999; Oschadleus and Underhill 2008; Echeverry-Galvis and Hau 2012, 2013; Johnson et al. 2012; Williams 2012; Peters et al. 2013). However, most tropical songbirds show either distinct molt cycles or arrest molt during breeding. Overlapping molt with breeding can compromise either feather quality or breeding success (Echeverry-Galvis and Hau 2012, 2013). Temporal separation of life-history stages does not necessarily imply that they occur as a mere sequence of states, such that completion of one process would trigger the beginning of the next (Wikelski et al. 2008; Williams 2012). There are many examples of species in which some processes are timed independently and sometimes more precisely than others. For example, Humboldt penguins (*Spheniscus humboldti*) and red-billed quelea (*Quelea quelea*) time molt precisely and annually, whereas breeding can occur at various times of year (Otsuka et al. 2004; Oschadleus and Underhill 2008). Conversely, in a Costa Rican hummingbird species, breeding activities were annual and synchronous, but molt occurred at various times of the year (Stiles and Wolf 1974).

Many life-history traits are under circannual control. Migration of higher-latitude species has been a classical topic of circannual rhythm research. A presumable correlate of migratory activity in captive birds, *Zugunruhe* (migratory restlessness), recurs in many species under constant conditions with free-running period lengths (Gwinner 1986, 1996, 2003). Circumstantial evidence indicates that similar patterns occur in tropical species (Styrsky et al. 2004; Helm and Gwinner 2006). For molt and breeding, evidence of circannual rhythms in tropical species is unambiguous (Gwinner and Dittami 1985; Chandola-Saklani et al. 2004; Goymann et al. 2012) (Fig. 13.1). A particularly instructive example for the endogenous control of circannual cycles comes from the aforementioned comparative study of blackcaps from central Europe and the Cape Verde Islands (Fig. 13.3). Hybrids between European blackcaps, which show a single reproductive cycle per year, and Cape Verdean blackcaps, which show two cycles per year, show circannual patterns that are intermediate, pointing to an inherited, biannual endogenous testicular rhythm (Berthold and Querner 1993). Clearly, there is enormous variation in the organization and timing of the annual cycle of birds. To consider the role of circannual rhythms and their possible *Zeitgeber*, we first give an overview over tropical environments before reviewing annual cycle patterns of birds.

13.3 The External Environment: Biomes in the Tropics

Given the diversity of tropical habitats, an identification of candidate environmental factors as *Zeitgeber* should start with a look at the typical tropical biomes and their degree of seasonality. A common misconception about the tropics is that they are a rather benign environment with relatively little variation in rainfall, temperature, and food availability. In reality, the biomes in the tropics are very diverse, ranging from little annual fluctuation to large differences between seasons and from high predictability to erratic changes in climate conditions. To describe these differences between biomes, the Colwell classification of temporal fluctuations in periodic phenomena (Colwell 1974) is very useful. Colwell distinguishes three parameters (predictability, constancy, and contingency), which are sufficient to describe aspects of physical or biological periodic phenomena. Predictability is defined as “a measure of the variation among successive periods in the pattern of a periodic phenomenon” (Colwell 1974, p. 1152). This predictability has two separable, additive components, constancy and contingency. Constancy “varies inversely with the amplitude of variation” of the trait in question (Colwell 1974, p. 1152), which means, when variation in a trait is high, constancy is low and vice versa. If we take the annual pattern of rainfall as an example, then constancy would be maximal when seasonality is low, that is, the rainfall is equally distributed throughout all months of a year (i.e., low intraannual variation in rainfall). High constancy implies high predictability in environments with little or no seasonality. Conversely, constancy would be lowest when there is a defined period of rain, and no rain during the rest of the year (i.e., high intraannual variation in rainfall, marked seasonality). Under these circumstances, predictability depends on the second component, contingency. Contingency is the degree to which the state of a variable at a particular time of year resembles the state in previous and subsequent years (Colwell 1974). Contingency is maximal if patterns are similar across years. In our example, if there is a pronounced rainy season during the same period of time in each year (e.g., if each year the rains start in January and stop in March), contingency is maximal, making the phenomenon highly predictable. If, on the other hand, there is a pronounced rainy season each year, but at different periods of time each year, then contingency and predictability are minimal. As a consequence, predictability is high with high degrees of constancy or high degrees of contingency, or a combination of both (for an example of the latter, see Colwell 1974). We believe that rigorous implementation of this classification can generate testable hypotheses for the conditions that will favor reliance on circannual rhythms (see e.g. Wingfield et al. 1992, 1993; Stevenson and Ball 2011 for physiological and photoperiodic applications). We use the terms predictability, constancy, and contingency to roughly classify the tropical biomes described below.

Following the recent biome classification of the World Wildlife Fund (Olsen et al. 2001), the biome of *tropical and subtropical moist broadleaf forests* in general represents the terrestrial biome with the lowest variability in annual temperatures and rainfall. This kind of forest is dominated by (semi-)evergreen deciduous tree species. Together with some tropical islands and the surrounding seas that serve as feeding grounds for many seabirds, these *moist broadleaf forests* come closest to the perception of the tropics as a benign and very constant environment. *Moist broadleaf forests* are highly predictable because they are characterized by high constancy and low contingency sensu Colwell (1974). *Tropical and subtropical coniferous forests* experience low levels of precipitation and moderate variability in temperature throughout the year and thus also offer relatively constant conditions throughout the year, albeit on a much lower level of "benigness." Also, these habitats are predictable (high constancy, low contingency).

Most other biomes in the tropics show large degrees of annual variation in rainfall or temperature and are characterized by at least two major distinctive seasons, the dry and the wet season. *Tropical and subtropical dry broadleaf forests*, *tropical and subtropical grasslands*, *savannahs and shrublands*, as well as *flooded grasslands and savannahs* , experience little variability in temperature, but organisms living in these environments have to cope with long dry seasons, during which there may be no rain at all and food availability may drop to similarly low degrees as in many higher-latitude biomes. In these biomes, wet and dry seasons typically occur on a relatively regular and predictable annual basis, and in this respect indeed resemble many higher-latitude biomes. Thus, these biomes are characterized by low constancy coupled with moderate to high contingencies sensu Colwell (1974). *Montane grasslands and shrublands* in the tropics (e.g., the Andes in South America, subalpine heaths in Africa, and the Asian Himalaya) not only show considerable seasonal variation in rainfall but in addition may also experience large fluctuations in temperature. Hence, seasonality in such biomes compares to similar alpine or even arctic biomes at higher latitudes. Because this biome is characterized by high contingency combined with low constancy sensu Colwell (1974), conditions are highly predictable.

Finally, *deserts and xeric shrublands* receive far less rain than other biomes. Further, the rains are typically irregular and as a consequence hardly predictable. Searing daytime heat may be followed by cold nights. These climatic conditions are very harsh, but support a rich array of habitats, which, because of the paucity and seasonality of available water, often are very ephemeral in nature. In the Colwell (1974) classification these biomes are characterized by low consistency and low constancy and are therefore poorly predictable.

In conclusion, it is hardly possible to speak of the tropics as such, because tropical biomes are very diverse. There is a great variation in predictability (constancy and contingency) of parameters such as rainfall, temperature, and food availability. As a consequence of this variance there are tropical biomes with a low degree of seasonality (high constancy) and others with a high degree of seasonality (low constancy). Biomes with a high degree of seasonality may be highly predictable (low constancy combined with high contingency) or erratic (low constancy combined with low contingency). Hence, there is no single solution as to how "tropical birds" may time the sequence of their respective life-history stages. Instead, replacing generalizations by environmental parameters characterizing the predictability of the habitat and the natural history of each species provides the basis for a better understanding of strategies and mechanisms of life-history stages.

13.4 Temporal Patterns of Life-History Stages in Free-Living Tropical Birds

The temporal patterns by which life-history stages recur in tropical birds can be loosely arranged by their degree of rhythmicity (along a gradient from arrhythmic to rhythmic). Arrhythmic patterns can be based either on continuous, *year-round* expression of one or more life-history stages (in an environment that is predictable because of high constancy), or on *opportunistic and erratic* occurrence during conducive seasonal conditions without any apparent periodicity (in an environment characterized by low constancy and low contingency). In species that show rhythmic patterns, an additional distinction can be made between roughly annual, *seasonal patterns* on the one hand, and nonannual, potentially "*free-running*" patterns on the other. The first group comprises species whose cycles have a long-term period length of 1 year, although annual precision of seasonal timing differs greatly between environments (expressed by the degree of contingency). In contrast, "free-running" patterns recur with a period that differs from 12 months, may or may not be synchronized within a population, and have no apparent reference to external cues. By definition, such "free-running" patterns should always be associated with environments characterized by high constancy and low contingency.

The distinction between arrhythmic, rhythmic, and "free-running" patterns is not always clear cut, but we find them nonetheless helpful for structuring the natural diversity of life histories. A specific caveat applies to the accuracy of classification of rhythmicity in tropical species. In contrast to higher latitudes, climatic conditions, especially those conducive for breeding, may differ on a small spatial scale in the tropics. Hence, when data are pooled for several populations, breeding seasons may appear to be extended or possibly arrhythmic (continuous), although in fact on a local scale they may be distinct and rhythmic. Similar caution applies to estimates of rhythmicity of populations. Under conditions that do not strictly limit the timing of a given life-history stage (i.e., in environments with high constancy), individuals within populations may follow their own rhythmic schedule, as has been described, for example, for the annual musth of elephants (Poole 1987) and tentatively for molt in tropical hummingbirds (Stiles and Wolf 1974). If considered on a population level, a species may thereby appear to be arrhythmic, although individuals may in fact be highly rhythmic but asynchronous.

13.4.1 Arrhythmic Patterns

Some species of birds in *tropical and subtropical moist broadleaf forests* may breed year-round. For example, because there is little seasonal fluctuation in rainfall and insect abundance in the Limoncocha forest of Amazonian Ecuador (high constancy and low contingency sensu Colwell (1974) several antbird species in this forest appear to breed year-round (Tallman and Tallman 1997). Continuous breeding has also been reported from cinnamon doves (*Columbigallina talpacoti*) at Sange Grande in Trinidad (Snow and Snow 1964), an area of the island with relatively constant rains throughout the year. Unfortunately, these and most similar data (reviewed by Immelmann 1971) refer to the population level, and therefore existing rhythmic patterns of individuals may have been overlooked. However, there are at least two tropical avian species for which continuous breeding has been reported on an individual basis: the Seychelle warbler (*Acrocephalus sechellensis*) and the bat hawk (*Machaeramphus alcinus*) .

On Cousin Island, the origin of Seychelle warblers, individuals express annual cycles with a distinct breeding season during the annual food peak. When birds were transferred to the neighboring island of Aride, which offers high insect food abundance throughout the year, individual pairs of Seychelle warblers started to breed continuously. Moreover, when molt occurred, it was overlapped by breeding (Komdeur 1996). Thus, when being transferred from an environment with low constancy but high contingency in food supply (Cousin Island) to an environment with high constancy and low contingency (Aride Island), Seychelle warblers started to breed year-round. A classic annual breeder can thus become an arrhythmic breeder if the environmental conditions permit this change in strategy. Opportunistic shifts between seasonal and year-around breeding have also been suggested for a number of Australian birds that breed in the riparian tropical savannahs of Australia (Astheimer and Buttemer 1999; Peters et al. 2013), and for some species that breed in mild temperate regions (Hahn et al. 1997).

In bat hawks a similar phenomenon has been observed. Normally, bat hawks in rural areas of Zimbabwe have a distinct breeding season with egg-laying during September and October, taking advantage of the fact that their main food, bats, breed during this time of the year (Hartley and Hustler 1993). At Mutare, a pair of urban bat hawks has been observed for several years and described to breed on a nonannual basis with a cycle of 10–11 months (Hartley and Hustler 1993). According to Hartley and Hustler (1993) this pair took advantage of a stable and regular food supply of bats attracted to the insects at electric city lights. Again, these birds seem to have shifted their breeding cycle when shifted from a habitat with high contingency and low constancy to a habitat with low contingency but high constancy.

A second way in which arrhythmic patterns of life history recurrence may arise relates to *opportunistic and erratic* timing. For example, in *deserts and xeric shrublands*, where rainfall occurs on an erratic and unpredictable basis, that is, low contingency and low constancy sensu Colwell (1974), birds do not follow an annual schedule but breed whenever suitable conditions occur. The prime example for this kind of opportunistic breeding has been the zebra finch (*Taeniopygia guttata*), an

Australian desert bird (Zann et al. 1995). These birds keep their reproductive system in a state of readiness to be prepared to breed once the rain has started to fall (Davies 1977). Interestingly, more recent work demonstrated that in contrast to zebra finches from such unpredictable environments, individuals from populations of more predictable habitats, that is, habitats with higher contingency sensu Colwell (1974), completely regress their gonads during nonbreeding (Perfito et al. 2007). Thus, a classic opportunistic breeder can become a seasonal breeder, if the environmental conditions permit this change in strategy.

Opportunistic breeding also occurs in other tropical species. The red-billed quelea is a complicated example (Disney and Marshall 1956; Disney et al. 1959; Ward 1965) because time patterns differ for different life-history stages. As mentioned, in contrast to the somewhat erratic patterns of breeding and migration, molt appears to be more rhythmic in quelea and other weavers with similar ecology. The completion of molt was synchronous in different populations, enabled by a tendency for birds to compensate for a late start by molting faster (Oschadleus and Underhill 2008). The initiation of reproduction is, at least in some populations, combined with migration, which in turn typically follows rain patterns. Hence, it is still debated which factors or, more precisely, which combination of environmental and endogenous factors are involved in the timing of breeding in this bird species (Jones 1989). We suspect that depending on the predictability of the local rainy season, opportunistic and annual, seasonal breeding may occur in populations of red-billed quelea.

Perhaps the best evidence for erratic patterns of breeding is the small ground-finch from the Galapagos Islands (Boag and Grant 1984; Schluter 1984). In these finches, initiation of gonadal growth and reproduction is initiated flexibly and most likely is induced by rainfall (Hau et al. 2004). Similar to opportunistic breeders in temperate regions (Berthold and Gwinner 1978; Hahn 1998; Deviche and Sharp 2001) and zebra finches in part of their Australian range (Astheimer and Buttemer 2002; Perfito et al. 2007), ground finches show low levels of reproductive hormones and completely regressed gonads outside the breeding season (Hau et al. 2004). Thus, small ground finches appear to be able to rapidly activate their reproductive system when environmental conditions become permissive. Such strategies are likely to be common in *desert and xeric shrubland* species, where environmental conditions for breeding are erratic and unpredictable, that is, contingency and constancy sensu Colwell (1974) are low.

13.4.2 Rhythmic Patterns

13.4.2.1 Annual Seasonal Patterns

Nonannual life cycles of tropical birds are the exception rather than the rule. By far the majority of species, even from relatively constant *moist broadleaf forests*, show seasonal life cycles on an annual schedule (e.g. Snow and Snow 1964; Ward 1969;

Fogden 1972; summaries in Immelmann 1971; Murton and Westwood 1977; Stutchbury and Morton 2001). Periodic patterns are even more pronounced, although sometimes variable between years, in birds living in highly seasonal tropical environments, including *dry broadleaf forests, grasslands, savannahs and shrublands, flooded grasslands and savannahs*, or *montane grasslands and shrublands*. In these often relatively predictable environments (i.e., low constancy, but moderate to high contingency sensu Colwell 1974), the timing of breeding typically reflects the ecological niche of birds (Immelmann 1971). In East Africa, for example, birds relying on insect food for their young tend to breed close to the onset of the rainy season, when insect abundance peaks. In comparison, many granivorous birds breed rather to the end of the rains, when the grasses are seeding (Brown and Britton 1980).

Some seasonal habitats seem to support two distinct breeding seasons, as indicated earlier for resident blackcaps on Cape Verde (Fig. 13.3). Similarly, in Colombia on the Andean plateau, two distinct rainy seasons support biannual breeding of individual rufous-collared sparrows (*Zonotrichia capensis*; Miller 1959). Thus, individuals of these species clearly show an annual cycle with distinct seasonality, but exploit suitable local conditions that support sufficient resources for two distinct breeding seasons.

13.4.2.2 "Free-Running" Patterns

An additional but poorly understood rhythmic pattern of life-history stage timing are "free-running" rhythms, as described for bat hawks. Such cycles recur with periodicities that differ from 12 months and bear no apparent relationship to any external cues. In the equatorial rainforest of Sarawak on Borneo, one of the most constant environments on earth (high constancy and little contingency), local populations of the chestnut-winged babbler (*Stachyris erythroptera*) and the little spider hunter (*Arachnothera longirostris*) showed a periodicity of breeding and molt of about 9 months (Fogden 1972). Thus, in the case of these two species, the individuals of a population seemed to express a synchronized life-history cycle of less than a year. It is not clear how these birds synchronized among each other, but probably social cues have an important role. Life-history cycles that are independent of the annual cycle can be quite common in pelagic tropical seabirds, which also experience little variation in seasonal food supply (high constancy and little contingency). For example, the brown boobies (*Sula leucogaster*; Dorward 1962), tropicbirds (*Phaeton* spp.; Stonehouse 1962), and sooty terns (*Sterna fuscata*; Ashmole 1963) of Ascension Island, or the Audubon's shearwaters (*Puffinus lherminieri*; Snow 1965), swallow-tailed gulls (*Creagrus furcatus*; Snow and Snow 1967), and brown pelicans (*Pelicanus occidentalis*; Harris 1969) of Galapagos all have life-history cycles of less than 12 months duration, suggesting that breeding and molt are not synchronized by an annual *Zeitgeber*, but that members of a population may be synchronized to each other by other cues.

13.4.3 Toward a More Differentiated View of Timing Patterns in Tropical Birds

Previous authors have suggested that the main difference in seasonality between higher latitude and tropical birds is that breeding seasons in tropical birds tend to be longer and that there is a greater variability among species and individuals with regard to when breeding occurs (Stutchbury and Morton 2001). We believe that this is, at least in part, an oversimplification stemming from limited available data, from misconceptions about seasonality in the tropics, and from confounding of more subtle patterns of life-cycle timing. As explained earlier, the extent of seasonality and predictability differs enormously between tropical biomes. Because data on annual cycles of tropical birds are scarce, those that have been available have formed the basis for far-reaching generalizations. For example, in the field of behavioral ecology and endocrinology there is a bias in considering mainly tropical birds from moist broadleaf forests, leading to generalizations on tropical birds (Stutchbury and Morton 2001) that, at best, may hold only for these relatively constant environments.

Furthermore, as already explained, the length of the breeding season may be overestimated when data from different populations, from individuals within populations, or from different years are pooled. For example, in contrast to the Colombian population of rufous-collared sparrows studied by Miller (1959; see above), Ecuadorian populations of this species breed on an annual seasonal basis (Moore et al. 2004, 2006). However, two populations that are separated by just 25 km breed at different times of the year (Moore et al. 2005). This difference is associated with a divergence in rainfall patterns on the eastern and western Andean slopes. Furthermore, other populations breed year-round (Class et al. 2011). Similar differences in local climate conditions exist, for example, in the eastern and western slopes of the Eastern Arc Mountains in East Africa, where the eastern slopes are under a strong influence of the Indian Ocean and receive sufficient rain and humidity throughout most of the year, whereas some of the western slopes fall dry outside the rainy season (Lovett 1990; Burgess et al. 2007). In both cases, markedly seasonal patterns could easily be mistaken for continuous or biannual breeding patterns.

Taking these difficulties in consideration, the breeding seasons of many bird species in highly seasonal tropical biomes may be even shorter than those in higher-latitude regions. For example, black coucals (*Centropus grillii*) live in *seasonally flooded savannahs* and breed only during a period of 3–4 months (Goymann et al. 2004b). Seasonality in these floodplains is surprisingly predictable. For example, black coucals in southwestern Tanzania started nesting at a similar time each year with low variation in onset of breeding, typically less than 3 weeks (median date of first nest, February 5; interquartile range, February 2–February 20; overall range, January 26–February 28; $n = 7$ years; W. Goymann, unpublished data). Most other birds in this floodplain breed during the same period of time. African stonechats, a *montane grassland* species, also breed during the rainy season for a period of about 3 months (Dittami and Gwinner 1985; Scheuerlein and Gwinner 2002). Their close relatives in Europe breed up to 4–5 months (Schwabl et al. 2005). Small groundfinches on Galapagos, or red-billed quelea in Africa, or many species in *deserts and xeric shrublands* may even have much shorter time periods available for breeding.

Oversimplifications of seasonality have also obscured the factors that influence sex hormone concentrations in tropical birds. It has been assumed that tropical birds generally have lower plasma levels of testosterone than higher-latitude species (Levin and Wingfield 1992; Wikelski et al. 1999; Stutchbury and Morton 2001), but systematic comparative studies suggest that it is the length of the breeding season that determines peak testosterone concentrations (Goymann et al. 2004a; Hau et al. 2008a, 2010). The shorter the breeding season, the higher is the testosterone peak also in tropical birds. For example, male black coucals and male rufous-collared sparrows express similar levels of testosterone as higher-latitude species (Moore et al. 2002, 2004; Goymann and Wingfield 2004). In African and European stonechats, peak plasma levels of testosterone are also very similar (Goymann et al. 2006), and year-round territorial birds in the tropics have been demonstrated to share very similar profiles of testosterone with year-round territorial species in the temperate zone (Goymann and Landys 2011). Again, not breeding in the tropics versus breeding at higher latitudes relates to testosterone, but the environmental conditions the bird lives in and its particular life history relate to hormonal patterns. Some of the conclusions regarding testosterone, latitude, and life histories can further be extended to other taxa including reptiles and amphibians (Eikenaar et al. 2012), suggesting that the patterns are strong and general.

In conclusion, we think that for understanding the timing of life cycles, the degree of seasonality in breeding, and other life-history stages, the predictability and the length of the breeding season, as well as the synchrony of breeding within a population, depend on the biome in which the species lives, because seasonal fluctuations in food abundance can be as high (and predictable) in tropical habitats as they are in higher-latitude areas.

13.5 *Zeitgeber* and Supplementary Cues for the Timing of Life-History Stages in Tropical Birds

Given the diversity of tropical environments, birds most likely have evolved many different mechanisms to time life-history stages and may use various proximate and ultimate environmental factors. Strong circannual rhythms could be particularly favorable when either constancy is high, so that they could free run and provide internal temporal structuring. They could also be advantageous in habitats with low constancy if seasonal changes in the environment are predictable, and if proximate cues provide reliable *Zeitgeber* information. In this section we discuss which kind of *Zeitgeber* tropical birds could use for timing.

13.5.1 Subtle Changes in Photoperiod

In general, photoperiod is considered to be the most important *Zeitgeber* for the synchronization of circannual rhythms. Although photoperiodism of tropical birds had been well described (Gwinner and Dittami 1985), for a long time many authors including Moreau (1936) and Ward (1969) considered it unlikely that the subtle change in daylength that occurs in tropical regions could act as a *Zeitgeber*. However, as early as 1959 it was demonstrated that rice (*Oryza sativa*) is sensitive to subtle differences in daylength of less than 15 min duration (Dore 1959). Then, Michaela Hau and colleagues discovered that spotted antbirds (*Hylophylax n. naevioides*) from Panama are able to respond to subtle photoperiodic cues and initiate gonadal growth after a slight increase in photoperiod of just 17 min (Hau et al. 1998; see also Beebe et al. 2005). Subsequently, other tropical birds have been demonstrated to be similarly sensitive to small changes in photoperiod, that is, the spotted munia (Chandola-Saklani et al. 2004) and the redheaded bunting (*Emberiza bruniceps*; Rani et al. 2005). In the yellow-green vireo (*Vireo flavoviridis*), minor differences in photoperiod experienced during the first days of life may synchronize juvenile molt, migratory fattening and *Zugunruhe* (migratory restlessness) in birds hatching over a period of 3 months (Styrsky et al. 2004). Even the northern temperate European starling (*Sturnus vulgaris*) is able to use a simulated photoperiod equivalent to the change in photoperiod at a tropical latitude (9°N or S) as a *Zeitgeber* to synchronize its circannual rhythm (Dawson 2007). Thus, birds may well be able to use slight changes in photoperiod to time their annual life-history cycle, and it has been suggested that this may be particularly useful for birds living in seasonally predictable tropical habitats, because changes in daylength could be used to time breeding in anticipation of the rainy season (Hau et al. 1998, 2008b; Wikelski et al. 2000).

13.5.2 Other Factors Related to Light

In tropical regions with pronounced annual rainy seasons, the change in cloud cover leads to large and at least sometimes predictable changes in light intensity (e.g. Muthiga 2006 for light intensity data from Kenya), which could be used to time seasonal events. Gwinner and Scheuerlein (1998) addressed this topic in the African stonechat. Although these birds show a very robust circannual rhythmicity (Fig. 13.1) (Gwinner and Dittami 1990), in nature breeding occurs annually and is timed to the rains around March to May (Dittami and Gwinner 1985). Experimentally changing the daylight intensity (i.e., mimicking the annual pattern in cloud cover during the rainy and dry seasons) while holding photoperiod constant led to a synchronization of annual reproduction and molt patterns. The gonads of African stonechats grew during the simulated late dry season, and the birds molted at the end of the simulated rainy season. In other species it has been shown that changes in light intensity have the potential to alter the perception of daylength (Bentley et al. 1998) suggesting that African stonechats could potentially use the same mechanism that has evolved to perceive changes in daylength to perceive changes in light intensity. Support for this idea comes from daily profiles of the hormone melatonin in African stonechats kept under the two light intensity conditions described previously (Kumar et al. 2007). Plasma hormone profiles differed slightly between birds kept under bright and nonbright light conditions, opening the possibility of a transduction mechanism involving melatonin.

A further reliable and predictive photic signal that is available even at the equator and is consistent from year to year independently of climate is the regular but synchronous variation in sunrise and sunset time (Gwinner and Dittami 1985; Borchert et al. 2005). Although daylength is constant at the equator, the times of sunrise and sunset vary by about 30 min over the year with two recurring annual maxima and minima (Fig. 13.4) (Borchert et al. 2005). This annual pattern in the timing of sunrise and sunset is caused by the elliptical shape of the Earth's orbit around the sun and the tilt of the Earth's axis relative to its orbit and corresponds to the difference between apparent solar time and the mean time ("equation of time"). Close to the equator, tropical trees synchronize flowering according to the annual change in the time of sunrise and sunset (Borchert et al. 2005). Further studies have suggested that the plants may actually measure the changes in insolation that accompany the

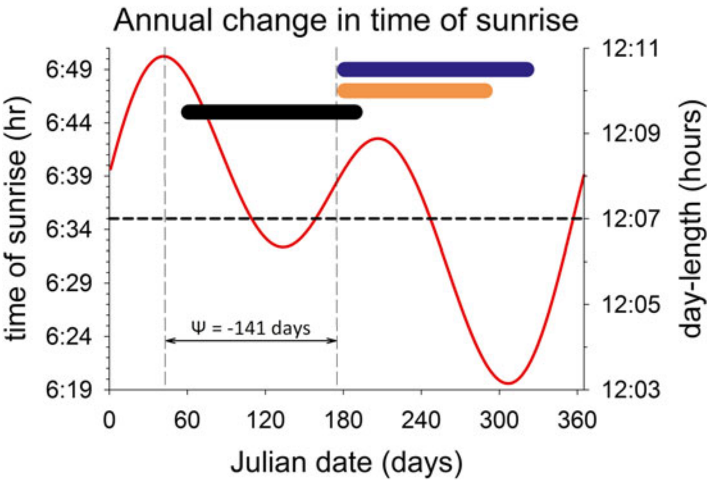


Fig. 13.4 Annual cycle in sunrise time. Annual change in the time of sunrise (*solid red line*) and subsequent sunset (not shown) in combination with a constant photoperiod (*horizontal dotted black line*) at Nakuru, Kenya (N 0°00′, E 36°00′). This annual change in sunrise and sunset is referred to as the "equation of time" in astronomy and is caused by the elliptical shape of the Earth's orbit around the sun and the tilt of the Earth's axis relative to its orbit. The *horizontal bars* indicate the period during which free-living African stonechats at Nakuru, Kenya, breed (*black bar*) and molt (*blue* = body molt, *orange* = flight feather molt) (Dittami and Gwinner 1985). The *arrow* between the two *vertical dashed lines* indicates the phase angle difference (**ψ**) between the day on which sunrise is latest (Julian day 41) and the mean date with the first occasion of molt (Julian day 182) in free-living African stonechats. (Reproduced from Goymann et al. 2012 with permission of the Royal Society)

change in sunrise and sunset pattern to achieve this stunning synchronization (Calle et al. 2010). To our knowledge, Heideman and Bronson (1994) were the first to experimentally test a *Zeitgeber* function of the simulated equatorial changes in sunrise and sunset on an animal, *Anoura geoffroyi*, a tropical phyllostomid bat. However, this bat responded neither to changes in sunrise and sunset times nor to changes in photoperiod, suggesting that this species is not at all responsive to photic cues (Heideman and Bronson 1994, Chap. 15). First evidence that birds could sense the change in sunrise and sunset pattern came from rufous-collared sparrows in Ecuador, which change their daily activity period according to the seasonal changes in sunrise and sunset times (I.T. Moore, unpublished data; cited in Hau et al. 2008b). Recently, we experimentally showed that African stonechats can use the annual change in the timing of sunrise and sunset (Fig. 13.4) as a *Zeitgeber* to synchronize their circannual rhythms (Fig. 13.5). In the absence of any change in photoperiod, stonechats entrained to the subtle shift of the solar day and assumed a phase relationship to the *Zeitgeber* that was similar to that of free-living birds from Kenya (Goymann et al. 2012). In contrast, individuals of a control group kept under the same photoperiod but without information about the annual change in the timing of sunrise and sunset showed free-running rhythms. This was the first experimental demonstration that a bird can use very subtle photic cues (simultaneous shifts in sunrise and sunset ranging between 0 and 3 min per week, depending on the slope of the equation of time) as a *Zeitgeber*.

An interesting experiment in spotted munias may relate to the effects of the simulated change in sunrise and sunset in African stonechats. Chandola-Saklani et al. (2004) demonstrated that spotted munias are capable of reacting to 15 daily increments of 1.5 min in photoperiod by advancing gonadal growth, but they do not respond to absolute long or short durations of light (i.e., absolute photoperiod). This puzzling finding may relate to the effect of the equation of time at the equator, where the time of sunrise and sunset changes along the year (i.e., synchronized increments and decrements of the timing of sunrise and sunset) without a change in daylength or absolute photoperiod. The effects of these synchronized increments and decrements in sunrise and sunset could potentially be supplemented by additional changes in the duration of twilight (Wever 1967) and changes in spectral light composition if these are a reliable predictor of upcoming environmental conditions.

13.5.3 Rainfall and Green Vegetation

Rainfall as a proximate cue to time the life cycle has been mainly discussed for species living in erratic unpredictable environments, such as *deserts or xeric shrublands* (Immelmann 1971). Existing evidence for stimulatory consequences, mostly on breeding, indicates that depending on the life history of a species these effects may be either directly related to rain or indirectly to its effects on the environment.

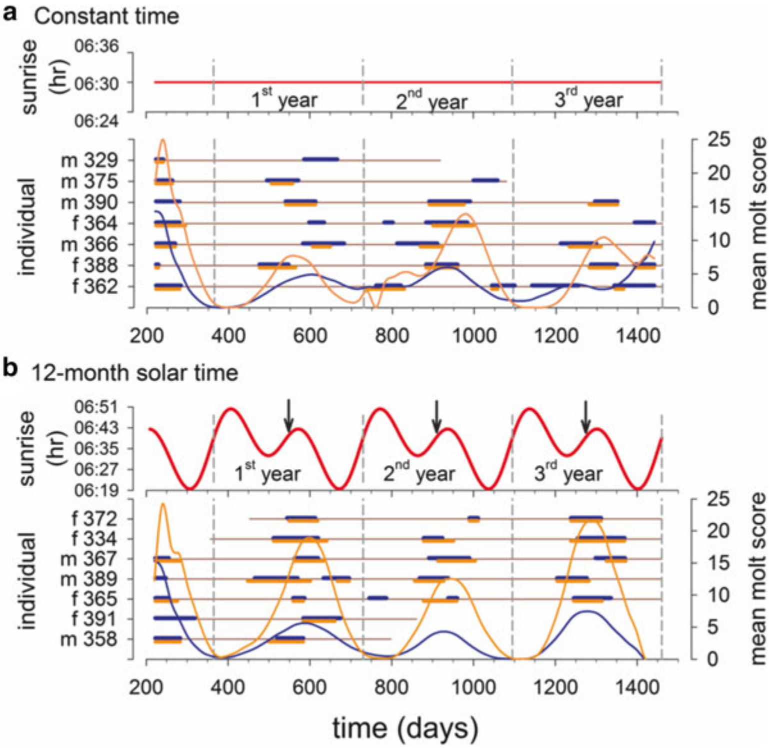


Fig. 13.5 Entrainment to the annual cycle in sunrise time. Body molt (*blue bars*) and flight feather molt (*orange bars*) of individual African stonechats maintained under an equatorial photoperiod for almost 3.5 years. Depicted are two groups of birds that received either (**a**) no change in the timing of sunrise and sunset (constant time conditions; *upper graph*) or (**b**) a simulation of the natural, 12-month cycle in the change sunrise and sunset times at the equator (12-month solar time conditions, *upper graph*; only change in sunrise shown). In all graphs the *thin* (*brown*) *lines* indicate the total time each individual bird stayed in the experiment. The *blue* and *orange curves* represent smoothed averages of body molt and flight feather molt scores of all individuals within one group (represented on the right *y*-axis), respectively. A sinusoidal curve with one approximate peak per year indicates synchronization to an annual *Zeitgeber* with the magnitude of the amplitude reflecting synchronicity among birds within one group. A curve with several peaks per year indicates the absence of synchronization to an external *Zeitgeber*. Because of differences in scoring method the amplitude of body molt (*blue*) is naturally smaller than the amplitude of flight feather molt (*orange*). The *time axis* represents consecutive days (1 = January 1, 2005), with the *dashed vertical lines* indicating the years. In **b**, the *black vertical arrows* in the *upper graphs* indicate the onset of molt in free-living stonechats at Nakuru, Kenya. Note that all birds in (**a**) and (**b**) were initially synchronized to a European photoperiod before they entered the experiment (with most birds still molting when entering the experiment around day 215). Bird IDs starting with “f” represent females, those starting with “m” represent males. (Reprinted from Goymann et al. 2012 with permission of the Royal Society)

Marshall and Disney (1957) have demonstrated that green grass, but not rain, stimulates red-billed quelea to continue breeding, as quelea started to build nests once offered green grass. However, according to Murton and Westwood (1977, p. 250) quelea may express an innate urge to build nests throughout the year whenever offered green grass, and the previous experiment did not demonstrate that the green grass actually would induce breeding in this species. The potential importance of green vegetation has been demonstrated in a northern temperate species, the wild canary (*Serinus canaria*). Birds on an island in the Atlantic Ocean start to develop their gonads and breed while daylength is still decreasing when stimulated by green vegetation and subsequent food availability (Leitner et al. 2003). Follow-up laboratory experiments have shown that wild canaries really use direct or indirect information from green grasses, because birds would react to the presence of living green plants, but not simulation of rainfall, artificial green vegetation, or plant extracts (Voigt et al. 2007, 2011). In similar vein, Hau et al. (2004) suggested that an increase in luteinizing hormone in small cactus finches (*Geospiza scandens* and *G. conirostris*) may be related to access to *Opuntia* cactus flowers. This suggestion is based on the observation that cactus finches with sufficient access to *Opuntia* flowers advanced breeding by 1–2 months and were able to breed even in years without rainfall (Grant 1996).

To our knowledge, the roles of rain and of green vegetation (direct or indirect: Ettinger and King 1981; Negus and Berger 1998) as a *Zeitgeber* for tropical birds have not been studied in detail, but this would certainly represent a subject worth investigating in further depth. For example, one of the authors studies coucals in the Usangu, a floodplain in southwestern Tanzania (Goymann et al. 2004b, 2005). This floodplain is highly seasonal with lush vegetation during and after the rains between January and May. With the exception of vegetation close to rivers and a permanent swamp, the area becomes very dry during the rest of the year, and the trees and shrubs shed all their leaves starting in June. In October, however, some shrubs and trees, that is, many acacias or baobab trees, start growing new leaves and flower about 2 months in advance of the first rains. It is quite possible that birds and other animals could use these vegetational predictive cues to time gonadal growth well in advance of the coming rains.

13.5.4 Food

Food has been suggested to act as both an ultimate and proximate factor for the timing of energetically expensive life-history stages, in particular breeding. Mechanistically, there are many conceivable ways in which this can be achieved: these could be direct ways, for example, through endocrine stimulation (Fidler et al. 2008) or through providing conducive energetic conditions. A seasonally steady and consistent increase in food abundance could thus be paralleled by gonadal development. Supportive evidence comes from a study of the yellow-vented bulbul (*Pygnotus goiavier*, Ward 1969). Based on monitoring the annual cycle of these birds on Singapore Island, Ward (1969) suggested that changes in the level of tissue protein may be driven by the availability of protein available from the environment. Such a mechanism related to "internal protein sensors" may be particularly useful for birds that do not fully regress their gonads during nonbreeding and can quickly grow them to full size once conditions are suitable enough for breeding, as has been found in opportunistic breeders such as the zebra finch (Davies 1977; but see Perfito et al. 2007, 2008).

Alternatively, stimulatory effects could be indirect through mediation of sensory systems. Either way, assessment of food availability would be one of the most direct "means" for a bird to judge whether environmental conditions are suitable for energetically demanding life-history stages. Indeed, the Seychelle warblers described earlier may have used food as a direct cue when, after being transferred from seasonal Cousin Island, they started breeding year-round on Aride Island. There, food is so abundant throughout the year that the gonads of birds probably do not regress at all (Komdeur 1996). However, for most other birds, the disadvantage of using food as a cue is that its occurrence may be rather sudden and does usually not provide much advance notice. This situation is particularly disadvantageous for species that completely regress their gonads: they would lose valuable time to recrudesce the gonads before being able to initiate nests. Thus, in most tropical birds, food availability may represent a "supplementary cue" (Wingfield and Kenagy 1991), important for the fine-tuning of seasonal behaviors in addition to a *Zeitgeber* that predicts the forthcoming breeding (or molting) season. Food thereby only stimulates reproduction when the reproductive window has been opened by circannual or environmental cues, as has been shown in an experiment with spotted antbirds. In male antbirds, presentation of live crickets advanced gonadal growth and song rate at the time when these birds were preparing to breed (Hau et al. 2000).

The possibility that food could also act as a *Zeitgeber* has been examined in a field experiment with African stonechats. When pairs received additional food before the breeding season, food supplementation advanced breeding but did not change subsequent molt or onset of breeding in the following year (Scheuerlein and Gwinner 2002). Hence, the normal circannual rhythm was not affected by food supplementation, but food only acted as a supplementary cue defining the precise onset of breeding activities. Alternatively, food supplementation advanced the circannual rhythm during the breeding life-history stage but other (unknown) cues shifted the circannual rhythm back so that the molt stage occurred at the "right" time of the year (Scheuerlein and Gwinner 2002).

13.5.5 Social Cues

Social cues must be important especially in species that nest in colonies and in which breeding is highly synchronized (Hahn et al. 1997; Helm et al. 2006). For example, Ward (1965) reports that in a colony of red-billed quelea 91 % of all nestlings hatch within a period of 3 days. Similar phenomena occur within colonies of other weavers (Hall 1970). In quelea and other weavers, these social cues may mainly supplement other environmental information to fine-tune breeding. In sooty terns that breed synchronously but do not follow an annual breeding schedule (Ashmole 1963), however, exchange of social information might represent the main synchronizing cue to coordinate reproductive activities within a colony. Social cues may be less important in species that breed in individual pairs. Gwinner et al. (1995) tested effects of breeding partners on captive African stonechats, but in these birds circannual rhythms were so strong that they persisted without modification, independently of the availability of a mate and of actual breeding performance. The role of social cues is in great need of more research. To our knowledge no studies so far have revealed the mechanisms by which social cues may time life history events of tropical birds, nor is there experimental evidence that social interactions could function as a *Zeitgeber* for circannual rhythms.

13.6 Conclusions and Outlook

Because of the large diversity of life-history strategies displayed by tropical birds, they are extremely useful to explore the "green end" of biological rhythms, that is, the ecological context of reliance on circannual (or other) rhythms and their various *Zeitgeber*. This knowledge is important for understanding the possible evolutionary consequences of timekeeping, including its impact on biodiversity. For example, if local environmental conditions favor a divergence of breeding seasons of two or more populations of a species living in close proximity (Moore et al. 2005), this may lead to allochronic isolation and speciation (Alexander and Bigelow 1960). Mechanistically, this could result from individuals of these different populations using the same *Zeitgeber* cues, but interpreting them in different ways. For example, the annual change in sunrise and sunset at the equator (Fig. 13.4) could act as a *Zeitgeber* in African stonechats but, depending on local environmental conditions, different populations align their activities with different phases of this cycle. Thus, Kenyan stonechats that start breeding around March (Dittami and Gwinner 1985) and northern Tanzanian stonechats which start breeding around October (Scheuerlein and Gwinner 2002, 2006) might use the same *Zeitgeber* differently.

To fully exploit this "green end" of biological rhythms, a more differentiated appreciation of the presence or absence of seasonality in the tropics and more data on annual cycles of individuals across species and biomes are needed. In this context, we consider Colwell's concept regarding the predictability of periodic phenomena (Colwell 1974) extremely useful. This concept, in combination with large-scale information about the biome in question and smaller-scale information about the particularities of local habitats and the life history of the species, will yield a more differentiated picture of the diverse life histories displayed by tropical birds in their manifold environments. It could also promote the generation of hypotheses for the evolution of circannual rhythms and guide the selection of powerful study systems. Promising recent developments in tracking technology (Bridge et al. 2011) will allow us to get a clearer picture of annual cycles and will yield data on individuals to resolve potential misconceptions about life-cycle timing and arrhythmic behaviours. Unfortunately, the link to endogenous mechanisms and the way they interact with *Zeitgeber* is still difficult, and more experiments under controlled laboratory conditions are needed to promote our understanding. Both approaches require a courageous long-term funding strategy that emphasizes the quality rather than quantity of publications resulting from such long-term commitments, and trusts and supports capable visionary researchers, as was the case for the ground-breaking work of one of the main pioneers of circannual rhythms, Ebo Gwinner.

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Chapter 14

The Circannual Clock in the European Hamster: How Is It Synchronized by Photoperiodic Changes?

Stefanie Monecke, Franziska Wollnik†, and Paul Pévet

Abstract In a seasonal environment, mammals time their reproductive phase so that the offspring are born in spring and summer. Two strategies have evolved to ensure accurate seasonal timing of reproduction, but both share a common *Zeitgeber*, the seasonal changes in photoperiod. The reproductive axis might be directly controlled, as in photoperiodic species, which require photoperiodic input to show seasonal changes in reproductive competence. In contrast, in circannual species photoperiodic changes act indirectly, namely, on an endogenous circannual clock that then times reproduction. This circannual clock generates self-sustained rhythms with a period length of about 1 year, and photoperiodic information is only needed to synchronize these rhythms. Concerning the mechanism that imparts the photoperiodic message internally, so far no differences between photoperiodic and circannual mammals have been reported. Recent results however, strongly suggest that the circannual European hamster (*Cricetus cricetus*) uses a fundamentally different mechanism than photoperiodic species. In the latter, photoperiod induces a change in the reproductive state via a melatonin-dependent pathway, whereas the circannual clock of the European hamsters can be synchronized via a melatonin-independent pathway. Instead, a circadian mechanism based on a specific organizational state is involved. Juvenile European hamsters use probably both pathways: the melatonin-dependent photoperiodic pathway for the short-term timing in the year of birth and the melatonin-independent circannual pathway for long-term timing of the seasonal events in the next year.

Keywords Activity • Circannual • *Cricetus cricetus* • Melatonin-independent • Photoperiodic time measurement

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14.1 Living in a Seasonal Environment

Sadly, Franziska Wollnik passed away and could not see this review published. Her ideas, work, and enthusiasm contributed substantially to our new model of circannual synchronization.

European hamsters (*Cricetus cricetus*) inhabit mainly agricultural areas in the temperate zones of the Eurasian continent between 45° and 60° N and 5° and 95° E. Survival in such a seasonally varying environment requires complex physiological adaptations and a precise timing (Visser et al. 2010). It is essential that the offspring is born at the most favorable seasons and that the animals prepare in time for the harsh conditions of winter with its food shortage but high energy requirements. The mating season of European hamsters lasts thus from April to early August (Vohralík 1974; Bekenov 1998; Monecke and Wollnik 2005). During this period a female can raise as many as two or three litters (Seluga et al. 1996; Franceschini and Millesi 2004) of about six pups each (Monecke and Wollnik 2008). The age at which they reach puberty (measured by the first descensus testis or first opening of the vagina, respectively) depends on the season of birth (Monecke et al. 2014) and varies between postnatal day 21 (males) or 32 (females) and an age of several months when gonads develop only after hibernation (Kirn 2004). The mainly herbivore food varies with the season (Surdacki 1964; Górecki and Grygielska 1975). The food stock for winter is built from late July on, when food availability is at the maximum. Although most energy reserves are stocked externally, hamsters build up fat deposits before winter (Siutz et al. 2012). Between September and March the animals hibernate in subterranean burrows (Karaseva 1962; Ružić 1976), a phenomenon that was already described for European hamsters in 1769 (Hildebrand 1769). The torpor bouts last typically about 3–6 days (Canguilhem et al. 1994; Waßmer and Wollnik 1997), during which body temperature falls close to ambient temperature so that energy expenditure is greatly reduced (Kayser 1964, 1971). Predation is by far the main reason for mortality (Kayser et al. 2003); however, as are most rodents, aged European hamsters are also susceptible to cancer (Ghadially and Illman 1965; Ernst et al. 1989; Brandes et al. 2004). Seasonality is well studied in this species, found in 48 of 53 tested physiological and behavioral parameters (Tables 14.1 and 14.2), in the size of 14 organs (Table 14.3), as well as in the histology or ultrastructure in 11 of 12 tested organs or cell types (Table 14.4). In 32 of 38 tested parameters (Table 14.5) the rhythms persisted even in a constant environment with a period length of about 1 year. Thus, they are driven by an endogenous circannual clock.

The European hamster is a generalist; it has a huge distribution area, the capacity to hibernate, and a high reproductive potential. In short it has all the physiological properties that are thought to be beneficial for the survival of a species (Geiser and Turbill 2009; Isaac 2009; Robinson et al. 2009). And indeed, it used to be a severe pest species (Nechay et al. 1977; Stubbe and Stubbe 1998). Nevertheless, today natural populations are dramatically declining all over the distribution area and are often at the brink of extinction (Nechay 2000; Weinhold and Kayser 2006; Sidorov et al.

Table 14.1 Seasonal variations in physiological parameters

	Parameter	J	F	M	A	M	J	J	A	S	O	N	D	Conditions/remarks	Literature
General physiology	Emergence and immergence date			↑	↑						↓			Free-ranging animals, several years and regions	Karaseva (1962), Ružić (1976), Weidling and Stubbe (1998) (review), Franceschini-Zink and Millesi (2008)
	Males reproductive			✓	✓	✓	✓	✓	✓					LDnat and free-ranging animals	Vohralík (1974), Bekenov (1998), Monecke and Wollnik (2005)
	Females reproductive				✓	✓	✓	✓	✓					LDnat and free-ranging animals	Vohralík (1974), Bekenov (1998), Monecke and Wollnik (2005)
	Daily activity rhythm					✓	✓	✓						LDnat, 20 °C	Wollnik et al. (1991), Monecke and Wollnik (2005)
	Daily temperature rhythm					✓	✓	✓						LDnat, 20 °C	Wollnik and Schmidt (1995)
	Sensitive phases to photoperiodic information	✓	▨	▨		✓	✓	✓	▨			✓	✓	LDnat => artificial SP/LP	Saboureau et al. (1999), Monecke and Wollnik (2004)
	Hibernation	✓	✓	✓						✓	✓	✓	✓	Natural conditions	Waßmer and Wollnik (1997), Wassmer (2004), Monecke et al. (2011b)
	Testis size				↑	↑	↑	↑						LDnat, 20 °C and natural conditions	Masson-Pévet et al. (1994), Monecke and Wollnik (2005)
	Pups per litter	▨	▨	No seasonal rhythm							▨	▨	▨	LDnat, 20 °C	Monecke and Wollnik (2008)
		▨	▨			↑					▨	▨	▨	Free-ranging hamsters, date of conception	Hufnagl et al. (2011)
	Successful matings	▨	▨	▨	▨	↑	↑			▨	▨	▨	▨	Female yearlings, from sawtooth light regime, recalculated for calendar dates	Monecke et al. (2011a)
		▨	▨	▨	▨		↑		↑	▨	▨	▨	▨	2-year-old females, from sawtooth light regime, recalculated for calendar dates	Monecke et al. (2011a)

(continued)

Table 14.1 (continued)

Parameter	J	F	M	A	M	J	J	A	S	O	N	D	Conditions/remarks	Literature
Frequency of aggressive interactions among males	▨	▨			↑	↑		▨	▨	▨	▨	▨	Free-ranging males	Millesi et al. (2004)
Water intake		↓				↑							From a region in which hamsters are “late”	Wendt (1991)
Food intake	↓				↑							↓	In sawtooth light and temperature regime	Miro et al. (1980)
		↓	↓			↑							From a region in which hamsters are “late”	Wendt (1991)
Body weight males				↓				↑					From a region in which hamsters are “late”	Wendt (1991)
		↓					↑	↑					Natural PP and temperature	Canguilhem et al. (1992), Masson-Pévet et al. (1994)
		↓			↑	↑	↑	↑	↑				Natural PP and temperature	Kayser and Schwartz (1960)
Body weight females		↓							↑				Natural PP and temperature	Canguilhem et al. (1992)
				↓			↑						From a region in which hamsters are “late”	Wendt (1991)
Calcium uptake	↑	↑				↓	↓	▨				↑	LDnat, 20 °C	Kayser and Haug (1969)
	↓	↓	↑	↑	↑	↑	↑	▨	▨	▨	↓	↓	LDnat, 20 °C / Nov 1–Mar 1 DD, 7 °C	Duval-Erny and Kayser (1964)
Calcium balance	↓	↓	↑	↑	↑	↑	↑	▨	▨	▨	↓	↓	LDnat, 20 °C / Nov 1–Mar 1 DD, 7 °C	Duval-Erny and Kayser (1964)
	↑	↑				↓	↓	▨			↑	↑	LDnat, 20 °C	Kayser and Haug (1969)

Daily rhythms	Amount of activity					↑	↑							Natural conditions/ free-ranging	Wendt (1989), Schmelzer (2005), Ziomek (2011)
						↑	↑	↑						LDnat, 20 °C	Wollnik et al. (1991), Monecke and Wollnik (2005)
	Phase angle of activity onset					↑	↑	↑						LDnat, 20 °C	Monecke and Wollnik (2005)
	Duration of activity					↓	↓	↓						LDnat, 20 °C	Monecke and Wollnik (2005)
	Apparent period length					↓	↓	↓						LDnat, 20 °C	Monecke and Wollnik (2005)
	Euthermic body temperature	↓	↓	↓		↑	↑	↑						LDnat, 20 °C and natural conditions	Wollnik and Schmidt (1995), Monecke et al. (2011b)
Neuronal level	Hypothalamic and cardiac adenylyl cyclase activity						↑						↓	Animals were transferred from LDnat to LD 12:12 or DD, respectively, before measure	Pleschka et al. (1996), Pleschka and Nürnberger (1997)
	β1- and β2-adrenergic receptors in the hypothalamus and heart						=						=	Animals were transferred from LDnat to LD 12:12 or DD, respectively, shortly before measure	Pleschka et al. (1996), Pleschka and Nürnberger (1997)
	Vasopression innervation					↑					↓			Natural conditions, sex differences	Buijs et al. (1986)
	Melatonin-binding site in the PT	↓			↑							↓	↓	Natural conditions	Skene et al. (1993), Masson-Pévet and Gauer (1994)
	Affinity of melatonin-binding sites in the PT	No seasonal rhythm												Natural conditions	Skene et al. (1993)

(continued)

Table 14.1 (continued)

	Parameter	J	F	M	A	M	J	J	A	S	O	N	D	Conditions/remarks	Literature
Hormonal level	Cortisol			No seasonal rhythm										Free-ranging females	Franceschini et al. (2007)
						↑	↑	↑	↑					Free-ranging males	Franceschini et al. (2007)
	Progesterone					↑	↑	↑	↑	↑				Free-ranging females, dates deduced from reproductive state	Franceschini et al. (2007)
								↑						Free-ranging animals	Krsmanovic et al. (1984)
	Testosterone						↑							Free-ranging animals	Krsmanovic et al. (1984)
					↑	↑	↑	↑	↑					Natural conditions	Buijs et al. (1986)
								↑						Natural conditions	Canguilhem et al. (1986)
	Gonadotroph activity of the prehypophysis	↑					↑				↓			Natural conditions	Petrovic and Kayser (1957)
	Content of adrenaline in the adrenal medulla		↓		↑									Natural conditions	Kayser and Aron (1950)
	Thyroid activity	↑					↓	↓	↓		↑			Natural conditions	Petrovic and Kayser (1958)
	Thyroxine (T_4)				↑	↑								Natural conditions	Canguilhem et al. (1986)
	Triiodothyronine (T3)			↑	↑	↑	↑							Natural conditions	Canguilhem et al. (1986)
Pineal gland	Melatonin					↓	↓	↓				↑	↑	Natural conditions; in pineal gland and plasma, females, various sampling protocols	Vivien-Roels et al. (1992, 1997), Garidou et al. (2003)
	5-Methoxytryptophol										↑	↑	↑	Natural conditions, females	Vivien-Roels et al. (1992), Ribelayga et al. (1998), Garidou et al. (2003)
	NPYergic innervation											↓	↑	Natural conditions	Møller et al. (1998)
	Tyrosine hydroxylase fibers										=		=	Natural conditions	Møller et al. (1998)
	Pineal protein										↑		↓	Natural conditions, females	Ribelayga et al. (1998)
	HIOMT activity										↓		↑	Natural conditions, females	Ribelayga et al. (1998)
							=					=		Natural conditions	Garidou et al. (2003)
	AA-nat mRNA						↑					↓		Natural conditions	Garidou et al. (2003)
	AA-nat activity						↑					↓		Natural conditions	Garidou et al. (2003)

Urinary excretion	Urine volume				↓	↓	↓	↓	↓					In sawtooth light and temperature regime	Canguilhem and Bloch (1966), Harberey et al. (1967)
	Sodium	↑			↓	↓	↓	↓	↓			↑	↑	In sawtooth light and temperature regime	Harberey et al. (1967)
	Potassium				↓	↓	↓	↓	↓	↓	↓			In sawtooth light and temperature regime	Harberey et al. (1967)
	Aldosterone			↑		↓	↓	↓	↓		↓			In sawtooth light and temperature regime	Canguilhem and Bloch (1966), Bloch and Canguilhem (1966)
	Chromogen Porter-Silber reaction (amount of adrenal steroids)	↑	↑											In sawtooth light and temperature regime	Canguilhem and Bloch (1966)
	aMT6s					↓	↓	↓						In LDnat, males and females	Monecke and Wollnik (2005)
	17-Ketosteroids	↓				↑	↑	↑				↓	↓	Natural conditions	Kayser and Schwartz (1960)
	Formaldehydrogenic steroids	↓	↓				↑						↓	Natural conditions	Kayser and Schwartz (1960)
	Catecholamines	No seasonal rhythm												In sawtooth light and temperature regime	Canguilhem and Bloch (1966)
	Creatinine	No seasonal rhythm												In LDnat, males and females	Monecke and Wollnik (2005)

In the month-columns (J–D), white areas indicate test periods; grey-shaded months were untested. Different results for the same parameter are given in separate lines. The boxes in the monthly columns of emergence and immergence dates indicates the range. ✓ present, ↑ maximum (plateau if several), ↓ minimum (plateau if several), = no difference found. *LD* ratio of light:darkness per day, *LDnat* natural variation of light and darkness, *DD* constant darkness, *SP* short photoperiod, *LP* long photoperiod

Sawtooth light regime is indicated when photoperiod and/or temperature were altered abruptly each 6 months to mimic summer or winter conditions. *PT* pars tuberalis, *NPY* neuropeptid Y, *HIOMT* hydroxyindole-*O*-methyltransferase, *AA-NAT* arylalkylamine *N*-acetyltransferase, *aMT6s* 6-sulphatoxymelatonin. Please note that the listed results might be subjected to regional modifications

Table 14.2 Seasonal variations of gene expression levels on the neuronal level tested in long (LP) or short (SP) photoperiod

	Parameter	LP	SP	Remarks	Literature
In the SCN	*Per2*	↑	↓		Tournier et al. (2007)
	Rev-erbα	↑	↓		
	AVP	↑	↓		
	clock	↑	↓	In both no daily rhythm	
	Bmal1	=	=	Daily mean similar, but daily rhythm only in LP	
	Light-inducible expression of Fos Protein	↓	↑	Longer in SP than in LP	Vuillez et al. (1996)
In the pars tuberalis	*Cry 1*	↓	↑	Phase change	Tournier et al. (2007)
	Per1	↑	↓		
	Rev-erbα	↓	↑	Phase change	
	MT1 melatonin receptor mRNA	↑	↓	No receptor mRNA found in SP	Dardente et al. (2003)
	β-TSH in MT1-expressing cells	↑	↓	For mRNA and protein	
	α-GSU in MT1-expressing cells	↑	↓	For mRNA and protein	
	Gonadotrophs	=	=		
	β-TSH RNA	↑	↓		Hanon et al. (2010)
	TSH receptor	↑	↓	Only one night value differed; in both PP no daily rhythm	
In the pars distalis	*β-TSH* RNA	=	=		
In the ependymal layer (lining third ventricle)	TSH receptor	=	=	In both PP no daily rhythm	
	Dio2	↑	↓	Daily rhythm only in LP	
	Dio3	↓	↑	Daily rhythm only in SP	

↑ indicates maximum, ↓ minimum, = stands for no difference; *SCN* suprachiasmatic nucleus. Different clock genes and clock-controlled genes (*Per1*, *Per2*, *Rev-erbα*, *AVP*, *clock*, *Bmal1*, *Cry1*) are listed for the SCN or the pars tuberalis; *β-TSH* β-thyroid-stimulating hormone, *GSU* α-glycoprotein subunit, *Dio2 Dio3* deiodinase 2 and 3, PP photoperiod

Table 14.3 Seasonal variations in organ weights according to (Reznik et al. 1976)

Organ	J	F	M	A	M	J	J	A	S	O	N	D
Hypophysis		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Thyroid glands		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Pancreas		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Adrenal glands		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Testes		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Heads of epididymes		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨

(continued)

Table 14.3 (continued)

Organ	J	F	M	A	M	J	J	A	S	O	N	D
Prostate gland		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Vesicular glands		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Bulbourethral gland		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Ovaries		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Parotid glands		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Mandibular glands		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Sublingual glands		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨
Zygomatic glands		▨	▨	▨	↑	▨	▨	▨	▨		▨	▨

Organs were taken from nonhibernating animals in January, May, and October
↑ indicates maximum

Table 14.4 Seasonal variations in the histology or the ultrastructure of glands and organs

Organ	Seasonal changes in histology or ultrastructure	Literature
Prehypophysis (anterior pituitary)	Yes	Kayser and Aron (1938, 1950)
Ovaries	Yes	Hilfrich et al. (1977)
Testes and accessory glands	Yes	Kayser and Aron (1938, 1950); Reznik-Schüller and Reznik (1973)
Leydig cells	Yes	Reznik-Schüller and Reznik (1974)
Parafollicular cells of the thyroid	Yes	Stoeckel et al. (1967)
Thyroid	Yes	Kayser and Aron (1938, 1950)
Parathyroid	Yes	Kayser and Aron (1950); Kayser et al. (1961)
Pancreas	Yes	Kayser and Aron (1950)
Adrenal cortex	Yes	Kayser and Aron (1938, 1950); Stoeckel et al. (1964a, b)
Adrenal medulla	No	Kayser and Aron (1950)
Bones	Yes	Kayser and Frank (1963)
Teeth	Yes	Kayser and Frank (1963)

2011; Rusin et al. 2013). Even in countries in which intense protection measures are in place, the species has lost about 75 % of its range within the past 15 years (Albert 2013; Reiners et al. 2014). Consequently, a severe loss of genetic diversity is observed (Banaszek et al. 2009; La Haye et al. 2012). The complex reasons for this decline are not understood (Monecke 2013) and modern agricultural practices (Weinhold and Kayser 2006) or climate change (Neumann et al. 2005) can only partly explain the present situation. More research on the physiology of the European hamster is needed to save it from extinction. The current review summarizes the present status quo with the focus on seasonal and circannual rhythms.

Table 14.5 Circannual variations in physiological parameters

		Constant conditions			
	Parameter	PP	Temperature (°C)		Circannual rhythm? Literature
General physiology	Reproductive state males	LD 16:08	20	Yes	Masson-Pévet et al. (1994)
	Hibernation	LD 10:14	20	Yes	Masson-Pévet et al. (1994)
		LL	20	No	Canguilhem et al. (1973)
		LL	7	Yes	Canguilhem et al. (1973)
		LD 16:08	20	Yes	Monecke et al. (2011b)
		LD 12:12	20	No	Canguilhem and Petrovic (1974)
		LD 12:12	15	No	Kempf et al. (1978)
		LD 12:12	7	Yes	Canguilhem and Petrovic (1974)
		DD	20	No	Canguilhem et al. (1973)
		DD	7	Yes	Canguilhem et al. (1973)
	Food intake	LD 12:12	15	Yes	Canguilhem et al. (1977)
	Body weight males	LL	20	Yes	Canguilhem et al. (1973)
		LL	7	Yes	Canguilhem et al. (1973)
		LD 16:08	20	Yes	Masson-Pévet et al. (1994)
		LD 12:12	23	Yes	Canguilhem and Bloch (1966)
		LD 12:12	20	Yes	Canguilhem and Petrovic (1974)
		LD 12:12	15	Yes	Canguilhem et al. (1977)
		LD 12:12	7	Yes	Canguilhem and Petrovic (1974)
		LD 10:14	20	No	Masson-Pévet et al. (1994)
		LD 08:16	15	Yes	Canguilhem et al. (1992)
		LD 08:16	7	Yes	Canguilhem et al. (1992)
		DD	20	Yes	Canguilhem et al. (1973)
		DD	7	Yes	Canguilhem et al. (1973)
	Body weight females	LD 16:08	20	Yes	Canguilhem et al. (1992)
		LD 08:16	15	Yes	Canguilhem et al. (1992)
	Ovarian weight	LD 12:12	20	Yes	Hilfrich et al. (1977)

Daily rhythms	Amount of activity		LD 15:09	20	Yes	Monecke and Wollnik, unpublished
			LD 12:12	20	Yes	Monecke and Wollnik, unpublished
			LD 09:15	20	Yes	Monecke and Wollnik, unpublished
			DD	20	Yes	Wollnik, unpublished
	Phase angle of activity onset		LD 15:09	20	Yes	Monecke and Wollnik, unpublished
			LD 12:12	20	Yes	Monecke and Wollnik, unpublished
			LD 09:15	20	Yes	Monecke and Wollnik, unpublished
	Body temperature		LD 16:08	20	Yes	Monecke et al. (2011b)
	Oxygen consumption		LD 08:16	23	Yes	Mletzko and Raths (1972)
			LD 08:16	6	Yes	Mletzko and Raths (1972)
Hormones	Testosterone		LD 12:12	15	Yes	Canguilhem et al. (1986)
	Thyroxine (T_4)		LD 12:12	15	Yes	Canguilhem et al. (1986)
	Triiodothyronine (T_3)		LD 12:12	15	Yes	Canguilhem et al. (1986)
	LH		LD 16:08	20	Yes	Sáenz de Miera et al. (2014)
Molecular level	Myosin isoenzyme expression in the left ventricle of the heart		LD 12:12	22	No	Morano et al. (1992)
	Turnover rate of brain serotonin	Pons Medulla	LD 12:12	15	Yes	Kempf et al. (1978)
		Hypothalamus	LD 12:12	15	No	Kempf et al. (1978)
		Anterior telencephalon	LD 12:12	15	No	Kempf et al. (1978)
		Amygdala	LD 12:12	15	Yes	Kempf et al. (1978)
		Hippocampus	LD 12:12	15	Yes	Kempf et al. (1978)
	Turnover rate of brain nor-adrenalin	Pons medulla	LD 12:12	15	Yes	Kempf et al. (1978)
		Hypothalamus	LD 12:12	15	Yes	Kempf et al. (1978)
		Anterior telencephalon	LD 12:12	15	Yes	Kempf et al. (1978)
	GABA	Olfactory bulb	LD 12:12	15	Yes	Ciesielski et al. (1985)
		Hypothalamus	LD 12:12	15	Yes	Ciesielski et al. (1985)
		Cerebellum	LD 12:12	15	Yes	Ciesielski et al. (1985)
	TSHβ mRNA	Pars tuberalis	LD 16:08	20	Yes	Sáenz de Miera et al. (2014)

(continued)

Table 14.5 (continued)

	Parameter		Constant conditions		Rhythmic?	Literature
			PP	Temperature (°C)		
	Dio2 mRNA	Paraventricular zone of the third ventricle	LD 16:08	20	Yes	Sáenz de Miera et al. (2014)
		Tuberoinfundibular sulcus	LD 16:08	20	Yes	Sáenz de Miera et al. (2014)
	Rfrp mRNA	Dorsomedial/ventromedial hypothalamic nuclei	LD 16:08	20	Yes	Sáenz de Miera et al. (2014)
	Kiss1 mRNA	Arcuate nucleus	LD 16:08	20	Yes	Sáenz de Miera et al. (2014)
Urinary excretion	Urine volume		LD 12:12	23	No	Canguilhem and Bloch (1966)
			LD 12:12	23	Yes	Harberey et al. (1967)
	Urinary sodium excretion		LD 12:12	23	Yes	Harberey et al. (1967)
	Urinary potassium excretion		LD 12:12	23	No	Harberey et al. (1967)
	Urinary aldosterone excretion		LD 12:12	23	Yes	Canguilhem and Bloch (1966)
	Urinary chromogen Porter-Silber reaction (urinary amount of adrenal steroids)		LD 12:12	23	No	Canguilhem and Bloch (1966)
			LD 12:12	20	No	Canguilhem and Petrovic (1974)
			LD12:12	7	Yes	Canguilhem and Petrovic (1974)
	Urinary catecholamines excretion		LD 12:12	23	No	Canguilhem and Bloch (1966)
	Urinary aMT6s excretion		LD 15:09	20	No	Monecke and Wollnik, unpublished
			LD 12:12	20	No	Monecke and Wollnik, unpublished
			LD 09:15	20	No	Monecke and Wollnik, unpublished

LH luteinizing hormone, *Dio2* type 2 thyroid hormone deiodinase, *RFRP* RF-amide related peptide, the kiss1 gene codes for kisspeptin. For further details see Table 14.1

14.2 The Circannual Clock and Its Resetting

It is essential that the seasonal cycle of physiological adaptations keeps entrained to the yearly environmental oscillation generated by the rotation of the earth around its tilted axis. The principal *Zeitgeber* in most species are the seasonal changes in photoperiod (Gwinner 1986; Goldman 2001; Gorman et al. 2001; Paul et al. 2008), the most predictable environmental signal. Basically, two strategies of entrainment to photoperiodic changes have evolved. Photoperiodic species as Syrian (*Mesocricetus auratus*) or Siberian hamsters (*Phodopus dsungorus*) react at all times of the year to changes in photoperiod with the appropriate change in physiology (Gaston and Menaker 1967; Elliott et al. 1972; Turek and Campbell 1979; Hoffmann 1982). These long-day breeders are reproductive whenever the photoperiod is longer than a critical value and their gonads regress whenever photoperiod falls below that value. In constant conditions they are only able to induce the development of gonads but not their regression endogenously. Circannual species such as sheep, sika deer, marmots, many hibernators (Pengelley 1974; Canguilhem 1985; Gwinner 1986; Boissin and Canguilhem 1988), and European hamsters (Masson-Pévet et al. 1994) are able to undergo both changes in the reproductive cycle (i.e., gonadal development and regression) endogenously without any environmental information, and changes in photoperiod are only needed at particular phases of the cycle to entrain the circannual rhythm to 1 year (Paul et al. 2008).

The anatomical basis of these timing mechanisms, especially for complete circannual rhythms, has yet to be elucidated. In mammals, lesions of different brain areas, among them the suprachiasmatic nuclei (SCN), which host the circadian clock, were ineffective to suppress circannual cycles (for review, Zucker 2001). An interesting pituitary-based circannual timing mechanism was proposed from results in Soay sheep (Lincoln et al. 2006), involving the pineal hormone melatonin in the rhythm generation process. However, European hamsters show perfect circannual rhythms even when they are pinealectomized (Masson-Pévet et al. 1994). We have recently discovered that the circannual clock seems to be located upstream (Sáenz de Miera et al. 2014) of the neuroendocrine pathway initiated in the pars tuberalis by the release of thyroid-stimulating hormone (TSHβ) and culminating in the activation of GnRH neurons for the photoperiodic control of seasonal reproduction. This pathway is also under circannual control (Sáenz de Miera et al. 2014).

In European hamsters the *Zeitgeber* signal (the changes in photoperiod) needs to fall into one of two sensitive phases to be effective. Such effective times are either the sensitive phase to short photoperiods between mid-May and mid-July (Saboureau et al. 1999) and thus, around the summer solstice, or the sensitive phase to long photoperiods around the winter solstice, which starts in mid-November and lasts until spontaneous gonadal development (Monecke and Wollnik 2004). Only during these phases does an experimental change in photoperiod (a shortening in the former and a lengthening in the latter) lead to an immediate change in the gonadal state. In natural conditions, the short-day signal is perceived as early as in mid-July, when the photoperiod falls below LD (ratio light:darkness) 15.5:8.5 (at ~48°N, critical photoperiod)

(Canguilhem et al. 1988). These data demonstrate the extreme sensitivity of the European hamster to changes in photoperiod during this sensitive phase, as in mid-July it has decreased from maximal values by ~30 min only, or by ~1 min per day.

The photoperiodic information is nevertheless perceived during all seasons, but similar to other circannual species (Duston and Bromage 1988; Randall et al. 1998; Nisimura and Numata 2001; Miyazaki et al. 2005, 2006), in European hamsters the direction and magnitude of a photoperiod-induced phase shift is a function of the endogenous phase during which a photoperiodic pulse is applied (Monecke et al. 2009). Such data can be plotted in a so-called phase-response curve (PRC). Thus, the entrainment follows the nonparametric model (Pittendrigh and Daan 1976). Although during most phases of the subjective year a 1-month short photoperiod (SP, LD 10:14) pulse has little or no phase-shifting capacities in animals kept in constant long photoperiod (LP, LD 16:08), it shifts the reproductive cycle by as much as up to ±180° in the middle of subjective summer, which corresponds to the timing of the sensitive phase to short photoperiod (Monecke et al. 2009). Similarly, a 1-month LP pulse in animals kept in constant SP is most effective in the middle of subjective winter when the animals are sensitive to LP; however, the magnitude of induced phase shifts is much lower compared with the effect of an SP pulse in subjective summer. For European hamsters it is thus the decrease in photoperiod that is the most important resetting signal (Monecke et al. 2009, 2010).

Circannual resetting follows in general the same principles as circadian resetting (see Chap. 16, this volume). (1) The shape of circannual PRCs resembles closely that of circadian PRCs. (2) The magnitude of seasonal phase shifts is dependent on the timing of the pulse. (3) A *Zeitgeber* stimulus simultaneously shifts the phase of the endogenous rhythm and changes its velocity (Beersma et al. 1999). In European hamsters, an LP pulse in subjective winter accelerates the cycle by advancing the onset of the reproductive phase and even more so the subsequent offset (Monecke et al. 2009). (4) Circannual resetting follows the limit cycle model (Johnson 1999; Johnson et al. 2004). According to this model, *Zeitgeber* stimuli simultaneously shift the state variable to another isochron and alter the oscillation's amplitude, in the most extreme case to zero, when the oscillation stops; this has been excellently proven for circannual rhythms (Miyazaki et al. 2007) and supported by results in European hamsters (Monecke et al. 2009) and sheep (Woodfill et al. 1994).

Other *Zeitgebers* than the photoperiod such as food, water, ambient temperature, and social cues are also known to alter seasonal traits (Paul et al. 2008), even though their interyear variation is so strong that they are unlikely to be the main *Zeitgeber* for circannual entrainment. For example, temperature can alter the period length of the circannual cycle in constant conditions (Mrosovsky 1985). During winter, high temperatures can advance and long-lasting cold temperatures can delay reproduction onset, as has been shown in ground squirrels (*Spermophilus lateralis*) (Barnes and York 1990) or European hamsters (Hufnagl et al. 2011). Seasonal temperature variations are able to entrain the body weight rhythm in European hamsters kept in constant darkness (Canguilhem and Petrovic 1974). When a 12-month temperature cycle was applied at the same time with a 6-month photoperiodic cycle, the animals followed the latter. Photoperiod is thus a stronger *Zeitgeber* than temperature (Canguilhem and Petrovic 1974).

14.3 Which Mechanisms Are Involved?

The current opinion of photoperiodism is summarized by Gorman et al. (2001): "There is no evidence to suggest that the fundamental mechanisms of photoperiodism are different in photoperiodic and circannual rhythms. Indeed both types of rhythms appear to depend on a circadian mechanism to measure day length, and the pineal gland is an important part of the photoperiodic mechanism in both." Although in principle this is still correct, we provide here new insights in the European hamster that certain aspects of photoperiodic and circannual resetting show fundamental differences.

The hormone melatonin is considered to play the key role in the synchronization process of seasonal rhythms by photoperiod. The production of melatonin by the pineal gland is limited to nighttimes, and transports thus a time message: its absence coincides with day, its presence with night, and its peak duration reflects the seasons, because it varies with the night length (Steinlechner 1992; Reiter 1993). Melatonin injections cause photoperiod-like changes in the reproductive state of intact or pinealectomized mammals (Bittman et al. 1983; Bartness and Goldman 1989; Pévet and Pitrosky 1997). Long-duration melatonin injections cause winter response (Steinlechner 2011) and season-specific infusions entrain circannual rhythms (Woodfill et al. 1994).

European hamsters show an extreme seasonality in the melatonin production, with long-lasting high-amplitude peaks in winter, and an extremely reduced production between mid-May and mid-July when nightly melatonin barely rises above daytime values (Vivien-Roels et al. 1992, 1997). That means, however, that the lack of a melatonin peak coincides precisely with the sensitive phase to short photoperiod (Saboureau et al. 1999; Monecke and Wollnik 2005), just when the animals are "waiting" for the short-photoperiod signal used for the principal resetting of the circannual clock (Monecke et al. 2009). Moreover, during the opposite sensitive phase when melatonin levels are high, the melatonin receptors are drastically reduced (Skene et al. 1993), which led to the question whether European hamsters can reset their circannual clock without melatonin. Indeed, recently we could demonstrate that pinealectomized European hamsters do entrain to changes in photoperiod in four physiological parameters (reproductive state, body weight, activity pattern, and body temperature) for as many as six cycles (Monecke et al. 2013). The discovery of the existence of such a melatonin-independent pathway to transduce photoperiodic information is fundamental after the role of melatonin in the transduction of the photoperiodic message was thought to be exclusive.

In view of these new insights, a thorough literature search revealed that such a melatonin-independent pathway might also exist in other circannual species. After pinealectomy or superior ganglionectomy, golden mantled ground squirrels (*Spermophilus lateralis*) (Hiebert et al. 2000), minks (*Mustela vison*) (Martinet and Allain 1985), wolves (*Canis lupus*) (Asa et al. 1987), edible dormice (*Glis glis*) (Jallageas et al. 1993), and Soay rams (Lincoln et al. 1989) entrained to (simulated) natural changes in photoperiod. Originally, it was concluded that entrainment must have been achieved by other seasonal *Zeitgebers* than photoperiod because most experiments were done outdoors. However, in view of the new data from European hamsters it is possible, and appears even likely, that the observed precise entrainment in pinealectomized or superior ganglion-ectomized circannual mammals was caused by photoperiod by the same melatonin-independent pathway as in European hamsters. Contrastingly, pinealectomized photoperiodic species are severely impaired in processing of photoperiodic information (Reiter 1974, 1975; Petterborg et al. 1981; Johnston et al. 1982).

Two observations in sham-operated and pinealectomized European hamsters kept in an accelerated photoperiodic regime (6-month cycle) showed that the melatonin-independent pathway acts on the circannual clock rather than directly on the gonadal axis as in a photoperiodic response (Monecke et al. 2013). (1) The animals entrained with a negative seasonal phase angle to the fast-changing *Zeitgeber* cycle compared with animals in a natural cycle. If it was a pure photoperiodic reaction, the reproductive phase would have been observed at the same photoperiods as outdoors. (2) In these conditions, a few animals were observed that initially entrained to each second cycle but later entrained to the 6-month cycle; this would not be possible if it were a photoperiodic reaction.

The discovery of a melatonin-independent pathway does not mean that melatonin is ineffective in European hamsters. For example, melatonin infusions are able to induce gonadal regression (Masson-Pévet et al. 1987); thus, the well-known pathway, which is called, in the following, the melatonin-dependent pathway, is functional. How it acts in intact European hamsters is not clear after the recent discovery of the melatonin-independent pathway. After the perception of the short photoperiod signal in mid-July (Saboureau et al. 1999), an increase in nightly melatonin production is observed (Vivien-Roels et al. 1992, 1997; Monecke and Wollnik 2005). This reappearing melatonin peak might then be interpreted as a short-day signal. However, because we could not observe the slightest impairment of entrainment in most pinealectomized animals in any of the measured parameters (Monecke et al. 2013), it seems rather unlikely that melatonin acts on the central circannual clock mechanism, especially as this would imply that it acts at the same time on the same structure as the melatonin-independent pathway. A possible alternative is that it acts on another structure, namely, directly on the gonadal axis as in photoperiodic species without affecting the circannual clock, and furthermore, that it acts at another time, namely outside the sensitive phases when neither melatonin (Vivien-Roels et al. 1992; Monecke and Wollnik 2005) nor its receptors in the pars tuberalis (Skene et al. 1993) are lacking. Because the circannual clock cannot be entrained between sensitive phases (Monecke et al. 2009), the proposed mechanism might serve as corrective measure during this time in case of a poor synchronization of the circannual clock. An effect of melatonin on the reproductive axis as in photoperiodic species would then ensure an appropriate timing of the seasonal physiology until the circannual clock can be reset in the next sensitive phase.

The question remains why we observe this extremely strong reduction in melatonin production between mid-May and mid-July. It is possible that melatonin would disturb the very precise perception or the processing of the resetting SP signal in mid-July, that is, at a season when day-to-day changes in photoperiod and advances in sunset are minimal (photoperiod decreases then by less than 1 min per day at 48°N). Whether melatonin can act during the sensitive phase to the long photoperiod around the winter solstice is also unclear: it is present, but its receptors in the pars tuberalis are dramatically reduced (Skene et al. 1993).

In spite of these open questions, it is clear that in European hamsters two pathways exist, a melatonin-independent one that is involved in the principal resetting of the circannual clock, and a melatonin-dependent one, which might (also) act directly on the reproductive axis as in photoperiodic species. Also, in the circannual Soay sheep some findings suggest that both pathways exist. After pinealectomy they are able to entrain to the natural year (Lincoln et al. 1989), but melatonin infusions are also able to phase shift and entrain the reproductive cycle (Woodfill et al. 1994).

14.4 The Interplay Between the Circadian and the Circannual Clock

It is well known that circadian rhythms can be altered by seasonal processes (Refinetti 2012). Furthermore, in the European hamster we could show that there is a complex interaction between the circadian and the circannual clock and a mutual control. The circadian clock in the suprachiasmatic nuclei (SCN) has a crucial role in photoperiodic time measurement (Goldman 2001). In the European hamster it is even the centerpiece of the melatonin-independent pathway for circannual synchronization and it drives the daily rhythms of activity and melatonin production. During 10 months of the year the melatonin production is rhythmic, but the activity pattern is extremely weak or even arrhythmic (Wollnik et al. 1991) and might thus transiently be uncoupled from the SCN, as suggested by the model of Bloch et al. (2013). Only between mid-May and mid-July, exactly during the sensitive phase to short photoperiods, the activity rhythm becomes very robust, the onset precise, and the phase angle φ of entrainment positive (activity starts before night onset) (Monecke and Wollnik 2005). The change between an imprecise “winter pattern” and a precise “summer pattern” occurs abruptly at the onset and offset of the sensitive phase. It should be stressed that the occurrence of the rhythmic summer pattern is endogenously driven by the circannual clock and that it is dependent on neither sex steroids nor a certain photoperiod, for several reasons: (1) Changes between summer winter pattern are also observed in different constant photoperiods and even in castrated animals kept in constant darkness (Wollnik and Monecke, unpublished data). (2) An animal that was out of phase with the natural year showed this pattern around the winter solstice before it reentrained (Wollnik et al. 1991). (3) The duration of the summer pattern is about 3 months shorter than the reproductive phase (Monecke and Wollnik 2005) (Fig. 14.1). However, in natural conditions the circannual clock induces only the onset of the summer pattern. The offset is induced by natural photoperiod when it falls below LD 15.5:8.5 in mid-July (at ~48°N) (Monecke and Wollnik 2005). Our current model of the seasonal entrainment by

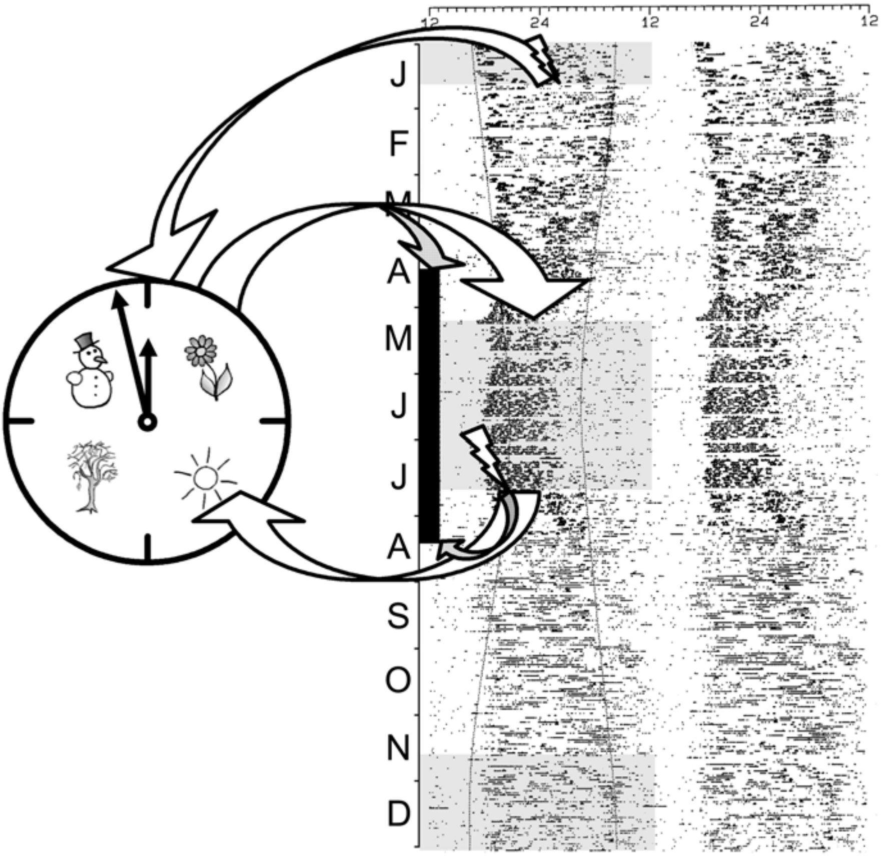

Fig. 14.1 Photoperidic time measurement and circannual resetting in the European hamster. Representative double plotted actogram of a female European hamster kept in natural photoperiods for an entire year. The *curved lines* indicate sunset and sunrise, respectively; *vertical black bar*, duration of the reproductive phase; *grey areas*, duration of the sensitive phases. Note the positive phase angle of circadian entrainment during the sensitive phase around the summer solstice. At its end the advance in sunset is recognized (*white flash*), the animal switches back to the winter pattern, gonadal regression is initiated, and the circannual clock (*left*) is reset. This circannual clock then times the onsets of the next year's reproductive and sensitive phase. Photoperiodic changes during the sensitive phase around the winter solstice contribute to a lesser extent to the resetting. Melatonin is, if at all, only a minor factor, in this process

this mechanism involving the circadian clock (visible in the activity pattern) and the circannual clock is presented in Fig. 14.1 (see also following explanation).

With an asymmetrical lengthening of the night phase it was experimentally proven that the SCN, which drives the daily activity rhythm, has a key role in the transduction of the photoperiodic message to the circannual clock in European hamsters (Monecke et al. 2006). When only lights-off was advanced by 8 h, all hamsters reacted instantly by the typical change in activity to the "winter" pattern. In contrast, none of the hamsters that were subjected to an 8-h delay of lights-on reacted immediately: they switched to the winter pattern only between 8 and 46 days after the transfer. The variability after such a strong external stimulus indicates an endogenously induced change rather than a reaction to the delayed lights-on. A remarkable linkage was observed between the behavioral level and the reproductive state: In each individual of both groups, the gonads started to regress after a very similar time span after the change in the activity pattern, indicating that gonadal regression was initiated at the very same day on which the activity pattern changed back to the winter pattern (Monecke et al. 2006). Consequently, gonadal regression started later and with a higher variability after a delay in lights-on. Interestingly, when European hamsters experienced in June an acute asymmetrical extension of the night into the morning, Fos-protein in the SCN was not inducible by a light stimulus in the additional hours of darkness (Vuillez et al. 1996), which might indicate that the SCN is insensitive to light in the morning.

It seems thus that European hamsters measure the changes in sunset time to obtain information about the season (Monecke et al. 2006) instead of being aboveground the whole day to read the full length of the photoperiod. The same is known from African stonechats (*Saxicola torquatus axillaris*), which use the clear seasonally varying but parallel changes in sunset and sunrise time as seasonal *Zeitgeber* in their equatorial habitat where photoperiodic changes are lacking (Goymann et al. 2012). That the European hamster measures the advances in sunset explains why activity onset has to precede dusk during the sensitive phase between mid-May and mid-July; that it does so in free-ranging animals is confirmed by several field studies (Wendt 1989; Schmelzer and Millesi 2003; Schmelzer 2005; Ziomek 2011). After the summer solstice, the advances in sunset relative to the stable activity onset indicate to the European hamster already in mid-July (Saboureau et al. 1999) that the days shorten below the critical value of 15.5 h of daylength (at ~48°N) (Canguilhem et al. 1988).

The change in the activity pattern, especially at the offset of the sensitive phase, is so abrupt that it is also an argument against the possibility that in intact European hamsters melatonin takes part in the circannual resetting mechanism. From Siberian hamsters it is known that it takes at least 3–14 days before the amplitude and duration of the melatonin peak start to increase after a transfer from LD 16:08 to LD 08:16 (Illnerová et al. 1984). Moreover, in the European hamster, as in many species, melatonin peaks in the second half of the night, and in long photoperiods it is sharply terminated by lights-on (Vivien-Roels et al. 1997). It seems thus, that for melatonin lights-on is a more potent environmental parameter in contrast to the lights-off signal for circannual resetting. And finally, pinealectomized animals show identical changes in the activity pattern and were not impaired in their entrainment ability (Monecke et al. 2013).

The changes in the activity pattern at the beginning and end of the sensitive phase are characteristic and can thus be used as a marker for the sensitive phase. These changes are reflected in two other circadian parameters. Oxygen consumption is elevated when the European hamsters are active. Before mid-July the peak shows a similar positive phase angle, φ, as the activity. Thereafter, the nightly peak of oxygen consumption delays and the phase angle φ becomes negative (Raths and Mletzko 1971; Mletzko and Raths 1972). In body temperature, the daily mean (Monecke et al. 2011b) and the strength of the daily rhythm (Wollnik and Schmidt 1995) are considerably higher between mid-May and mid-July than in the rest of the year.

Such characteristic activity or temperature patterns at a certain season are unknown in typical photoperiodic hamster species (Gattermann 1985; Scherbarth and Steinlechner 2008; Weinert et al. 2009). In contrast, they are mostly found in circannual species and if so only around the solstices similar to European hamsters. This might be a hint that other circannual species (see examples following) have a similar circannual resetting mechanism.

Most obvious similarities are seen in circannual species sharing similar photoperiodic constraints as the European hamsters, that is, a lack of photoperiodic information during a part of the year because of hibernation or living above the polar circles. Golden mantled ground squirrels (*Spermophilus lateralis*) show identical gonad-independent changes in the activity pattern in constant conditions (Lee et al. 1986; Lee and Zucker 1995; Freeman and Zucker 2000).

Arctic ground squirrels (*Urocitellus parryii*) are diurnal during their active season. Similar to European hamsters, they show changes in the activity pattern in early June and mid-July. Between these dates the activity offset is clearly delayed, and activity ends precisely each day at the same time (Williams et al. 2011a). After mid-July, activity offset advances with the shortening of days. Furthermore, body temperature of males and nongestating females shows a rise in mid-May and stays on this elevated level until mid-July (Williams et al. 2011b).

Diurnal reindeers (*Rangifer tarandus tarandus*) experience a kind of opposite photoperiodic schedule and show the opposite responses. During the polar day between mid-May and mid-July, their circadian activity rhythm is lost. A unimodal precise activity pattern, similar to the summer pattern in European hamsters, is expressed during the short days between mid-November and mid-January (van Oort et al. 2005, 2007), that is. also for a 2-month period but around the winter solstice. The reproductive period is well synchronized between individuals; thus, they might use the opposite solstices for entrainment.

Alhough complete yearly actograms or temperature recordings are mostly missing, some similar changes in circadian parameters could be found in other species. They have in common that they occur, as in the European hamster, between mid-May and mid-July:

- Badgers (*Meles meles*) are predominantly nocturnal, but between mid-May and end of July emergence from the sett occurs before sunset and the return after sunrise; this seems not to be caused by short nights around the summer solstice but by an increased activity duration (Kowalczyk et al. 2003).
- In European ground squirrels (*Spermophilus citellus*) the phase angle of activity onset is quite constant over the active season because activity onset varies and parallels sunrise time. Only between mid-May and mid-July it is vice versa, and

the time of activity onset stays constant, but the phase angle varies (Hut et al. 1999; Everts et al. 2004).

- Cape ground squirrels (*Xerus inauris*) show very precise and constant emergence and immergence times between late May and late July; thereafter, timing is more variable and emergence time advances and immergence time delays progressively (Scantlebury et al. 2012). However, because the squirrels' habitat is in the Southern Hemisphere, this time interval comprises the winter solstice. For this species the photoperiodic signal in winter seems more reliable than in summer when animals frequently enter the burrow in daytime because of heat (Scantlebury et al. 2012).
- Although the phase angle of the activity onset is usually negative in flying squirrels (*Glaucomys volans*), they entrain with a positive phase angle between mid-May and mid-July. The magnitude depends on the latitude of recording (Daan and Aschoff 1975).
- Common voles (*Microtus arvalis*) show an abruptly increased nightly feeding activity between mid-May and mid-July and a change in the diurnality index (Hoogenboom et al. 1984).
- Red deer (*Cervus elaphus*) show elevated amounts of activity and increased diurnality between mid-May and mid-July (Georgii 1981; Georgii and Schröder 1983; Arnold et al. 2004). Moreover, heart rate peaks in mid-May (Arnold et al. 2004). Activity increases later for a second time at the rut season.
- During most of the active season, the phase angle of activity onset is positive in brown bears (*Ursus arctos*), except between mid-May and mid-July, when it becomes negative (Ware et al. 2012). However, further activity parameters change abruptly during other seasons.
- Similar to European hamsters (Monecke et al. 2011b), in Alpine marmots (*Marmota marmota*) euthermic temperature also increases after the hibernation period and reaches a high plateau between May and July before it decreases again toward the next hibernation phase (Arnold et al. 2011).

In all these species the circadian output is changed for a 2-month period around one solstice, mostly toward a more precise rhythm. Although it has not been proven for any of the species, it is worth considering the possibility that they synchronize their circannual cycle also around a solstice and that they use a similar mechanism as European hamsters involving a special circadian organization. These changes in the circadian organization occur in mammalian species living at various latitudes at the same (or exactly opposite) season. Thus, circadian organization may also in other circannual species indicate a functional state, that is, a phase in which the circannual clock is sensitive for the resetting photoperiodic signal. If this hypothesis is true, there would be another fundamental difference between circannual entrainment and photoperiodic synchronization apart from the degree of melatonin dependency, namely, the season when changes in photoperiod are effective. The critical photoperiod in all photoperiodic species tested so far ranges between LD 12:12 and LD 13:11 (Gaston and Menaker 1967; Hoffmann 1982; Rhodes 1989), which occurs in natural conditions around the equinoxes. In contrast, a special circadian organization, as seen in some circannual species, is observed around a solstice.

14.5 Both Pathways in Action: The Ontogeny of European Hamsters

The offspring of a circannual species in which females can have several litters per year faces two major challenges: (1) they need to know at which season they are born, either to become reproductive soon or to start the preparation of hibernation; and (2) the circannual clock, which times the onset of the next year's reproductive phase, needs to be synchronized for the first time. It seems that these are two different processes (Monecke et al. 2014).

Juvenile European hamsters show an activity pattern that is identical to the adult summer pattern until postnatal day 78 (Monecke et al. 2014), whether they are born before, during, or after the occurrence of the summer pattern (or the sensitive phase) in adults. Only thereafter do they switch to the appropriate activity pattern of the season. This special activity pattern in juveniles might thus also reflect a sensitive phase. It serves probably the same purposes as in adults, namely, the crucial resetting of the circannual clock, even though it might occur at other seasons than in adults.

However, at the first switch to the winter pattern, when the circannual clock is synchronized for the first time, the onset of the subsequent (i.e., next year's) reproductive phase is triggered. This mechanism thus cannot time a possible reproductive phase in the year of birth because gonads might start to develop as early as postnatal day 21 in pups of early-born litters (Kirn 2004) and thus before the switch in the activity pattern. Moreover, in contrast to adults, which reach sexual quiescence about 2 months after the change in the activity pattern, most litters reach sexual quiescence around or even before the activity pattern changes to the winter pattern (Monecke et al. 2014). Consequently, the seasonal events in the year of birth have to be timed by another mechanism. It might be a pure photoperiodic mechanism involving maternal programming prenatally (Horton and Stetson 1992) and the melatonin pathway postnatally. The requirements for that exist at least. Female European hamsters produce, even around the summer solstice, small amounts of melatonin, which was shown by the urinary excretion of 6-sulphatoxymelatonin (Monecke and Wollnik 2005); this might be sufficient to transport the seasonal message to the embryos. Moreover, in juveniles the urinary excretion of 6-sulphatoxymelatonin is considerably higher than in adults, so that it is likely that the former produce melatonin even between mid-May and mid-July (Monecke et al. 2014).

14.6 Conclusions

This chapter gave an overview of the resetting of the circannual reproductive cycle in adult and juvenile European hamsters. Although it follows, in general, in all major points the current concept of seasonal timekeeping, it differs in fundamental details from photoperiodic species. (1) In contrast to the latter, the resetting of the circannual clock is largely melatonin independent in European hamsters.

Other circannual species probably also use such a melatonin-independent pathway for seasonal entrainment. (2) In European hamsters, a special circadian organization seems to be necessary to measure daylength (advances in sunset). Some of such seasonal changes in circadian parameters are found in other circannual but not in photoperiodic species. Further research is necessary to investigate whether these mark also a functional state as in European hamsters. Neither in European hamsters nor in other circannual species does melatonin seem to be ineffective as transducer of the photoperiodic message, but some important aspects suggest that melatonin may not act on the circannual clock mechanism. Instead, it might act in many circannual species directly on the gonadal axis as in photoperiodic species. We propose that two distinct processes ensure accurate seasonal timekeeping in circannual species: a circannual circadian-based but melatonin-independent one and a photoperiodic melatonin-dependent one. The extent to which each is used might be dependent on both species and age.

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Chapter 15
Circannual Clocks in Tropical Bats and Heritable Variation in Seasonal Reproductive Timing in Temperate Zone Mice

Paul D. Heideman

Abstract Two continuing puzzles in clock biology are the synchronizing mechanisms (zeitgebers) for endogenous circannual clocks in tropical organisms and the neuroendocrine sources of heritable variation in annual timing in natural populations. Tropical species of mammals are often as highly seasonal as temperate zone mammals. In many bats, these may be regulated by an endogenous circannual clock whose zeitgeber is unknown, while rodents may be more likely to rely on direct stimulation or inhibition of reproduction by current nutrient sources and environmental stressors. Heritable physiological variation must underlie the microevolution of seasonal timing in relationship to annual clocks and annual cycles. The nature of this normal variation in clock output pathways is largely unknown. In a population of field mice from Virginia, USA, data from selection lines and a random-bred control line have been used to determine heritable variation in neuroendocrine systems, metabolism, and behavior in relationship to heritable variation in reproductive responses to winter photoperiod. Variation at multiple neuroendocrine sites (e.g., density of melatonin binding, number of immunoreactive gonadotropin-releasing hormone neurons, amount of luteinizing hormone secretion, differences in metabolic rate) may combine and interact to cause heritable variation in winter phenotype.

Keywords Circannual rhythm • Heritable variation • Rodent • Seasonal reproduction • Tropical Chiroptera

Abbreviations

AMDR	Average daily metabolic rate
BMR	Basal metabolic rate
C	Control

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FSH	Follicle-stimulating hormone
GnIH	Gonadotropin inhibitory hormone
GnRH	Gonadotropin-releasing hormone
LD	Long photoperiod
LH	Luteinizing hormone
MBH	Mediobasal hypothalamus
NR	Nonresponsive
R	Responsive
RFRP	RF-amide-related peptide
SCN	Suprachiasmatic nucleus
SD	Short photoperiod
T_3	Thyroid hormone
TSH	Thyroid-stimulating hormone

15.1 Introduction

The nature of entraining cues to seasonal rhythms in tropical mammals and the amounts, kinds, and sources of variation in seasonal clock function are important questions in mammalian physiology and ecology. Most mammals are tropical, including ancestral humans. In nonhuman mammals there is evidence of ecologically meaningful variation both within and across populations in seasonality (Bronson and Heideman 1994; Heideman and Pittman 2009; Prendergast et al. 2001). The situation is less clear for human seasonality (Bronson 2004; Foster and Roenneberg 2008; Prendergast et al. 2009; Wehr 2001), but variation in human responses could account for the diverse findings for humans (Bronson 2004; Prendergast et al. 2009). Humans certainly contain medically important variation (Stearns 2012), which should include variation in human responses to seasonal cues (Bronson 2004). Thus, for both mammalian evolutionary ecology and human biology, these two enigmas are important: (1) What are the entraining cues for the calendars of tropical mammals? and (2) What is the nature of heritable variation in seasonal clock function? Because seasonal rhythms tend to affect most other systems, it is valuable to understand both the basis of seasonality and variability in seasonality. Heritable variation in seasonal clocks and clock outputs is the raw material for evolution of seasonal timing.

In this chapter, I review these two enigmas. My focus is on long-term, multiyear studies conducted with my collaborators and students on potential environmental regulators of seasonal reproduction in tropical mammals as well as heritable variation in seasonality in temperate zone rodents, in both the field and the laboratory. I use the terms “seasonal clock” and “seasonal calendar” interchangeably to refer to the neuroendocrine pathway(s) that result in a seasonal pattern. I use the terminology of Zucker et al. (1991) for rhythms of type I (“hourglass timer” restarted annually by an environmental cue), type II (endogenous circannual rhythm with an entraining cue), or type III (direct responses to a seasonal event, with no internal timer or clock). Central to these questions is the necessity for seasonal calendars to

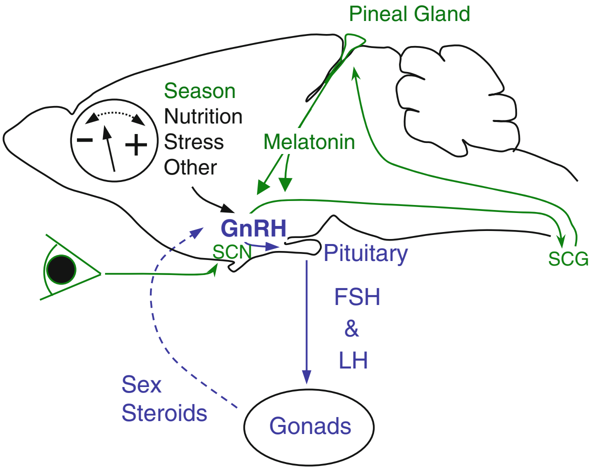

Fig. 15.1 The hypothalamic-pituitary-gonadal (HPG) axis (in *blue*) of the reproductive system is regulated by multiple inputs, including season (photoperiod pathway, in *green*, beginning with the eye at left), nutrition, and stress (in *black*). Genetic variation could occur in the photoperiodic pathway, in nonphotic inputs to gonadotropin-releasing hormone (*GnRH*) neurons, in GnRH neurons themselves, or elsewhere in the HPG axis, and each might be altered by microevolutionary change. At the neuroendocrine level, microevolutionary changes may include numbers of neurons or hormone-secreting cells, activity of these cells, abundance of receptors or binding of receptors in target cells, and strength of response to receptor binding, represented in the figure by an adjustable rheostat. *FSH* follicle-stimulating hormone, *GnRH* gonadotropin-releasing hormone, *LH* luteinizing hormone, *SCG* superior cervical ganglion, *SCN* suprachiasmatic nucleus. (Modified from Heideman and Pittman 2009)

send information to the hypothalamus in the brain to regulate the reproductive axis: hypothalamus, anterior pituitary gland, and gonads (Fig. 15.1).

15.2 Seasonal Calendars of Tropical Mammals

Most terrestrial mammals are tropical, and even in the tropics a seasonal component to reproductive timing is common, especially in species with long life expectancy, such as bats (Bronson 1989; Bronson and Heideman 1994). Bats comprise more than one-fourth of all species of mammals, and 80 % of these species are tropical. Most tropical bat populations reproduce with some seasonal variation in timing, including populations living on or near the equator (Heideman 2000; Racey 1982; Racey and Entwistle 2000). Selective forces favoring seasonal reproductive patterns include seasonal variation in ease of foraging, seasonal cycles in food resources, predator satiation or seasonal predator avoidance, advantages of intraspecific cooperation (e.g., clustering of juveniles for warmth), and intra- and interspecific competition (Bronson and Heideman 1994; Heideman 2000; Racey and Entwistle 2000) (and see Goymann and Helm, this volume).

15.2.1 Seasonal Rhythms in Wild Populations of Tropical Bats

To understand any clock or calendar, we need to understand its accuracy and precision in normal use. In other words, how good is the clock? We need both laboratory data and quantitative field data covering multiple years. Unfortunately, studies with data on precise timing of the same events in the same population over multiple years are slow, expensive, and still rare. Because of the difficulty of estimating birthdates for mammals, data are often presented as monthly percentages of individuals that are in categories such as pregnant, lactating, or nonreproductive. Such data are problematic in mammals because they do not allow precise quantitative estimation of average timing and variability in timing (Heideman and Utzurrum 2003). The most useful data for mammals are the dates for particular events (e.g., birthdates or weaning dates) or quantitative measures that can be used to predict birthdates, such as estimates of embryo size (Heideman 2000).

From the studies available, we know that the seasonal clocks of mammals can be as predictable and regular as those of mammals in the temperate zones. Some populations of bats within 10°N latitude of the equator are able to achieve high synchrony and high seasonality (Heideman 1995). Multiparous adult females in a population of the fruit bat *Haplonycteris fischeri* at 9°N latitude on Negros Island, Philippines, produced pups in a narrow temporal window of about 5 weeks or less in each year (Heideman 1988, 1989b). Timing of births on Negros Island differed by as much as 2 months from timing on two other islands in the Philippines at nearly the same latitude and only 100–200 km apart, but was indistinguishable from the timing of births in a population much farther north on Luzon at 18° latitude (Heideman 1988). Among years, there was statistically significant variation in mean timing, but the differences for *H. fischeri* between years were no more than 3 weeks (Heideman 1988, 2000). Three other species of fruit bats (*Harpyionycteris whiteheadi*, *Nyctimene rabori*, and *Ptenochirus jagori*) in the same location on Negros had similarly narrow ranges of timing of births, with statistically significant variation in mean timing among years of up to 3 weeks (Heideman 2000). In data collected on Negros Island over 6 different years for another species, *Rousettus amplexicaudatus*, the average timing of births differed by less than 3.5 weeks across the 6 years, although with statistically significant differences in timing among years (Heideman and Utzurrum 2003). Pups of *H. fischeri* born during a drought year had significantly higher mortality than in a wet year, suggesting that the timing of birth in relationship to the wet season has fitness consequences (Heideman 1988). Historical rainfall records showing variability of wet–dry seasons indicate that the timing of births for *H. fischeri* would fall in dry periods in about one-third of years. Interestingly, for three species (*H. fischeri*, *P. jagori*, and *R. amplexicaudatus*), "primigravid" (first-time pregnant) females differed statistically in timing of births from adults, with later births by about 1–2 months for all three species (Heideman and Utzurrum 2003; Heideman 1989a, b, 1995). Some other species of fruit bats in the Philippines have been reported to have broader (even much broader) peaks with lower synchrony (Heideman and Utzurrum 2003; Heideman 1995). More recently, Bumrungsri et al. (2007) reported significant synchrony and seasonality of reproduction in a population of *C. brachyotis* from 13°N latitude in Thailand, with statistically significant differences between years in the average timing of lactation and pregnancy. In a 4-year study of a population of *Saccopteryx bilineata* at 10°N latitude at La Selva Costa Rica, Greiner et al. (2011) reported timing of estrus occurring only in December. In a 3-year study of the nectarivore *Anoura geoffroyi* at 10°N latitude from Trinidad, births occurred in late November and early December in each year (Heideman et al. 1992). Data in that study were not adequate to test for significant differences among years. In a 4-year study of the fruit bat *Phyllostomus hastatus* at 10°N latitude from Trinidad, Porter and Wilkinson (2001) reported narrow birth peaks of about 1 month in each of 4 years, with significant differences in timing both among years and among caves on the island within single years.

Considered together, these and other data allow five inferences about the annual calendars of tropical bats. First, the level of synchrony and similarity of seasonality among years can be comparable to seasonally reproducing temperate zone populations of bats and other mammals. Tropical bats are able to time reproductive events within narrow windows of time: within 1–2 months in each year (Greiner et al. 2011; Heideman 2000; Porter and Wilkinson 2001; Racey 1982; Racey and Entwistle 2000). The annual calendars of tropical bats within 10° latitude of the equator can be as precise and accurate as the annual calendars used by temperate zone bats (the latter possibly entrained by photoperiod) (Beasley et al. 1984b; Beasley and Zucker 1984). Second, because the timing of births and lactation can vary significantly among years (Bumrungsri et al. 2007; Heideman and Utzurrum 2003; Heideman 1988, 1995; Porter and Wilkinson 2001), the output of the annual calendars of at least some tropical bats is not solely dependent upon an environmental cue such as photoperiod that provides the same information in every year. For example, Porter and Wilkinson (2001) proposed that social cues modify the timing of births in the tropical bat *Phyllostomus hastatus*. Third, because first-year primigravid females of some species give birth at a significantly different time than multiparous females, but in their second and later reproductive years synchronize with other multiparous females (Heideman and Utzurrum 2003; Heideman 1988, 1995), we can presume that there are developmental events that can adjust the timing of events controlled by tropical seasonal clocks. Fourth, the timing of reproduction is not tightly linked to any single point on the annual calendar, but can be tied to different times of year. Individuals in different populations at the same latitude can have different timing of reproduction (Heideman and Utzurrum 2003; Heideman 1988, 1995; Porter and Wilkinson 2001), presumably using the same neuroendocrine annual calendar. In support of the same inference, primigravid females have different peaks of birth than multiparous adults (Heideman and Utzurrum 2003; Heideman 1988, 1995), although these first-time mothers, just 1 year later, adjust their timing of births to that of other multiparous adults. Fifth, these differences in timing are not removed by gene flow. For example, populations of *H. fischeri* only 100–200 km apart have different average timing of births (Heideman 1989b), indicating significant variation between nearby populations.

15.2.2 Environmental Cues and Events That Entrain or Drive Seasonal Rhythms in Tropical Bats

Entraining cues for seasonal rhythms must be assessed in the laboratory. The problem for field studies is that any circannual reproductive and biological rhythm *must* be correlated with any annual environmental change (Heideman 2000). For type I and type II rhythms, a single environmental cue at any time of year would be sufficient, in principle, to allow all remaining events in that year to be controlled by an internal timer (type I rhythms) or internal circannual clock (type II rhythms). Only for type III rhythms is it necessary that environmental events occur at the same time as the physiological activity they control. Detailed field data over multiple years can, however, help to eliminate potential entraining cues. However, those field data are useful only to falsify potential connections, in cases where reproductive timing and environmental cues do not match when compared over multiple years. For example, evidence that the timing of births changes significantly among years (Bumrungsri et al. 2007; Heideman and Utzurrum 2003; Heideman 1988, 1995; Porter and Wilkinson 2001) rules out any cue that is exactly the same in each year (such as photoperiod) as the sole entraining agent for a rhythm. Even so, that evidence does not rule out photoperiod as one of two or more cues that act in combination.

Data on whether seasonal rhythms of reproduction in tropical bats are type I, II, or III rhythms are limited. Species of tropical and temperate zone bats that have been held in constant conditions in the laboratory and found to have endogenous circannual rhythms of reproduction include, from the temperate zone, *Antrozous pallidus*, at 38°N latitude (Beasley et al. 1984a; Beasley 1985–1986), and from the tropics *Anoura geoffroyi* (Heideman and Bronson 1994; Heideman 2000) and *Phyllostomus hastatus* (Porter and Wilkinson 2001), both at 10°N latitude. Thus, bats, including tropical bats, can have type II rhythms, but more tests of tropical seasonal rhythms are needed.

What might be the environmental cues that entrain or drive seasonal rhythms in tropical bats? Entraining cues would need to connect sensory input containing the information to the reproductive system. Seasonal photoperiodic information has been hypothesized to affect the reproductive system through one or more of three inputs (Fig. 15.2a–c). Any of these three are potential inputs for nonphotoperiodic seasonal

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Fig. 15.2 (continued) RFRP), inducing gametogenesis and sex steroid secretion, respectively. **c** Melatonin may act directly or indirectly on kisspeptin neurons in the arcuate nucleus or anterior periventricular nucleus that in turn stimulate GnRH secretion (Dardente 2012; Tsutsui et al. 2013). Of these three hypotheses, mechanism **a** has the most detailed support (Dardente 2012; Ikegami and Yoshimura 2012). Any one, two, or three of these mechanisms may be involved in photoperiodic inputs to seasonal reproduction. *DIO* deiodinase, *FSH* follicle-stimulating hormone, *GnIH* gonadotropin inhibitory hormone, *GnRH* gonadotropin-releasing hormone, *KISS* kisspeptin, *LH* luteinizing hormone, *MBH* mediobasal hypothalamus, *Mel* melatonin, *PT* pars tuberalis of the pituitary stalk, *RFRP* RF-amide-related protein, *SCN* suprachiasmatic nucleus, *T* testosterone, T_3 active thyroid hormone, T_4 inactive thyroid hormone, *TSH* thyroid-stimulating hormone. *Large dashed rectangle*, MBH; *small dashed oval*, anterior pituitary; *larger dashed oval*, gonads. Receptors and receptor binding are indicated by shaped *boxes* that fit with Mel, TSH, or shapes indicating GnRH (*circles*), kisspeptin (*hexagons*), RFRP (*triangles*), testosterone (T), Mel, or TSH

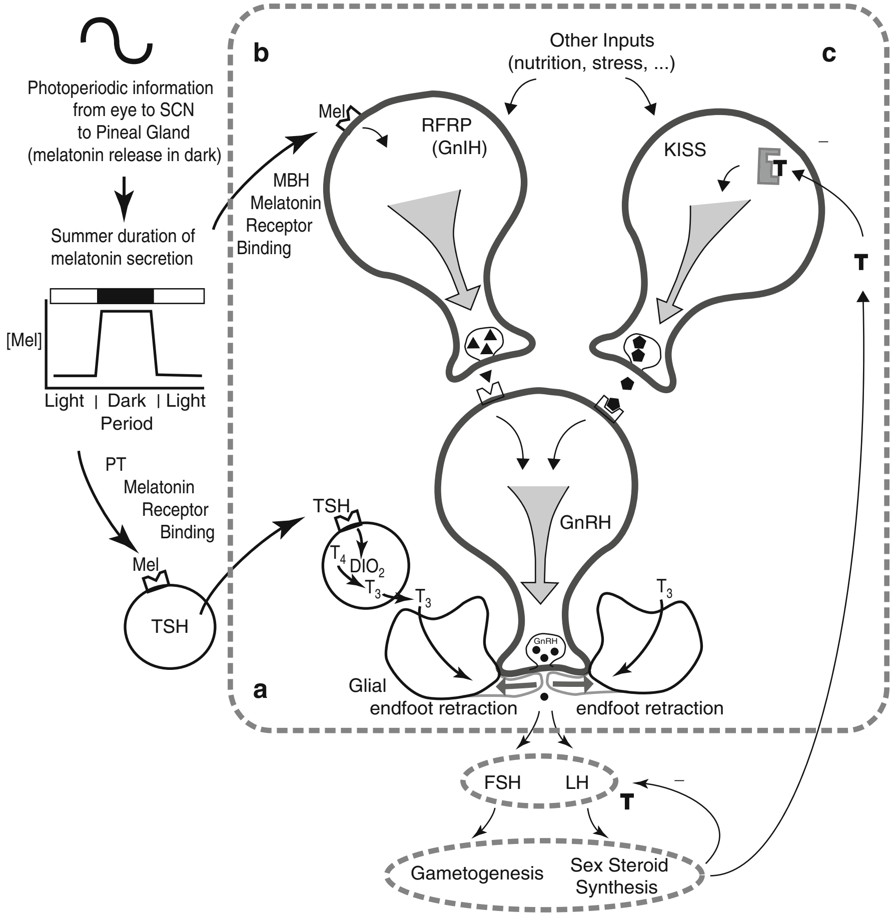

Fig. 15.2 Three hypothesized mechanisms of action of photoperiod in the mediobasal hypothalamus (MBH; large *dashed rectangle*) to regulate the reproductive system of mammals (**a**, **b**, **c**). As the first stage for all three mechanisms, photoperiodic information from the eye passes through a circuitous pathway to the pineal gland, causing melatonin secretion only during the dark period. The duration of the melatonin signal directly indicates the duration of the night, thereby providing a physiological indicator for season. In mammals with 6-month gestation periods, a shortening photoperiod is stimulatory, whereas a long photoperiod is stimulatory in photoperiodic rodents. (**a**) A stimulatory pattern of melatonin secretion causes TSH release from cells in the pars tuberalis of the pituitary gland, increasing TSH levels, but only in the area of the pituitary stalk and MBH. The paracrine action of TSH induces the enzyme deiodinase-2 in cells in the MBH to convert inactive T_4 to active thyroid hormone T_3. In the median eminence of the basal hypothalamus, T_3 induces glial endfoot retractions that are interposed between GnRH terminals and capillary basement membrane, allowing increased release of GnRH. In the absence of T_3, glial endfeet extend beneath GnRH terminals to block GnRH access to capillaries. The increase in GnRH secretion causes FSH and LH release from the anterior pituitary (*small dashed oval*), which cause gametogenesis and sex steroid secretion, respectively, in the gonads (*larger dashed oval*). (**b**) In an alternative or parallel pathway (Tsutsui et al. 2013), a stimulatory melatonin signal acts on RFRP (GnIH) neurons to alter their inhibition of GnRH neurons of neurons that secrete GnRH. An increase in the frequency of GnRH pulses causes FSH and LH release from the anterior pituitary (potentially also regulated by

cues as well. In the temperate zone and high latitudes of the tropics (>15°N or S latitude), photoperiod is the typical zeitgeber used to entrain circannual clocks (Bronson 1989; Bronson and Heideman 1994), but seasonal photoperiodic change decreases in the tropics, down to approximately 1 h of annual variation at 10°N and S latitude and zero hours of change on the equator. In theory, photoperiod is a potentially useful cue down to 5°N latitude, and perhaps even closer to the equator (Hau et al. 1998). Temperate zone hamsters can respond reproductively to a pattern of changing photoperiod equivalent to 5° latitude, although timing was imprecise (equivalent to ± perhaps 1–2 months) (Heideman and Bronson 1993). One species of subtropical bat, *Pteropus poliocephalus*, from latitude 28° S has been shown to have reproductive responses to photoperiod, along with changes in melatonin secretion in short nights versus long nights (McGuckin and Blackshaw 1992; O'Brien et al. 1993), but the changes were so slight that O'Brien et al. (1993) concluded that photoperiod is likely to be only a minor environmental cue controlling seasonal reproduction. Several species of temperate zone bats have been shown to respond reproductively to changes in photoperiod or to melatonin (*Scotophilus heathi*, in vitro only; Singh and Krishna 1995; *Antrozous pallidus*; Beasley et al. 1984b), but other temperate species might not (Racey and Entwistle 2000).

Below latitudes of approximately 10–15°N, mammals appear not to rely upon photoperiod to entrain seasonal rhythms (Bradshaw and Holzapfel 2007; Bronson and Heideman 1994). The tropical species *Anoura geoffroyi* from a highly seasonal population at latitude 10°N is capable of producing nocturnal melatonin secretion that is longer in duration in long nights than in short nights but appears not to use photoperiod or melatonin to regulate their reproduction (Heideman and Bronson 1994; Heideman et al. 1996). Photoperiod and melatonin treatments did not modify the 7-month period of the seasonal endogenous rhythm of testis size in *Anoura geoffroyi*, nor did changes in food quality, changes in humidity, or attempts to simulate seasonal changes in sun position, lunar cycles, or polarized light (Heideman 2000). However, sun position (which varies in the sky and on the horizon even on the equator), lunar cycles, and changes in polarized light were not simulated in a convincingly natural fashion (Heideman 2000). There is evidence that tropical birds can use nonphotoperiodic solar cues to regulate reproductive rhythms (Goymann et al. 2012; Gwinner and Scheuerlein 1998), which suggests that nonphotoperiodic solar cues might be usable by mammals as well.

In principle, nonphotoperiodic calendar inputs could regulate seasonal reproduction in tropical mammals using any of the three inputs hypothesized to link photoperiod to reproduction. All three ultimately alter the output of gonadotropin hormone-releasing neurons. Nonphotoperiodic inputs could regulate any of these: release of thyroid-stimulating hormone (TSH) within the brain to activate events that allow GnRH release (Fig. 15.2a), action on RF-amide-related peptide (RFRP, also referred to as gonadotropin inhibitory hormone, GnIH) neurons to regulate GnRH secretion (Fig. 15.2b), or action on kisspeptin neurons that stimulate GnRH secretion (Fig. 15.2c). Methods are available to test these pathways in wild-source tropical mammals for changes in activity during seasonal reproductive transitions.

15.2.3 Environmental Cues and Events That Entrain or Drive Seasonal Rhythms in Tropical Rodents

Tropical species of rodents have many populations that are seasonal in reproduction (Bronson and Heideman 1994), but the limited evidence suggests that many have type III rhythms. Many tropical rodents may be affected by seasonal events that directly inhibit or stimulate reproduction. For example, when a highly seasonally reproducing population (8°N latitude) of the Venezuelan cane mouse *Zygodontomys brevidauda* was tested in the laboratory, neither photoperiod nor melatonin affected reproduction (Bronson and Heideman 1992; Heideman and Bronson 1990). Photoperiod altered the duration of melatonin secretion, showing that this population had the ability to detect seasonal changes in photoperiod (Bronson and Heideman 1992). Finally, even treatments such as food restriction and neonatal testosterone that have unmasked or uncovered photoperiodic responses in other rodents did not affect reproductive timing in *Z. brevicauda* (Bronson and Heideman 1992). It appears that this population reproduces whenever environmental conditions and food are adequate. A highly seasonal population of Costa Rican (10°N latitude) cloud forest mice (*Peromyscus nudipes*) did not alter fertility in response to changes in photoperiod (Heideman and Bronson 1992). Instead, in the dry season, poorer nutrition and reduced body mass appear to suppress embryonic implantation. Females ovulate and mate throughout the year, but only during the wet season, when body mass is high, are the resulting embryos allowed to implant and develop (Heideman and Bronson 1992). These two tropical populations are both highly seasonal in the production of young, but use underlying opportunistic strategies in what appear to be type III rhythms (Bronson and Heideman 1992; Heideman and Bronson 1992).

At present, we need more laboratory tests of the types of circannual rhythms and possible entraining cues for tropical mammals.

15.3 Heritable Variation in Seasonal Clocks

Heritable variation in phenotype is a critical element in understanding seasonal calendars. There is good evidence for variation among natural populations (reviewed by Bronson 1989; Bronson and Heideman 1994) and for heritable variation within natural populations (reviews by Blank and Desjardins 1986; Blank 1992; Bronson 1989; Bronson and Heideman 1994; Heideman and Pittman 2009; O'Callaghan et al. 1992; Prendergast et al. 2001). We know much less about the underlying physiological causes and heritability of physiological variation in circannual calendars and calendar outputs.

To understand the physiological and genetic causes of variation in seasonal calendars in natural populations, we must have data on variation within and between natural populations. Inbred laboratory rodents can indicate target genes and physiology to test, but laboratory strains of rodents lose natural variation while accumulating mutations that would not persist in nature. For example, some laboratory populations of rodents have been shown to be reproductively nonphotoresponsive because of defects in the circadian clock that block normal clock function (reviewed by Majoy and Heideman 2000). In contrast, individuals from recently wild-derived populations that are reproductively nonphotoresponsive appear to have fully functional and normal circadian clocks (e.g., our reproductively nonphotoresponsive selection line of white-footed mice; Majoy and Heideman 2000). In fact, these reproductively nonresponsive individuals retain photoperiodic control of some traits, such as body mass, even though they largely dissociate fertility from calendar control (Heideman 2004).

15.3.1 Heritable Variation in Seasonal Timing Within Populations and the Potential for Evolutionary Change

When individuals within a population differ in seasonal reproduction, are the differences based upon different responses to clock outputs, or are the seasonal clocks different? In other words, do some individuals have seasonal clocks that are damaged such that seasonal alarms never go off? Alternatively, do some individuals have a reproductive axis that is insensitive to seasonal alarms—insensitive to seasonal neuroendocrine chemical signals that occur through each year? In both scenarios, genetic differences among individuals or populations should be the result of genetic variation within neurons and hormonal systems, either in the clock itself or in the responses to the clock. For reproduction, a connected series of at least six different cell types have direct effects on fertility. For each of these cell types in the pathway from clock outputs through the reproductive axis, it is possible to have heritable variation in (1) the expression of the receptor proteins for chemical messages, (2) the numbers of cells of that type, (3) the strength of the response to receptor binding, and (4) the amount of chemical message released in response to a particular stimulus (see Fig. 15.3 for a model illustrating effects of some categories of variation on fertility).

To identify sources of variation in these pathways in a single natural population, my laboratory has tested for neuroendocrine genetic variation in a life history trait, winter reproductive timing (Heideman and Pittman 2009), that is known to be genetically variable in multiple populations and species of temperate zone rodents (Bronson and Heideman 1994; Prendergast et al. 2001). Here, I review this heritable variation in clock function in relationship to seasonal reproduction and other seasonal physiological changes.

15.3.2 Environmental Heterogeneity in Reproductive Timing

Winter reproductive suppression in temperate zone rodents is commonly induced by the environmental signal of the short photoperiod of winter. The long winter nights allow a long duration rise in melatonin each night (Goldman 2001). Rodents use

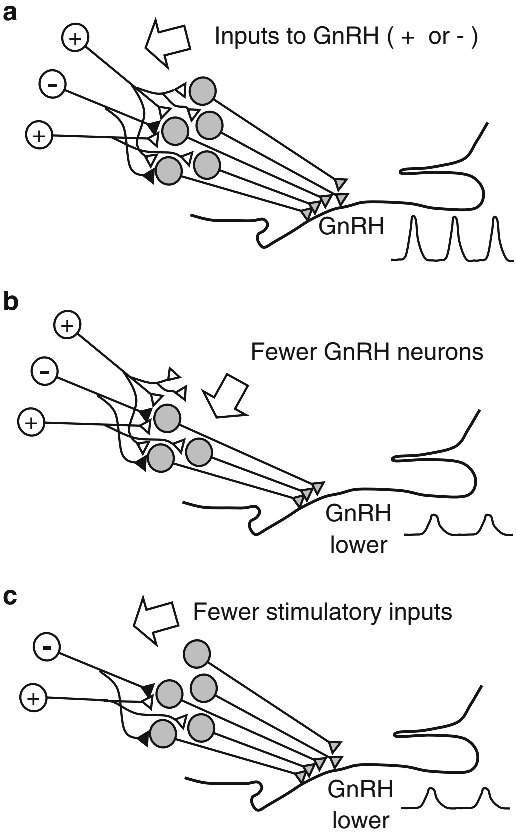

Fig. 15.3 A conceptual model of genetic neuroendocrine variation leading to variation in function of the reproductive axis. Each panel shows a sagittal view of the base of the hypothalamus with GnRH neurons projecting to the median eminence; anterior is to the left. (**a**) GnRH neurons (*gray*) receive multiple stimulatory inputs (labeled + with *white axon terminals*) and inhibitory inputs (labeled – with *black axon terminals*) from neurons. In this model, GnRH pulse output, and therefore the function of the reproductive axis, depends in part upon the summed stimulatory and inhibitory input to GnRH neurons. (**b**) Individuals with fewer GnRH neurons, each receiving the same summed stimulatory and inhibitory input as in (**a**), secrete less total GnRH. The result is lower amplitude or lower frequency of GnRH pulses. In this case, the reduction in GnRH neurons is predicted to cause an individual to be less likely to maintain reproductive function under any conditions; conversely, an increase in number of GnRH neurons is predicted to cause an individual to be more likely to maintain reproductive function under any conditions. (**c**) Alternatively, individuals with a reduction in neural stimulatory input (white axon terminals) to GnRH neurons will have reduced stimulation of GnRH neurons, resulting in reduced secretion of GnRH. As in (**b**), the result is lower amplitude or lower frequency of pulses of GnRH. Not shown: heritable variation in receptor abundance or internal cell signaling could also result in heritable variation in reproduction function. (Modified from Heideman and Pittman 2009)

both the duration of elevated melatonin and the direction of lengthening/shortening change of nightly melatonin as seasonal cues (Prendergast et al. 2009). Temperate zone rodents appear to rely directly on the changing photoperiod signal as their primary indicator of season (Bronson and Heideman 1994) using either type I or II rhythms (Prendergast et al. 2009). A functioning circadian clock is necessary for rodents to have calendar responses to changes in photoperiod (Goldman 2001). One hypothesis for the cause of variation in seasonal reproduction is variation in the ability of the photoreceptor cells and circadian clock to detect changes in photoperiod and produce a melatonin signal that indicates the season (Majoy and Heideman 2000; Prendergast et al. 2001). An alternative hypothesis is that variation in seasonal reproduction is not caused by alterations in the seasonal clock but rather by variation in the sensitivity of the reproductive axis to a seasonal melatonin signal. An additional hypothesis is that heritable variation is likely to occur at multiple points in complex physiological pathways (Heideman and Pittman 2009). My students, collaborators, and I have addressed these hypotheses by assessing variation in the circadian system and reproductive system in mice from a natural population of white-footed mice, *Peromyscus leucopus*, founded from individuals captured in 1995 near Williamsburg, VA, USA (latitude 37°N, longitude 76°W) (Heideman et al. 1999a).

15.3.2.1 Animal Model

The following description of our population and selection lines is summarized from other publications (Avigdor et al. 2005; Broussard et al. 2009; Heideman and Pittman 2009). White-footed mice are found throughout most of eastern North America. Populations vary highly in the pattern of timing of reproduction, including populations with continuous reproduction (some southern populations), summer reproduction (some northern populations), winter reproduction (some southern populations experiencing seasonal drought with winter rains), and fall/spring reproduction (some populations at intermediate latitudes, including our study population (Terman 1993; Bronson and Heideman 1994). Females can produce several litters per year, with a 3-week gestation period, producing litters ranging in size from two to eight offspring. Offspring become independent at 3–4 weeks of age, and reproductive maturity can occur at age 46–60 days under permissive conditions. Mortality from predation is generally high, and particularly in winter, mortality may be highest when mice must expose themselves to forage for food or seek mates. Typical wild individuals probably live 1–4 months, with few wild individuals achieving even 1 year in age (Schug et al. 1991), but individuals in the laboratory may remain healthy and fertile for 2–3 years. Because this species does not hibernate, wild individuals must be active and must find food throughout the year. There is a trade-off for white-footed mice in costs and benefits of suppressing reproduction in the winter. Suppressing reproduction can increase the probability of survival to a more favorable season. Thus, suppressing reproduction in harsh seasons may bring a fitness advantage (Heideman et al. 2005). However, if winter mortality is high, then suppressing reproduction may lead to death before the chance to reproduce. If so, then reproduction in harsh seasons may produce sufficient offspring to outweigh the risk costs and energetic costs of reproduction (Heideman et al. 2005).

High levels of variation in winter breeding may be a result of heterogeneous selection pressures. Individual home ranges of mice may vary in food abundance and risk of predation. Because *P. leucopus* are generalist granivores and omnivores that live in a wide range of habitats, mice that are near-neighbors in a population may experience widely varying winter food abundance and foraging conditions. Abundant acorn "mast" with superabundant acorns under oak trees occurs in mast fruiting years, which may contrast with far lower densities of small seeds under pines or shrubs in nearby home ranges of mice (Elias et al. 2004; McCracken et al. 1999; Scarlett 2004). Winter foraging time and risk may be highly variable from one home range to another, and additional variation occurs from year to year. Selection pressures for or against winter reproduction may be highly heterogeneous within populations, favoring winter breeding in some home ranges or in some years but winter suppression in other home ranges or years (Heideman et al. 2005). This kind of variable selection can maintain genetic variation within a population.

It is unsurprising that populations of *P. leucopus* (and its close relative, *Peromyscus maniculatus*; Blank and Desjardins 1986; Blank et al. 1988; Blank and Freeman 1991; Blank 1992; Desjardins et al. 1986; Korytko et al. 1995; Korytko et al. 1998; Mintz et al. 2007) have been found to hold substantial genetic variation. In the laboratory, individuals range from fully fertile in short photoperiod to infertile in short photoperiod (Heideman and Bronson 1991; Heideman et al. 1999a; Lynch and Gendler 1980). In the source population for our colony, for example, approximately 20–50 % of adults are in reproductive condition during mid-winter (Heideman et al. 1999a; Terman 1993). In white-footed mice, there appears to be widespread, genetically based variability in the photoneuroendocrine pathway that regulates reproduction (Heideman and Bronson 1991; Heideman et al. 1999a; Prendergast and Nelson 2001). In North America, species in the genus *Peromyscus* are particularly well studied (*P. leucopus*), and see papers by Blank and collaborators on *P. maniculatus* (Blank and Desjardins 1986; Blank et al. 1988; Blank and Freeman 1991; Blank 1992; Desjardins et al. 1986; Korytko et al. 1995; Korytko et al. 1998; Mintz et al. 2007). Many other reports suggest that this level of variation may be common among temperate zone small rodents that are granivores or omnivores and remain active in winter (Prendergast et al. 2001).

15.3.2.2 Selected and Control Lines of Mice

We developed bidirectional artificial selection lines and one unselected control line to study the sources of physiological variation in reproductive responses to winter (Heideman et al. 1999a). Forty-eight wild-caught mice were bred to establish a parental laboratory generation. Pups in the parental generation were conceived and born in long photoperiod (16L:8D; LD), transferred to short-day photoperiod (8L:16D; SD) within 3 days of birth, weaned at 21–23 days of age, and singly housed in polyethylene cages with wire tops and pine shavings until 70 ± 3 days of age. At 70 days of age, mice were assessed for reproductive condition and body mass. From 208 individuals in the parental generation, a subset was chosen at random to form breeding pairs initiating an unselected control (C) line. From the remainder, individuals that were relatively reproductively immature were used to found a line that was relatively strongly suppressed reproductively by short photoperiod, termed responsive (R) to photoperiod (Heideman and Bronson 1991; Heideman et al. 1999a). Individuals that were relatively unsuppressed, with large gonads, were used to found a line that was weakly suppressed reproductively by short photoperiod, termed nonresponsive (NR) to photoperiod. After several generations, selection criteria were made more stringent. By the third generation (Heideman et al. 1999a), most mice in the R line were reproductively suppressed when raised in short photoperiod, whereas most mice in the NR line had gonads typical of individuals raised in long photoperiod (Heideman et al. 1999a). Neither line became truly nonresponsive to short photoperiod; even after selection, both the R line and NR line responded to SD with an identical decrease in body mass and food intake (Heideman et al. 2005). In addition, both lines had smaller reproductive organs in SD than in LD. In the NR line, however, most individuals mature reproductively even in SD (see Avigdor et al. 2005; Heideman et al. 2005).

Experiments to identify potential neural and endocrine sources of variation between the lines began after the third generation. Additional information on the selection lines and their reproductive phenotypes are available elsewhere (Broussard et al. 2009; Heideman et al. 1999a, 2005; Heideman and Pittman 2009).

15.3.3 Evidence for Variation in Circadian Clock Function Among Lines

The potential neuroendocrine causes of differences in reproduction between the R and NR selection lines fall into two categories. First, the neuroendocrine circuitry that measures photoperiod could be variable between lines, resulting in different outputs to the reproductive axis. Second, the sensitivity of the reproductive axis to suppression could be different between lines, resulting in stronger reproductive suppression in SD in the R line despite receiving the same outputs from the neuroendocrine circuitry that measures photoperiod. In mammals, this photoperiodic input is carried from the retina in the eye through the retinohypothalamic tracts to the hypothalamus (Ebling 2005) (Fig. 15.1). The hypothalamic pathway includes the suprachiasmatic nucleus (SCN) and paraventricular nucleus, from which neurons connect to the superior cervical ganglia, and finally back via sympathetic neurons to the pineal gland (Ebling 2005). The pineal gland produces melatonin only at night. The pattern of change of the melatonin signal and duration of elevated melatonin regulates fertility. As noted earlier, variation might occur in more than 6 major cell types, with potential variation in numbers, receptor expression, internal cell responses to receptor–ligand binding, and amount of chemical signal released per episode of cell stimulation. Figure 15.3 shows a model for regulation of reproduction by heritable variation in cell numbers or the combination of stimulatory and inhibitory inputs to a particular cell type (Heideman and Pittman 2009).

As noted briefly earlier, photoperiod may affect reproduction via three hypothesized pathways. A short-day pattern of melatonin alters thyroid hormone production localized to the mediobasal hypothalamus (MBH) (Nakao et al. 2008; Ono et al. 2008). Active thyroid hormone (T_3) causes retraction of glial end feet that allows access of GnRH neuron terminals to the pituitary portal system (Dardente 2012; Ikegami and Yoshimura 2012). Melatonin can affect RFRP (GnIH) neurons in the dorsomedial nucleus to regulate GnRH neuronal activity. Finally, melatonin or T_3 may act on kisspeptin neurons in the anteroventral periventricular nucleus or arcuate nucleus (Dardente 2012; Tsutsui et al. 2013) (Fig. 15.2). GnRH regulates secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary, which regulate gametogenesis and production of sex steroid hormones, respectively, in the gonads. RFRP neurons have been proposed to act on pituitary cells that secrete FSH and LH in addition to RFRP effects on GnRH neurons. Individuals can differ at any of these sites or at downstream sites. Any allele that blocks or partially suppresses these photoperiodic inputs to the reproductive axis would alter responsiveness to winter photoperiod. In the single wild population that we study, there is evidence for heritable variation at many points in the pathway (summarized schematically in Fig. 15.4).

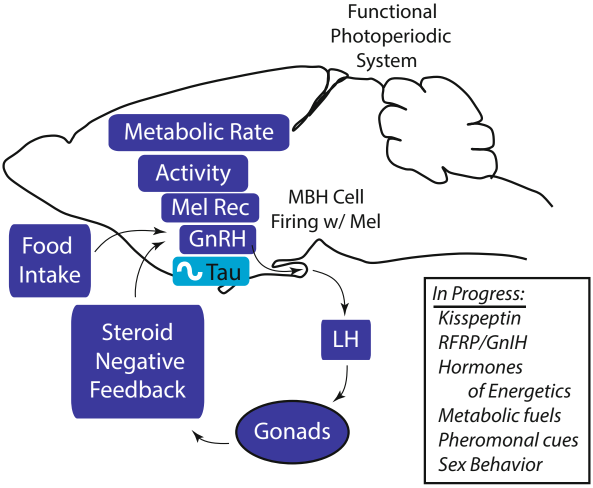

Fig. 15.4 Sources of heritable variation related to reproductive photoresponsiveness identified in a single, natural-source population of a small mammal (*Peromyscus leucopus*, white-footed mouse) from 37°N latitude in Virginia, USA. *White text* in *dark blue boxes* indicates significant heritable differences between selection lines. *Black text* indicates no evidence for heritable variation. *Black text* "Tau" in the *light blue box* indicates heritable variation in a circadian rhythm trait (free-running period, or tau) that appears to be unrelated to reproductive photoperiodism. Testing in progress is indicated by italicized words in the *text box*. Additional details are provided in the text. *GnRH* gonadotropin-releasing hormone, *LH* luteinizing hormone, *MBH* mediobasal hypothalamus, *Mel Rec* melatonin receptor binding, *RFRP/GnIH* RF-amide-related peptide/gonadotropin inhibitory hormone

15.3.4 Evidence for Variation in the Circadian Clock and Outputs to the Reproductive Axis

Mice in our NR selection line differed significantly from R mice in a circadian rhythm trait (free-running period longer in NR mice than R mice) (Majoy and Heideman 2000). A long free-running period can cause photoperiod nonresponsiveness in laboratory rodents. However, this difference appears not to be the cause of nonphotoresponsiveness in our mice, as housing nonresponsive mice in constant dark did not induce reproductive photoresponsivenss (Majoy and Heideman 2000). In vitro, both selection lines held neurons in the preoptic area of the hypothalamus that altered neuronal firing rate in response to melatonin treatment, but responses of neurons from the two lines were not significantly different (Fetsch et al. 2006). The selection lines had melatonin receptors in the same regions of the brain, but differed in the amount of iodomelatonin binding (Heideman et al. 1999b). The NR line had significantly higher levels of iodomelatonin binding than the R line, suggesting a potential source of variation in reception of the SD signal. However, this heritable difference suggests greater photoperiodic inputs based on season to the nonresponsive line (Heideman et al. 1999b), a difference that we cannot explain.

Both lines have retained the capacity to detect and respond functionally to an SD. Both lines had lower body mass and lower food intake in SD relative to LD (Heideman et al. 2005). Both lines had higher brown adipose tissue (BAT) in SD than in LD (Reilly et al. 2006). Thus, the seasonal calendar is functional and intact in both lines.

15.3.5 Evidence for Variation in Sensitivity of the Reproductive Axis to Environmental Inputs

The lines differ in reproductive traits even in a long photoperiod. The NR line has had larger gonads than the R line when raised in either a summer-like long photoperiod or a winter-like short photoperiod (Avigdor et al. 2005). Both lines are inhibited reproductively by SD; the important difference is that despite inhibition by SD, the NR line in SD has sperm counts and testes size similar to those of control line mice in LD (Avigdor et al. 2005). In contrast, our R line has high sperm counts and larger testes only in LD. Why?

One possibility is that some mice are genetically prone to maintain fertility under any stressors. If so, we hypothesized that if NR mice had more GnRH neurons available to stimulate the reproductive axis, then NR mice might be more resistant to reproductive inhibition by SD. Our finding matched that hypothesis. In both long and short photoperiods, our NR line had 50 % more immunoreactive (IR) GnRH neurons than the R line (Avigdor et al. 2005). Furthermore, in the C line, there was significant heritability in the number of IR-GnRH neurons (Heideman et al. 2007), confirming that this is a heritable trait in this population. Given these heritable differences in GnRH neurons, it is possible that seasonal clock output to GnRH neurons in both lines suppresses fertility in some mice (represented by our R line) but is insufficient to prevent fertility in others (our NR line) (Fig. 15.3b).

Pituitary reproductive hormones are secreted in response to GnRH from the hypothalamus. Thus, if the variation observed in numbers of IR-GnRH neurons affects the reproductive axis, we should predict similar variation in the secretion of LH or FSH (Figs. 15.1 and 15.3). Consistent with this prediction, ovariectomized females with replacement estradiol from the NR line had higher circulating levels of LH than similarly treated females the R line in both long and short photoperiod (Heideman et al. 2010). In both lines, short photoperiod suppressed levels of LH. However, females in the NR line had levels of LH in winter-like photoperiods that matched the R line even in long, summer-like photoperiods (Heideman et al. 2010). This result suggests that the heritable variation in numbers of IR-GnRH neurons produces a functional outcome on fertility. We have not been able to test for variation in FSH because we have not been able to validate a FSH assay for our mice.

In ongoing research, we are testing for heritable variation in kisspeptin neurons, RF-amide-related peptide (RFRP) neurons, and additional hormonal systems. Our preliminary data from these experiments suggest that heritable variation occurs in additional neuroendocrine systems that affect reproduction. Our current working hypothesis is that multiple, independent sources of heritable variation contribute additively to produce variation in the seasonal calendar outputs for reproduction (Heideman and Pittman 2009), but not variation in the seasonal calendar itself.

15.3.6 *Evidence for Variation in Phenotypic Plasticity in Food Intake and Metabolism in Relationship to Reproduction*

Although heritable variation is an important component of variation in nature, phenotypic plasticity and gene-by-environment interactions modify the effect of genes alone. Phenotypic plasticity is the ability of an animal of a particular genotype to alter phenotype in response to environmental inputs. An example of phenotypic plasticity is the response of our R line to short photoperiod: they suppress fertility in SD, but are fertile in LD. An example of a gene-by-environment interaction is the fact that mice in our R line are subfertile or infertile in SD but the NR line is not.

In our population, two other traits are also phenotypically plastic, both with a gene-by-environment interaction. In some (but not all) experiments, males in the NR line were found to eat approximately 45 % more food than males in the R line (Heideman et al. 2005; Kaseloo et al. 2012). Food intake was correlated with testis size in SD, but not in LD (Heideman et al. 2005; Kaseloo et al. 2012), suggesting a gene-by-environment interaction of selection-line-by-photoperiod in the relationship between food intake and fertility. Metabolic rate was correlated with food intake and showed another gene-by-environment interaction. At a thermoneutral temperature (28 °C), basal metabolic rate (BMR) and average daily metabolic rate (AMDR) were higher in NR than in R mice (Kaseloo et al. 2012). Food intake, BMR, and ADMR were correlated with testis size in short, winter-like photoperiods. However, none of these measures were correlated with testis size in long, summer-like photoperiods, indicating both genetic and environmental effects on metabolism and food intake (Kaseloo et al. 2012). In a follow-up study, there were gene-by-environment interactions of these variables in relationship to temperature (Kaseloo et al. 2014).

Because food intake is correlated with reproductive development in short, winter-like photoperiod, but not in long summer photoperiods, perhaps restriction of food could suppress fertility of our NR line in SD but not LD. Consistent with this prediction, we found that slightly restricted access to food in the NR line decreased testis size in SD, but not LD (Reilly et al. 2006). Restriction treatment mice were not deficient in calories or nutrition; in fact, mice in restricted access treatments gained weight, and did so at the same rate as controls fed ad libitum. In other words, males in the NR line are capable of strong reproductive inhibition in SD, but they require a second environmental signal, slightly restricted access to food (Reilly et al. 2006). This finding is further evidence that variation between lines is not caused by a broken seasonal clock, but rather by lower sensitivity of the NR line to the seasonal signal of short photoperiod.

Finally, recent results indicate that the different genetic strains (our NR and R lines) are most phenotypically different from each other when temperatures are warm and when food is abundant (Kaseloo et al. 2014). Under those benign conditions, NR mice have larger reproductive organs, are more active, and have higher active and resting metabolic rates. As temperatures are made colder experimentally, the two selection lines tend to converge in food intake, metabolic rate, and possibly fertility (Kaseloo et al. 2014). It may be that in sufficiently cold temperatures, or with sufficiently restricted access to food, the two lines may become functionally identical in short, winter photoperiods, but this has yet to be tested.

15.4 Conclusion

Our experiments on the single population of wild white-footed mice that we study have identified significant heritable variation in a surprisingly large number of locations in the neuroendocrine pathway regulating seasonal reproduction (Fig. 15.4). Interestingly, the variation appears never to be from damage to any important neuroendocrine system. For example, there are differences in melatonin receptor binding, but both selection lines can respond to melatonin at the neuronal level (Fetsch et al. 2003), both have seasonal physiological responses to short photoperiod (Heideman et al. 2005), and the circadian clock is functional in both lines (Majoy and Heideman 2000). There are heritable differences in numbers of IR-GnRH neurons (Avigdor et al. 2005; Heideman et al. 2007) and levels of LH (Heideman et al. 2010), but animals in both lines are fully fertile in LD (Heideman, unpublished data). There are heritable differences between lines in metabolic rate and activity in SD at thermoneutral temperatures (Kaseloo et al. 2012), but in SD when animals are exposed to cold temperatures typical of winter, metabolic rate and activity converge on the same value, with fewer and smaller differences (Kaseloo et al. 2014). In this wild-source population, we see functional variation that modifies how individuals respond to seasonal change, but not by damaging important neuroendocrine systems. NR mice and R mice show the same photoperiodic reduction of food intake and body mass (Heideman et al. 2005), despite being differently sensitive reproductively. Our observations of gene-by-environment interactions suggests that the genetic differences among individuals may be mainly genetic differences in phenotypic plasticity, rather than differences in reproduction that would be identical in all winter conditions.

Our comparisons of the NR and R selection lines are not comparisons of two genotypes, one R and one NR. In fact, our results are equally consistent with the hypothesis that the two selection lines retain heritable variation in reproductive photoresponsiveness. Even after ten generations of selection, both lines produced some offspring of the opposite phenotype (Heideman and Pittman 2009). An important question is whether the variable neuroendocrine traits we have observed are correlated with each other or, alternatively, are independent contributors to variation in fertility. In a current experiment, we are measuring multiple neuroendocrine traits in the same individuals to test for independence of variation across multiple neuroendocrine traits. Independent combinations of different variants might produce phenotypes that, at one extreme, are fully inhibited reproductively by a short, winter-like photoperiod. At the other extreme would be phenotypes that are not suppressed in SD except by multiple inhibitory inputs from multiple environmental cues (e.g., only by the combination of SD, cold, and restricted food). Most individuals might fall in the middle, with intermediate responses. Intermediates might be partially reproductively suppressed by SD alone, but may alter phenotype in either direction by additional environmental inputs.

What about tropical mammals that reproduce seasonally? In tropical mammals, we cannot study heritable variation in seasonal regulation of reproduction until we know the nature of seasonal calendar inputs that regulate fertility. Clues to these seasonal calendar inputs might be found by testing tropical mammals at seasonal reproductive transitions for changes in TRH secretion in the pars tuberalis and changes in deiodinases, and active thyroid hormone in the mediobasal hypothalamus.

What might we predict about the amount natural variation in neuroendocrine regulation by seasonal clocks in other species of mammals? Only two populations have been studied in depth: ours and that of Blank and collaborators (*Peromyscus maniculatus*; Blank and Desjardins 1986; Blank et al. 1988; Blank and Freeman 1991; Blank 1992; Desjardins et al. 1986; Korytko et al. 1995; Korytko et al. 1998; Mintz et al. 2007). The latter population has been studied mostly for phenotypic variation, with the degree of underlying genetic variation uncertain. Most of the experiments by Blank and colleagues have examined neuroendocrine phenotypic variation in a single wild-source population that is known to be genetically variable for reproductive photoresponsiveness, but without genetically distinct lines to provide direct evidence of heritable variation. In both populations there appears to be variation in some of the same portions of the pathway (e.g., GnRH neurons), although often not the same differences. In both the tropics and the temperate zone, different geographic populations often have substantially different patterns of timing. In combination, the evidence suggests that substantial heritable intraspecific variation in reproductive regulation may be common in mammals. In some cases, especially when natural selection is variable from year to year or place to place, high levels of heritable variation may be expected even within single populations.

In laboratory rodents, nonresponsiveness to photoperiod is sometimes caused by alleles that inactivate or damage the circadian clock, rendering photoperiodic time measurement inaccurate or impossible (Smale et al. 2005). In sharp contrast, in wild populations there is little evidence that this is true (Heideman and Pittman 2009). Rather, in wild populations nonresponsiveness to photoperiod may be trait specific (i.e., affecting one trait, such as reproduction but not another, such as body mass), and phenotypically plastic with gene-by-environment interactions.

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Chapter 16
Circannual Rhythms in Insects

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Abstract Although many insects adapt to seasonal changes by photoperiodism, a small proportion of insect species use a circannual rhythm for seasonal adaptations. The circannual pupation rhythm of the varied carpet beetle *Anthrenus verbasci* shows a periodicity of approximately 40 weeks under constant conditions, and the change in photoperiod acts as a zeitgeber. The circannual rhythm of *A. verbasci*, of which the larval duration varies from one to several years, probably plays an important role for synchronizing the pupation and breeding times with spring each year. There are only slight differences in the critical daylength for circannual entrainment among geographically distinct populations in Japan, and they pupate in the same period under natural conditions in Osaka. Therefore, *A. verbasci* can adapt to seasonal changes in different regions without changing the parameters of the circannual rhythm. Long-term endogenous rhythms have also been reported in oviposition and pupation of some ant species. These insects are considered to refer not only to external cues but also to the phase of an endogenous clock for maintaining appropriate seasonality.

Keywords *Anthrenus verbasci* • Geographic variation • Phase-response curve • Photoperiodism • Seasonal adaptation

16.1 Introduction

Many species adapt to seasonal environmental changes by a direct response to photoperiod, that is, photoperiodism, but some species are known to use a circannual rhythm for seasonal adaptations (Gwinner 1986; Goldman et al. 2004; Paul et al. 2008).

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Circannual rhythms regulating various physiological and behavioral functions have been reported in a variety of organisms. However, our understanding of circannual rhythms is still poor compared to that of photoperiodism and circadian rhythms.

Insect species that have a life cycle shorter than a year seem to have no reason to use a circannual rhythm. Some of these insects enter diapause in a specific developmental stage of one generation to avoid the production of an additional generation in the season unfavorable for growth and reproduction, such as winter. For diapause induction and termination, many temperate insects use photoperiod and temperature as reliable seasonal cues from the environment (Danilevskii 1965; Danks 1987; Saunders 2010).

Some insect species of which the life cycles take two or more years may adopt a circannual rhythm for seasonal adaptations (Saunders 2010). The circannual rhythm in insects was first reported in the varied carpet beetle *Anthrenus verbasci* by Blake (1958, 1959, 1960, 1963). *A. verbasci* is widely distributed in temperate regions and known as a pest that feeds on woolen goods, dried animal products, and zoological specimens (Griswold 1941; Hinton 1945). The primary natural habitat of this beetle is the nests of birds (Yokoyama 1929; Hinton 1943, 1945; Woodroffe 1953). Adults emerge, mate, and lay eggs from spring to early summer. Larvae grow by feeding mainly on dried animal matter during warm seasons. Pupation occurs in spring. The duration of the life cycle varies from one to several years depending on the local climate and nutritional conditions (Yokoyama 1929; Griswold 1941; Blake 1958; Kiritani 1958; Miyazaki et al. 2009a). Blake (1958, 1959) showed that pupation of this species occurs with a periodicity of approximately 40 weeks under constant conditions. Similar long-term rhythms have been reported in pupation of another carpet beetle, *Anthrenus sarnicus* (Coombs and Woodroffe 1983; Armes 1990), and in queen oviposition and larval pupation of some ant species (Kipyatkov 1993, 1995). Chronobiological analyses, however, have not been conducted in detail in these reports. Since 1996, we have been running further tests and new experiments based on the present chronobiological views in the circannual rhythm of *A. verbasci*. In this chapter, with a central focus on the results obtained in *A. verbasci*, we outline the characteristics of the circannual rhythm in insects and discuss its adaptive significance.

16.2 Periodicity Under Constant Conditions

Constant 24-h photoperiods have been routinely used as seasonally constant conditions under which the circannual rhythm should be demonstrated to persist for at least two cycles with periods different from 12 months (Gwinner 1981a, 1986). Nisimura and Numata (2001) observed the pupation rhythm of *A. verbasci* with a period of approximately 37 weeks, when larvae within 1 week after hatching were continuously reared on bonito powder under a constant photoperiod of 12-h light and 12-h darkness (LD 12:12) at 20 °C and 66 % relative humidity (Fig. 16.1a). In circadian rhythms, the daily periodicity found in the once-in-a-lifetime event such as hatching,

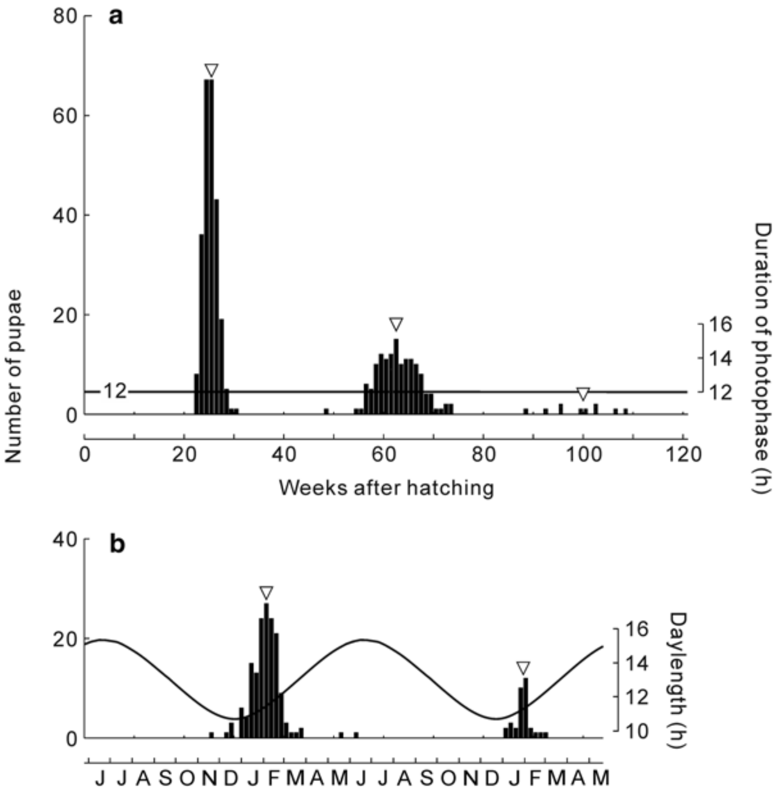


Fig. 16.1 Self-sustainability (**a**) and entrainability (**b**) in the circannual pupation rhythm of *Anthrenus verbasci* at 20 °C. Larvae were kept under light–dark (LD) 12:12 (**a**) or under naturally changing daylength (**b**). *Triangles* indicate median of each pupation group. *Solid line* in each panel indicates the duration of photophase or the natural daylength, including 1 h of twilight in Osaka, Japan (35°N). ((**a**) Modified from Nisimura and Numata 2001; (**b**) modified from Miyazaki et al. 2006)

pupation, and eclosion in insects can be explained by the concept of "gate," which was first introduced for the allowed time for eclosion in the fruit fly *Drosophila pseudoobscura* (Pittendrigh 1966). According to this concept, the gate is opened periodically by a circadian rhythm and only sufficiently developed individuals can hatch, pupate, or eclose during the period of gate opening (Saunders 2002). As described in more detail later, the pupation rhythm in *A. verbasci* has some similarities to the circadian eclosion rhythm of *D. pseudoobscura*, although the time scale is not days but years. Therefore, *A. verbasci* has an endogenous circannual rhythm and the gate for pupation is controlled by this rhythm (Blake 1958, 1959; Nisimura and Numata 2001). Larval growth inhibition by a low-nutrient diet has little or no influence on the timing of pupation in each circannual cycle but markedly changes the percentage of pupae in each cycle (Miyazaki et al. 2009a).

Most physiological processes depend strongly on temperature. However, temperature compensation of period length is needed to accurately measure time and is known as a key characteristic of biological rhythms (Saunders 2002; Johnson et al. 2004). This property was first clearly shown in eclosion of *D. pseudoobscura* in circadian rhythms (Pittendrigh 1954) and in pupation of *A. verbasci* in circannual rhythms (Blake 1958, 1959). Temperature compensation is relatively easy to examine in insects, as compared to mammals or birds, because they are poikilotherms. Nisimura and Numata (2001) kept larvae of *A. verbasci* under LD 12:12 at various constant temperatures. Larvae began to pupate 21–27 weeks after hatching as the first pupation group at temperatures between 17.5 and 27.5 °C, and the second group pupated about 40 weeks after the first. High temperatures did not result in earlier pupation. Other developmental processes of *A. verbasci*, for example, the rate of larval development, the proportion of pupation, and the lengths of egg and pupal stages, depend on temperature as usual physiological processes do (Griswold 1941; Blake 1958; Kiritani 1958). Therefore, the timing of pupation of *A. verbasci* is clearly and specifically temperature compensated. Moreover, in *A. sarnicus*, three pupation peaks with intervals of 32–42 weeks were observed at both 15 and 25 °C, although the first and third peaks at 25 °C consisted of only two to four pupae (Armes 1990). This observation suggests that temperature compensation of the period also exists in the pupation rhythm of *A. sarnicus*.

There is a pronounced influence of photoperiod on the degree of persistence and the period length of the circannual rhythm of *A. verbasci*, as in other organisms with circannual rhythms (Gwinner 1986). The pupation rhythm was clear under LD 12:12 but was obscure under LD 15:9 and LD 16:8 and arrhythmic under LD 14:10. Although a clear rhythm was observed under LD 13:11, the circannual period was 5 weeks longer than under LD 12:12 (Fig. 16.2) (Nisimura and Numata 2003). There is still no convincing interpretation of these photoperiodic effects on the degree of persistence and the period length (see also Gwinner 1981a, 1986).

Many circadian rhythms in insects do not persist under constant light (Saunders 2002). Miyazaki and Numata (2010) examined whether the circannual pupation rhythm of *A. verbasci* persists under constant light. Rhythmic pupation did not occur under constant light when hatching larvae were continuously reared under these conditions. However, the pupation rhythm oscillated with a circannual pattern under constant light when hatching larvae had been exposed to LD 12:12 for 8 weeks before transfer to constant light. Therefore, preexposure to LD 12:12 was required for this circannual rhythm to be exhibited under constant light. As described next, the photoperiod is a zeitgeber for this circannual rhythm. It is possible that the initial circannual phase of newly hatched larvae varies among individuals, but exposure to LD 12:12 synchronizes the circannual phase of the larvae, and the circannual rhythm of pupation is exhibited under constant light.

Although the circannual rhythm in pupation of *A. verbasci* is detected only in populations, an individual insect can also display a circannual rhythm. Blake (1958) reported not only the pupation rhythm but also the alternation of the active (molting) and resting periods in the larvae of this species. Figure 16.3 shows molting, pupation, and body weight in seven larvae of *A. verbasci* that were individually reared under LD 12:12 at 22.5 °C (Nisimura and Numata, unpublished data). Although results in more individuals are necessary for conclusions, it seems that the alternation

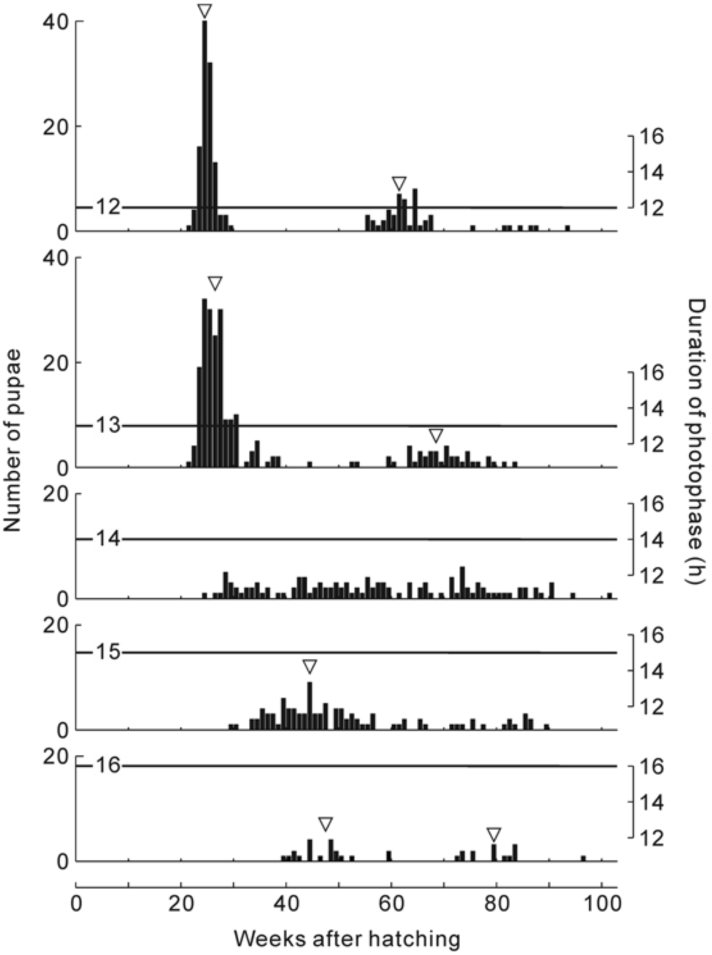
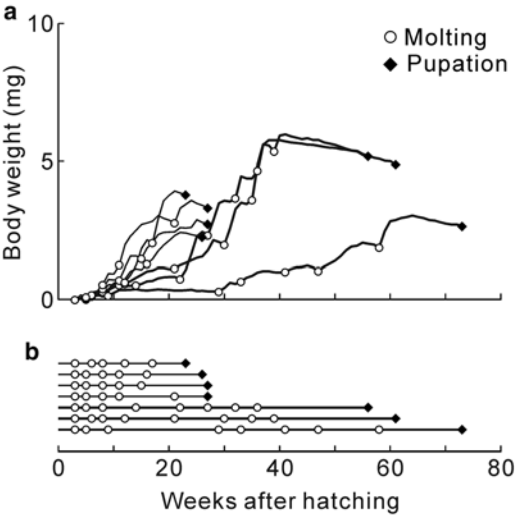


Fig. 16.2 Pupation of *Anthrenus verbasci* under various constant photoperiods at 20 °C. *Triangles* indicate median of each pupation group. *Solid line* in each panel indicates the duration of photophase. (Modified from Nisimura and Numata 2003)

of the active and resting periods was observed in terms of molting and the increase of the body weight. Thus, observation and analysis of the circannual rhythm in an individual would be feasible in the frequency of molting and the increase of body weight in *A. verbasci*.

In some species of ants, a queen stops oviposition and resumes it after an interval of several weeks or months, and such cycles are spontaneously repeated under constant photoperiod and temperature. Many of these rhythms show great variation in the endogenous period over several years (Kipyatkov 1993, 1995). In the red wood ants *Formica aquilonia* and *Formica polyctena*, for example, an endogenous period of the oviposition rhythm varied between 90 and 525 days (mean, 212 days)

Fig. 16.3 Change in body weight (**a**) and the timing of molting and pupation (**b**) of *Anthrenus verbasci* larvae individually reared under LD 12:12 at 22.5 °C. Each *solid line* shows the growth of an individual



and between 60 and 345 days (mean, 179 days), respectively, even under optimal conditions (Kipyatkov and Shenderova 1990). Considerable intraindividual variability in the period is also reported in circannual rhythms of other animals (Gwinner 1986). In *F. aquilonia* and *F. polyctena*, the mean periods of oviposition rhythms did not depend on temperature between 20 and 25 °C and did so only slightly between 17 and 30 °C. Moreover, the mean periods were not different between short-day and long-day conditions (Kipyatkov and Shenderova 1990). It is considered of value to examine whether the stability of the period of the endogenous rhythm to temperature and photoperiod also exists in queens of other ant species.

16.3 Response to Seasonal Environmental Cues

Timing of pupation in *A. verbasci* and *A. sarnicus* is modulated by naturally changing daylength (Blake 1960, 1963; Armes 1990; Nisimura and Numata 2003; Miyazaki et al. 2006). The circannual period of *A. verbasci* is considerably shorter than 1 year, but continuous exposure to naturally changing daylength entrains the pupation rhythm to 1 year (Fig. 16.1a, b). Such entrainment is attributed to phase resetting of the circannual rhythm in response to the zeitgeber of photoperiodic changes but not to the direct regulation of development and diapause by classical photoperiodism (Nisimura and Numata 2001, 2003); this is similar to the entrainment of circadian rhythms to a daily cycle achieved by resetting of a circadian clock in response to light.

The phase of circadian rhythms advances or delays depending on the phase at which a zeitgeber stimulus is applied. A phase-response curve (PRC) is a plot of these phase shifts as a function of the phase of the stimulus. PRCs provide useful information regarding the entrainment of the rhythm and the underlying mechanism. In most cases, a circadian PRC to light is constructed by administering a light pulse to a circadian rhythm under constant darkness (Saunders 2002; Johnson et al. 2004). From systematic experiments in the eclosion rhythm of *D. pseudoobscura*, Winfree (1970) realized that the resetting pattern of the circadian clock can be divided into two major types. He called these types type 1 and type 0, when pulse lengths are relatively short and long, respectively. Type 1 PRCs have low amplitude and a continuous transition between delays and advances (Fig. 16.4a). Type 0 PRCs have high amplitude and a breakpoint at the transition between delays and advances (Fig. 16.4b).

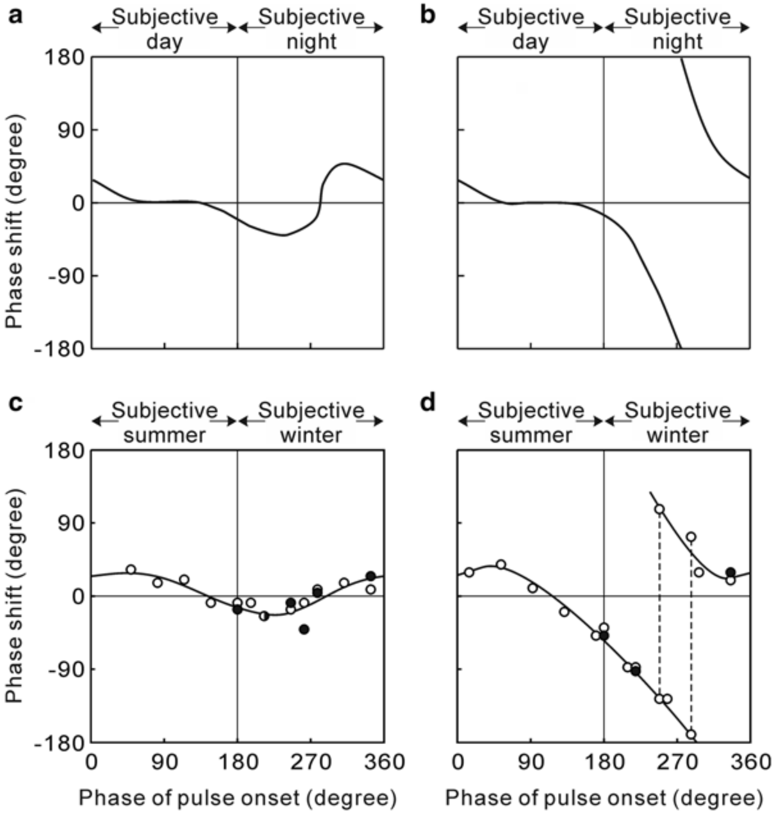


Fig. 16.4 Comparison of phase-response curves for circadian and circannual rhythms. (**a**, **b**) Phase-response curves in circadian rhythms, type 1 (**a**) and type 0 (**b**). (**c**, **d**) Phase-response curves in the circannual pupation rhythm of *Anthrenus verbasci*, one curve for 2-week long-day pulses (**c**) and one curve for 4-week long-day pulses (**d**). Larvae were kept under LD 12:12 at 20 °C and exposed to LD 16:8 for 2 weeks (**c**) or 4 weeks (**d**) at various phases in the circannual rhythm. The circannual period under continuous LD 12:12 is shown in terms of angle degrees (0–360°), and the initial phase under LD 12:12, i.e., the beginning of this experiment, is represented as 180°. *Open* and *closed circles* represent the phase shifts in the first and second pupation group after pulse perturbation, respectively. *Broken lines* in (**d**) show the split into advanced and delayed groups. (**c**) From Miyazaki et al. 2007; (**d**) from Miyazaki et al. 2005)

In addition, Winfree (1970) found that when a light pulse of intermediate length was applied at the transition between delays and advances in the circadian PRC, the eclosion of *D. pseudoobscura* became arrhythmic. Phase resetting dependent on pulse lengths and arrhythmicity evoked by a singular stimulus have also been confirmed in circadian rhythms of various species (Saunders 2002; Johnson et al. 2004).

To construct PRCs for a circannual rhythm, Miyazaki et al. (2005, 2007) kept *A. verbasci* larvae under LD 12:12 and exposed the larvae to LD 16:8 for 2 or 4 weeks (long-day pulse) during various phases of the rhythm. A long-day pulse clearly caused a phase advance or delay of the circannual rhythm in a phase-dependent manner. Circannual PRCs to 2-week and 4-week long-day pulses resembled type 1 and type 0 PRCs of circadian rhythms, respectively (Fig. 16.4c, d). Moreover, Miyazaki et al. (2007) found that a 4-week long-day pulse administered at the transition between delays and advances in the circannual PRC can evoke arrhythmicity in pupation of *A. verbasci*. These results demonstrated that the circannual rhythm of *A. verbasci* is entrained to an environmental cycle by the same mode of phase resetting as circadian rhythms and indicated that the circannual rhythm of *A. verbasci* is derived from a biological clock obtained for adaptation to annual environmental changes, that is, a circannual clock (see Miyazaki et al. 2012 for details).

Phase responses of circannual rhythm in *A. verbasci* are observed not only under constant conditions but also under naturally changing daylength. Miyazaki et al. (2006) applied 4-week long-day pulses between early August and late November under natural daylength at a constant temperature of 20 °C and observed phase shifts in the first pupation group. A long-day pulse applied on 4 August, 1 September, and 29 September caused 3-, 5-, and 5.5-week delays, respectively. A pulse applied on 27 October and 24 November caused 2- and 3-week advances, respectively. A pulse applied on 29 September also induced much less synchronous pupation than other conditions. Thus, the circannual phase of *A. verbasci* changes at least from early August to late November under natural daylength, as under LD 12:12.

Temperature and more infrequently photoperiod serve as environmental factors to accelerate or cease egg-laying and pupation in ant species (Kipyatkov 1993). The regulation of the life cycle by external factors is very important for ants with endogenous periods that deviate considerably from 1 year with great variability. However, it has still not been clarified whether temperature and photoperiod directly regulate the initiation and cease of oviposition and pupation or act to reset the phase of the endogenous rhythm as zeitgebers.

16.4 Photoperiodic Time Measurement for Entrainment

As described here, the photoperiod considerably affects the periodicity and phase setting of circannual rhythm in *A. verbasci*. Therefore, the photoperiodic time measurement system is involved in the regulation of the circannual rhythm. This system seems identical to that used for usual photoperiodism in insects without circannual rhythms, because it is probable that a circannual clock was originated later than the basic form of the photoperiodic time measurement system in the course of evolution.

A critical daylength is one of the important characteristics in insect photoperiodism. Behavioral and physiological responses are shown depending on whether the daylength is above or below this value (Danilevskii 1965; Danks 1987; Saunders 2002, 2010). In the Osaka population of *A. verbasci*, when larvae were kept under constant photoperiods, the critical daylength for synchronous pupation was between 13 and 14 h (Fig. 16.2) (Nisimura and Numata 2003). Similarly, a clear phase delay, which is pivotal for the appropriate entrainment of a circannual rhythm with a period considerably shorter than 1 year, was induced when the photoperiodic change exceeded a critical value in the photophase between 13 and 14 h (Miyazaki and Numata 2009). It is, therefore, considered that the photoperiodic time measurement system of *A. verbasci* in Osaka adopts this critical value as a criterion for determining whether the daylength is long or short.

The photoperiodic time measurement system generally involves a circadian clock. The Nanda–Hamner protocol, in which organisms are subjected to light–dark cycles with a fixed short photophase followed by a variable scotophase to give cycle lengths (T) of up to 72 h or more, has often revealed the involvement of a circadian system in photoperiodic responses. If short-day responses are induced when T is a multiple of 24 h, but not induced when T is not a multiple of 24 h, it is considered that a circadian system is involved in photoperiodic time measurement (Saunders 2002; Goldman et al. 2004). By this protocol, Miyazaki et al. (2009b) examined whether a circadian clock is involved in photoperiodic entrainment of the circannual rhythm of *A. verbasci*. Exposure to LD 12:36 or LD 12:60, for which T is a multiple of 24 h, had effects similar to exposure to LD 12:12. In contrast, exposure to LD 12:24 or LD 12:48, for which T is not a multiple of 24 h, had effects clearly different from exposure to LD 12:12. These results show the involvement of a circadian system in photoperiodic entrainment of the circannual rhythm of *A. verbasci*.

16.5 Adaptive Significance

Circannual rhythms with a period deviating significantly from 12 months and with relatively weak self-sustainability appropriately function only in the presence of seasonal zeitgebers entraining the rhythms to the natural annual cycle (Gwinner 1986). Gwinner (1981b, 1986) stated that as yet there appears to be no convincing answer to the question whether external seasonal factors could not do the same job alone. Naturally and more seriously, such a question also arises in insects that are more susceptible to environmental factors than are homeotherms. In insects inhabiting temperate regions, other systems that depend exclusively on external proximate factors such as temperature and photoperiod are predominantly more common than circannual rhythms (Danilevskii 1965; Danks 1987; Saunders 2010). Unfortunately, we cannot convincingly explain why a small proportion of insect species have evolved a circannual rhythm for the control of their life cycle. However, at least, we can discuss some significance of the circannual rhythm in the control of the life cycle.

The circannual clock probably allows the organism to better predict and adapt to seasonal changes and increase the chances of survival and reproductive success

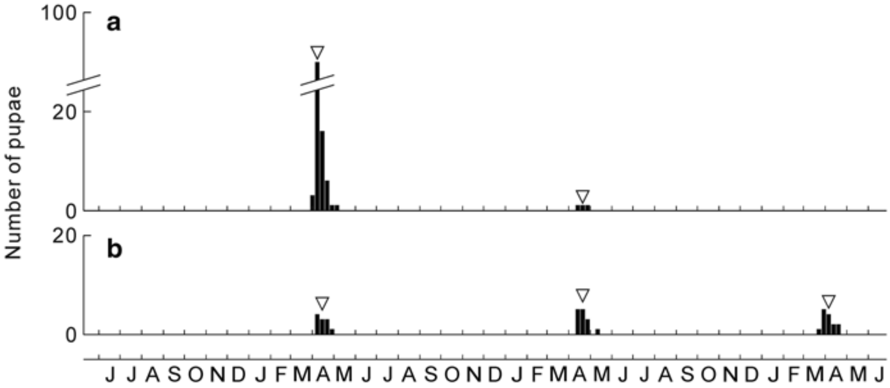


Fig. 16.5 Effects of dietary nutrition on the pupation of *Anthrenus verbasci* under natural daylength and temperature in Osaka, Japan. Dried bonito powder (**a**) or feathers of the domestic pigeon (**b**) were provided as larval food. *Triangles* indicate median of each pupation group. (Modified from Miyazaki et al. 2009a)

(Gwinner 1981a, 1986). *A. verbasci* can refer not only to ambient photoperiod and temperature but also to the phase of a circannual clock and thereby may show more accurate seasonal responses in the field than other insects that do not have this clock (Miyazaki et al. 2012). Although the pupation peak of *A. verbasci* exists in early February under natural photoperiod at 20 °C (see Fig. 16.1b), pupation occurs in April under natural photoperiod and temperature in Osaka because of the suppression of development by low temperature during winter (Fig. 16.5) (Nisimura and Numata 2003). Nisimura and Numata (2003) placed larvae of *A. verbasci*, within a week after hatching, at various times of the year under natural temperature and photoperiod in Osaka. The results showed that larvae that had hatched between October and mid-April (i.e., broadly speaking in “winter”) could not grow to pupae, whereas larvae that had hatched between late April and September (i.e., in summer) could pupate in March or April, of the next year or the year after. In addition, pupal weight was relatively high (approximately 4 mg) in individuals that had hatched between late April and July, but only approximately 2 and 1 mg in individuals reared from August and September, respectively. Therefore, it was suggested that in *A. verbasci*, synchronous pupation and adult emergence in spring achieve the sufficient growth of offspring larvae during warm seasons so that the larvae overwinter and the relatively large adults with high fecundity emerge. Armes (1990) also reported that all the first-instar larvae of *A. sarnicus* introduced into the pigeon loft in southern England between November and February died within a few weeks, but larvae introduced between March and September could successfully develop to pupation between May and July, of the next year or the year after. Thus, *A. verbasci* and *A. sarnicus* should synchronize the breeding time with the beginning of the warm season to enhance the survival and reproductive rates, and therefore the circannual clock regulating pupation timing is likely a beneficial adaptive mechanism.

A circannual clock is the repetitive system oscillating for a number of years. This clock may facilitate production of consistent seasonal responses every year. If the rate of larval development of *A. verbasci* differs considerably among individuals in natural environments, it is considered that the gate controlled by the circannual clock is of advantage in restricting pupation to the appropriate season each year. In rearing experiments under outdoor conditions in Osaka, however, most individuals pupated in the next spring (Fig. 16.5a) (Nisimura and Numata 2003; Miyazaki et al. 2009a). In these experiments, bonito powder was provided as a sufficient larval diet, as in our other experiments. Dried bonito is favorable for growth of *A. verbasci* larvae (Kiritani 1958). Under natural conditions, however, such nutrient-rich food is unlikely to be available frequently. In bird nests, *A. verbasci* larvae feed on feathers and other dry animal materials (Hinton 1943; Woodroffe 1953). The nutrient quality of these foods is probably poorer, except for the carcasses of dead birds. When larvae of *A. verbasci* were supplied with pigeon feathers as food under outdoor conditions in Osaka, larval development was slower than on bonito powder, and the pupation times varied over 3 years or more. Although most larvae did not develop to pupae and died, similar numbers of the surviving larvae pupated around April each year for 3 years (Fig. 16.5b). There was also a larva that pupated in spring of the fourth year (Miyazaki et al. 2009a). Therefore, it is probable that the larval development of *A. verbasci* in the natural habitat often varies and takes 1, 2, or more years to complete. In nutrient-poor and unstable environments where the duration of the life cycle differs considerably among individuals, the gate for pupation controlled by a circannual clock would have greater significance as a seasonal adaptation.

With higher latitudes, insects are subjected to longer, colder winters and shorter, cooler summers. Because the developmental rate of insects is correlated with environmental temperature, insect species that are univoltine at lower latitudes may extend the duration of the life cycle over a number of years at higher latitudes (Saunders 2009). In fact, the life cycle of *A. verbasci* takes 2 years to complete in southern England even though favorable food for growth is supplied (Blake 1958, 1960), whereas it takes 1 year in central Japan (Yokoyama 1929; Nisimura and Numata 2003). The significance of internal time-of-year information provided by the circannual rhythm seems higher with higher latitudes because of shorter warm seasons and the longer life cycle.

In some insects living in further high latitudes, however, expression of seasonal traits regulated by long-term endogenous rhythms may decline under constant laboratory conditions. Many ant species distributed in the temperate climatic zone spontaneously resume oviposition and development under constant conditions. In the ant species penetrating to extremely high latitudes, such as the northernmost areas of the Palaearctic region, spontaneous reactivation under constant conditions is possible but could not be sufficient to maintain the colony. These species require a long exposure to low temperature for complete restoration of oviposition and development as a result of adaptation to the climate with a long and cold winter. Under such environments, endogenous rhythms in ants effectively control the physiological process and change the internal tendency for seasonal traits (Kipyatkov 1993, 1995), suggesting that persistence of endogenous seasonal rhythms becomes weak and dependency on external seasonal factors becomes strong in insects living in extremely high latitudes, although it has been shown only in some ants.

16.6 Geographic Variation

Insect species distributed in different areas often show geographic variation in the seasonal timekeeping mechanism (Danilevskii 1965; Saunders 2002, 2009). In many insects, for example, the critical daylength in photoperiodism for induction of winter diapause increases by approximately 1.0–1.5 h for every 5° of latitude, because diapause must be induced earlier in cooler climate zones (Danilevskii 1965; Danks 1987). Adjustment of the pupation timing in *A. verbasci* by photoperiod is attributed to phase resetting of the circannual rhythm rather than induction and termination of larval diapause (Nisimura and Numata 2001, 2003). To examine whether geographic variation exists in the seasonal timekeeping mechanism of *A. verbasci*, Matsuno et al. (2013) examined, under various environmental conditions, the pupation time in four geographically distinct populations in Japan: Takanabe (32.1°N), Osaka (34.7°N), Sendai (38.3°N), and Sapporo (43.1°N) populations.

The initial phase and endogenous period of the circannual pupation rhythm under continuous LD 12:12 differed only slightly among the four populations. When larvae were exposed for 12 weeks to various longer photophases between 13 and 16 h and then transferred to LD 12:12, in all procedures the first pupation peak significantly was delayed, compared to that under continuous LD 12:12. Figure 16.6 shows results in the Osaka population. The magnitude of the delay depended on the photophase duration of 12-week exposure. Exposure to LD 14:10, LD 15:9, or LD 16:8 caused 9-week delays. Exposure to LD 13:11 and LD 13.5:10.5 caused 3- and 6-week delays, respectively. Therefore, LD 13:11 and LD 13.5:10.5 induced intermediate delay responses.

The degree of the delay evoked by the same stimulus depended on the geographic population. Exposure to LD 16:8 induced the maximum delays of 9–10 weeks in all four geographic populations. This maximum delay was regarded as 100 %, and the critical daylength was calculated as the photophase duration under which the magnitude of the delay was 50 % (Fig. 16.7a). The critical daylength was 12.8 h in the Takanabe population, 13.2 h in the Osaka population, and 13.6 h in the Sendai and Sapporo populations. Thus, there was a correlation between the critical daylength for entrainment of the circannual rhythm in *A. verbasci* and habitat latitude, but the correlation was much weaker than that obtained in photoperiodism for diapause induction in other insects, for example, larval diapause of the rice stem borer *Chilo suppressalis* (Kishino 1970) (Fig. 16.7b).

The gate for pupation of *A. verbasci* is set approximately a half year after transfer from long days to short days by a phase shift in the circannual rhythm (Nisimura and Numata 2001; see also Fig. 16.6), and the development and pupation are suppressed by low temperature during winter (Nisimura and Numata 2003). These findings

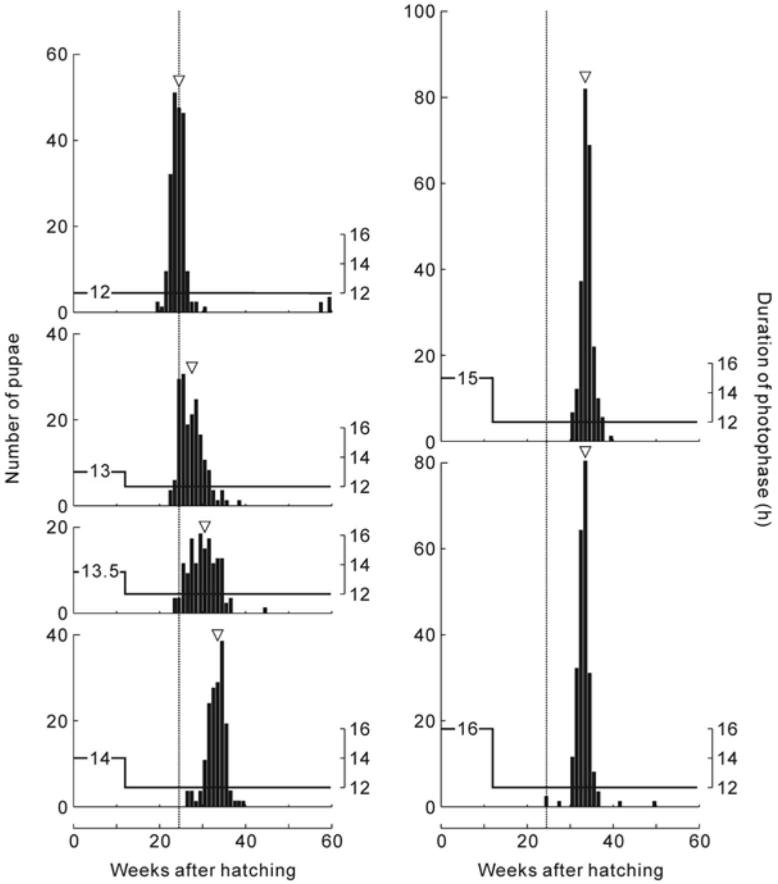


Fig. 16.6 Effects of photoperiodic changes on the timing of pupation in the Osaka population of *Anthrenus verbasci* at 20 °C. *Triangle* indicates median of each pupation group. *Vertical dotted lines* show median of the first pupation group under continuous LD 12:12. *Solid line* in each panel indicates duration of photophase. (Modified from Matsuno et al. 2013)

suggest that even though the geographic cline in the critical daylength is slight, *A. verbasci* can avoid pupation and adult eclosion during autumn and winter. Therefore, selective pressures on the critical daylength for phase delay of the circannual rhythm of *A. verbasci* might be weak compared to those on the critical daylength for diapause induction in many other insects. In fact, when newly hatched larvae were reared from spring under outdoor conditions in Osaka, in all four geographic populations many larvae survived and showed a pupation peak in April of the following year (Fig. 16.8) (Matsuno et al. 2013). Thus, *A. verbasci* appears capable of adapting to seasonal changes in different geographic regions without changing the parameters of the circannual rhythm. Such a mechanism may be one factor contributing to the geographically widespread distribution of *A. verbasci* (Griswold 1941; Hinton 1945).

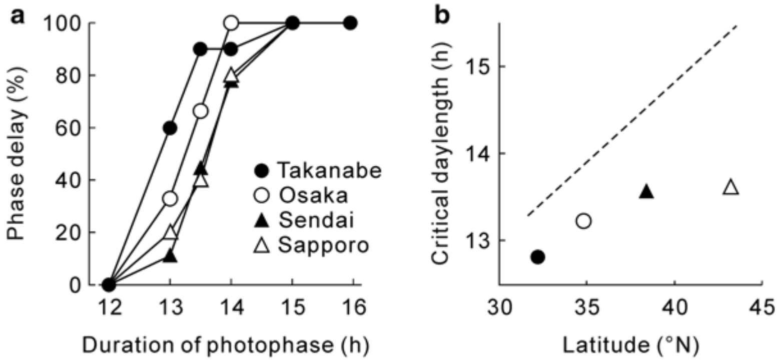


Fig. 16.7 Responsiveness to photoperiod for the timing of pupation in Takanabe, Osaka, Sendai, and Sapporo populations of *Anthrenus verbasci* at 20 °C. (**a**) Relationship between the photophase before transfer to LD 12:12 and the degree of phase delays. (**b**) Relationship between the habitat latitude and the critical daylength for phase delay. Phase delays were calculated from the first pupation peak when larvae were exposed for 12 weeks to various longer photophases and transferred to LD 12:12, compared to the first pupation peak under continuous LD 12:12 (see Fig. 16.6). The magnitude of phase delay induced by LD 16:8 exposure was regarded as 100 %, and the critical daylength was regarded as a photophase duration under which the magnitude of the delay was 50 %. The *broken line* designates the critical daylength for induction of larval diapause in *Chilo suppressalis* at 25 °C (Kishino 1970). (Modified from Matsuno et al. 2013)

16.7 Conclusions and Perspectives

The circannual pupation rhythm of *A. verbasci* shows self-sustainability, temperature compensation of the period, and entrainability to a zeitgeber, and the shapes of the circannual PRCs in this rhythm resemble those of circadian PRCs. These results indicate that a circannual clock of *A. verbasci* is the endogenous oscillator that has some parallels with a circadian clock (Miyazaki et al. 2012). It is likely that this circannual clock arose in the evolutionary process during which the life-cycle duration has been extended to a few or several years, and involved, as part of the input pathway for entrainment, a photoperiodic time measurement system almost identical to that used for usual photoperiodism exhibited by many temperate insects (Miyazaki et al. 2009a, b, 2012). Because this circannual clock sets the gate for pupation approximately a half year after the decrease of photoperiod in late summer and development is suppressed by low temperature during winter, even though the geographic cline in the critical daylength is slight, pupation adequately occurs in spring or early summer in many local areas (Matsuno et al. 2013). In this way, *A. verbasci*, and probably *A. sarnicus*, accomplish synchrony with the seasons by the circannual rhythm. The circannual clock of *A. verbasci* is also likely to regulate other developmental and physiological parameters, including larval molting. Therefore, the primary role of the circannual clock(s) may be to maintain the appropriate relationship between a temporal sequence of physiological stages and natural local seasons.

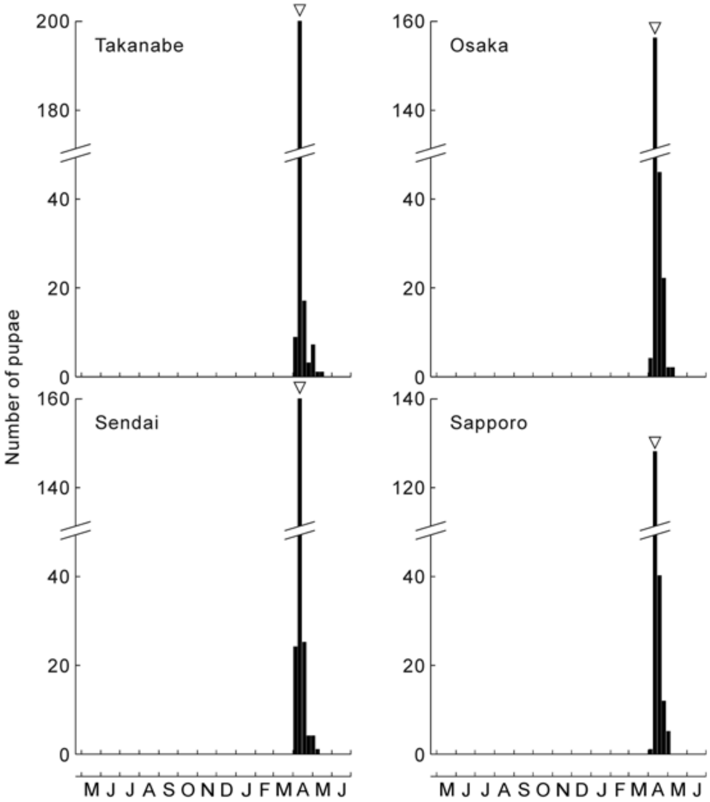


Fig. 16.8 Pupation of Takanabe, Osaka, Sendai, and Sapporo populations of *Anthrenus verbasci* under natural daylength and temperature in Osaka, Japan. Newly hatched larvae were reared from spring, and many larvae showed a pupation peak in April of the following year regardless of the geographic population. *Triangles* indicate median of each pupation group. (Modified from Matsuno et al. 2013)

It is a future subject to uncover in more details the physiological mechanisms and functions of the circannual rhythm of *A. verbasci*. One possible way would be to identify molecular candidates involved in this circannual rhythm by utilizing omics approaches such as RNA-seq and then reveal the precise role of the candidates by RNA interference, which inhibits gene expression in various physiological responses, or by genome editing technology such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) to disrupt the candidate genes.

The circannual clock is an internal provider of the approximate time of year, and its oscillation is affected by external time cues. Therefore, annual rhythms displayed by many organisms can be regarded as a consequence of the interaction between internal and external time-of-year information. The understanding of the adaptive value of circannual rhythms depends on the understanding of this interrelationship (Gwinner 1986). Concerning seasonality in ants, Kipyatkov (1993, 1995) described it as follows. (1) Many species of tropical and subtropical ants do not clearly show annual rhythms in ontogenetic events, but in some other species endogenous rhythms may have an important role in the regulation of seasonal development under conditions of tropical and subtropical climates. (2) In species distributed in lower latitudes of the temperate zone, reactivation induced by the endogenous rhythms occurs in autumn but approaching cool weather makes the resumption impossible until warm seasons. (3) In species distributed in much higher latitudes, true reactivation caused by cold weather is developed and reactivation induced by the endogenous rhythms is incomplete. This broad overview on the relationship among habitat latitudes in ant species, influences of external seasonal factors, and the role of endogenous rhythms in the control of the life cycle may be helpful to investigate the ecological and evolutionary aspects of circannual rhythms in insects, although more investigations are required.

Carpet beetles and ants are holometabolous insects, but circannual rhythms may exist in hemimetabolous insects, although the evidence is still insufficient (Mao and Henderson 2007; Saunders 2010). Investigations of insects of which the life cycles take several or more years require a long time and are scare compared to investigations of insects that have a life cycle shorter than a year. Therefore, how far seasonal strategy with circannual rhythms is distributed over insect species is still veiled. To clarify the physiological and adaptive significance of circannual rhythms in insects, further research is needed across various insect species.

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Glossary

Amplitude Difference between the maximum or minimum value of a biological oscillation from its mean.

Biological clock Self-sustained oscillator which generates a biological rhythm in absence of external periodic input.

Biological rhythm A cyclical, repeated variation in a biological function.

Circa-rhythms Endogenous rhythms, which free-run in constant conditions with a period slightly deviating from the environmental cycle to which they entrain in nature.

Circadian rhythm An endogenous biological rhythm with a period length of around 24 h, which under natural conditions is entrained to the 24 h day. From the Latin *circa* and *dies*, “about a day”.

Circalunar rhythm An endogenous biological rhythm with an endogenous period length close to one lunar month; under natural conditions it entrains to the lunar cycle (29.53 days) and is correlated with every second spring or neap tide event in marine organisms.

Circannual rhythm An endogenous biological rhythm with a period length of about 1 year, which under natural conditions is entrained to the solar year (i.e., 365.25 days).

Circasemilunar rhythm An endogenous rhythm with a period length of around half a month, which under natural conditions is entrained to the cycle of spring or neap tides (14.77 days).

Circatidal rhythm An endogenous biological rhythm with a period length of around a tidal or bitidal cycle; under natural conditions it entrains to the locally prevailing tidal cycles (periods of 12.4, 24.8 h or otherwise).

Clock gene Gene involved as a component of the molecular mechanism that produces a circadian oscillation.

Clock-controlled gene A gene whose expression is regulated directly by the core oscillator mechanism.

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D Abbreviation for "darkness".

Endogenous rhythm An oscillating system capable of self-sustained oscillations, i.e., of generating periodic repetitions under conditions that provide no external information about the period length it assumes.

Entrainment The process by which a biological oscillator assumes the same period length as that of the driving oscillator (*Zeitgeber*), such as the light/dark cycle; usually the phase relationship is stabilized.

Free-running period (τ) The period length of a free-running biological rhythm, i.e., the duration of the time taken between two consecutive recurrences of a phase marker (e.g., the maximum or onset of a biological process).

Free-running rhythm The endogenous rhythm exhibited under constant conditions.

Internal desynchronization State in which two or more previously synchronized variables within the same organism have ceased to exhibit the same period length and/or phase angle.

L Abbreviation for "light".

Masking The phenomenon whereby an external factor directly affects the expression of an overt rhythm temporarily, without affecting the period length or phase of that rhythm (in contrast to "entrainment").

Melatonin A hormone produced rhythmically e.g., in vertebrates, by the pineal gland with high levels at night.

Neap tide Tide with the minimum range between high and low tides, which occurs every 14.77 days around the first and last quarters of the moon, when the sun–-earth axis and the moon–-earth axis form a right angle.

Oscillator A system capable of producing a regular fluctuation of an output around a mean. In chronobiology, an oscillator usually refers to a molecular mechanism within a cell capable of generating self-sustained rhythms.

Overt rhythm An observable rhythm that is usually directly or indirectly regulated by the circadian clock, but could also be driven directly by environmental fluctuations.

Pacemaker Structure capable of sustaining its own oscillations and of regulating other oscillators.

Period length Time after which a defined phase of the oscillation reoccurs, i.e., time taken for a full cycle.

Phase A particular reference point within the cycle of a rhythm (e.g., wake-up time or maximum).

Phase angle Position of a phase point (e.g., wake-up time) relative to the period (expressed in fraction of the period) or relative to a given time of day if the cycle is entrained (more strictly referred to as "phase angle difference").

Phase response curve The profile of phase shifts in response to a *Zeitgeber* stimulus as a function of the oscillator phase at which the stimulus is applied.

Phase shift A single, persistent change in phase brought about by the action of a *Zeitgeber*.

Photoperiod The duration of the light fraction in a 24-h cycle; depends on time of year and latitude.

Photoperiodism The response to the length of day or night by organisms, a physiological mechanism for seasonal adaptation.

Rhythm Regular temporal or spatial repetitions of patterns.

Spring tide Tide with the maximum range between high and low tides, which occurs every 14.77 days around the new moon or the full moon when the earth, sun and moon are situated in an approximately straight line.

Synchronization Process or state in which two or more oscillations assume the same phase angle and the same period length.

Temperature compensation Stability of the period length of biological clocks under different constant ambient temperatures, in contrast to normal physiological Q10 principles.

Zeitgeber From the German "time giver", often translated as "synchronizing cue". A periodic environmental signal that entrains a biological rhythm; in circadian context light is the predominant *Zeitgeber*, but other circa clocks may be responsive to other *Zeitgeber*. A "*Zeitgeber*" does not induce a rhythm but determines its period length and sets its phase angle.

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