

THINK AGAIN

Insights & Perspectives

Circalunar clocks—Old experiments for a new era

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Abstract

Circalunar clocks, which allow organisms to time reproduction to lunar phase, have been experimentally proven but are still not understood at the molecular level. Currently, a new generation of researchers with new tools is setting out to fill this gap. Our essay provides an overview of classic experiments on circalunar clocks. From the unpublished work of the late D. Neumann we also present a novel phase response curve for a circalunar clock. These experiments highlight avenues for molecular work and call for rigor in setting up and analyzing the logistically complex experiments on circalunar clocks. Re-evaluating classic experiments, we propose that (1) circalunar clocks in different organisms will have divergent mechanisms and physiological bases, (2) they may have properties very different from the well-studied circadian clocks and (3) they may have close mechanistic and molecular relations to seasonal rhythms and diapause.

KEYWORDS

biological clocks, chronobiology, ecophysiology, evolution, lunar rhythm, marine biology, reproductive rhythm

INTRODUCTION

When in 2017 the Nobel Prize in Physiology or Medicine was awarded to Jeffrey C. Hall, Michael Rosbash and Michael W. Young for ‘discoveries of molecular mechanisms controlling the circadian rhythm’ (press release from the Nobel Assembly at Karolinska Institute), it underscored the growth and immense progress that the study of biological clocks has experienced in the past decades. However, the statement also highlighted how little we still know about the molecular clockworks underlying ‘non-circadian’ biological clocks. Circatidal, circalunar and circannual clocks are still ‘nature’s enigmatic clocks’.^[1] Currently, a new generation of biologists is setting out to discover the molecular underpinnings of these clocks, supported by technological advances such as high throughput sequencing or genome editing. But while we depart for new ground, we should not abandon the many classic experiments that have been carried out on circatidal, circalunar and circannual clocks since the early 20th century. They not only remind us of the experimental rigor required to address complex phenomena such as long-term biological rhythms. Careful re-evaluation of

this work also produces hypotheses along which we can design experiments to be conducted with today’s advanced technologies.

The little we know about the molecular basis of circalunar clocks has been comprehensively reviewed before.^[2–4] In this review, we throw the spotlight on major mechanistic concepts which come from a large body of now classic, largely behavioral laboratory experiments. We underline the implications and prospects that these concepts and experiments have for guiding new molecular work. By integrating classic work, we cast hypotheses on the molecular basis of circalunar clockworks. Firstly, we suggest that circalunar clocks will *not* be all build along the same mechanistic principles, implying that they arose several times in the course of evolution. In contrast, circadian clocks likely also have multiple evolutionary origins, but seem to be built along similar principles.^[5] Secondly, circalunar clocks may have some properties that differ markedly from circadian clocks. We illustrate this with an unpublished experiment by the late Dietrich Neumann, a pioneer of circalunar clock research. Thirdly, we propose that circalunar clocks may have close mechanistic and molecular relationships with seasonal timing mechanisms. In some species circalunar clocks may even be

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derived from a diapause-like mechanism. Formulation and evaluation of these hypotheses requires basic understanding of biological clock concepts and terms, which we develop in the following section.

Cycles, rhythms and clocks—Basic terminology and classification

Biological clocks are endogenous time-keeping mechanisms, which allow an organism to anticipate regular environmental cycles and adjust their physiology and behavior accordingly. There is an important distinction between *cycles*, *rhythms* and *biological clocks*. The term *cycle* is somewhat indiscriminately used for both environmental cycles, for example, night and day, as well as cycles in organisms' behavior or physiology, for example, the sleep-wake cycle. In contrast, the term *rhythm* is reserved for recurring behavioral or physiological events in organisms. Rhythms can be *direct responses* to environmental cycles. In this case they are exogenously driven and do not result from a biological clock. Alternatively, they can be the manifestation of an endogenous *biological clock*, allowing for anticipation of the exogenous cycle. This can be demonstrated in so-called *free-run experiments* (see below).

Biological clocks and rhythms are cyclic phenomena and borrowing from physics are described as *oscillators*, which among other characteristics have a specific *period* and *phase* (Figure 1A). The *period* of a clock or rhythm is the time that it takes to reach the same point in the oscillation again, for example, the time between one peak of activity and the next (Figure 1A). The *phase* of a clock or rhythm describes when the specific phenomenon happens relative to an external time frame (Figure 1A). For example, nocturnal and diurnal animals both have a rhythm with a 24-h period, but they differ in their phase of activity.

In nature there are four highly regular environmental cycles, which all result from the movements of earth and moon in relation to the sun: tides, night and day, the lunar cycle and the seasons (Figure 1B, C). For each of these cycles, corresponding biological clocks have evolved. As night and day and the seasons depend on the earth's movements related to the sun, the corresponding clocks can be considered 'sun-related' clocks (Figure 1D). The tides and the lunar cycle depend largely on the movement of the moon relative to earth. The corresponding clocks can therefore be considered 'moon-related' clocks (Figure 1D). Tides and the lunar cycle are closely intertwined. Most notably, the amplitude of the tides changes throughout the lunar cycle and is largest just after full moon and new moon during the so-called spring tides (Figure 1C). Organisms which require a specific tidal situation, such as spring tides, often have clocks that allow them to time half a lunar cycle—these are called circasemilunar clocks (Figure 1C, D).

The periods of circadian and circatidal clocks are in the range of hours (Figure 1D). These short-period clocks usually control an organism's 'daily business', for example its activity, feeding or UV protection.^[6–8] Given that a circatidal period is very close to half a circadian period, there has been a long debate on whether or not these two clocks may rely on the same molecular clockwork,^[9–10] with a recent molecular twist that suggests both is partially true.^[11] The long-period biological clocks—circalunar clocks and circannual

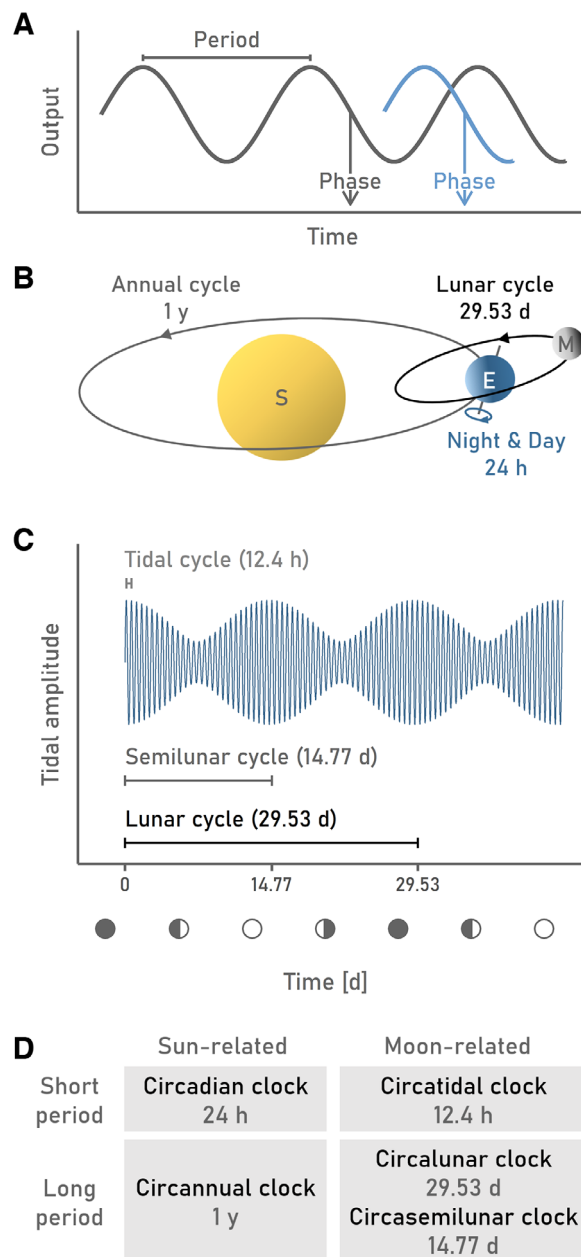


FIGURE 1 Features of biological rhythms and the tides. (A) Biological rhythms are described as oscillations. The time between two successive occurrences of the same state is the *period* of the rhythm. The time when a specific state occurs relative to an external time frame is the *phase* of the rhythm—for example, nocturnal versus diurnal activity. (B) All regular environmental cycles come about by the movements and positions of earth (E), moon (M) and sun (S). Revolution of the earth around the sun causes the annual cycle. Rotation of the earth around its own axis causes night and day. Revolution of the moon around the earth causes the lunar cycle. The tides come about by a complex interaction of the moon's and sun's gravitational influence on the earth's water bodies, combined with the earth's rotation around its axis. (C) The period of the tidal cycle is 12.4 h. The amplitude of the tides changes throughout the lunar cycle. It is particularly large twice a lunar cycle, namely during the spring tides just after full moon and new moon. Spring tides repeat in a semilunar cycle. (D) Biological clocks can be classified based on their period and their major geophysical influences.

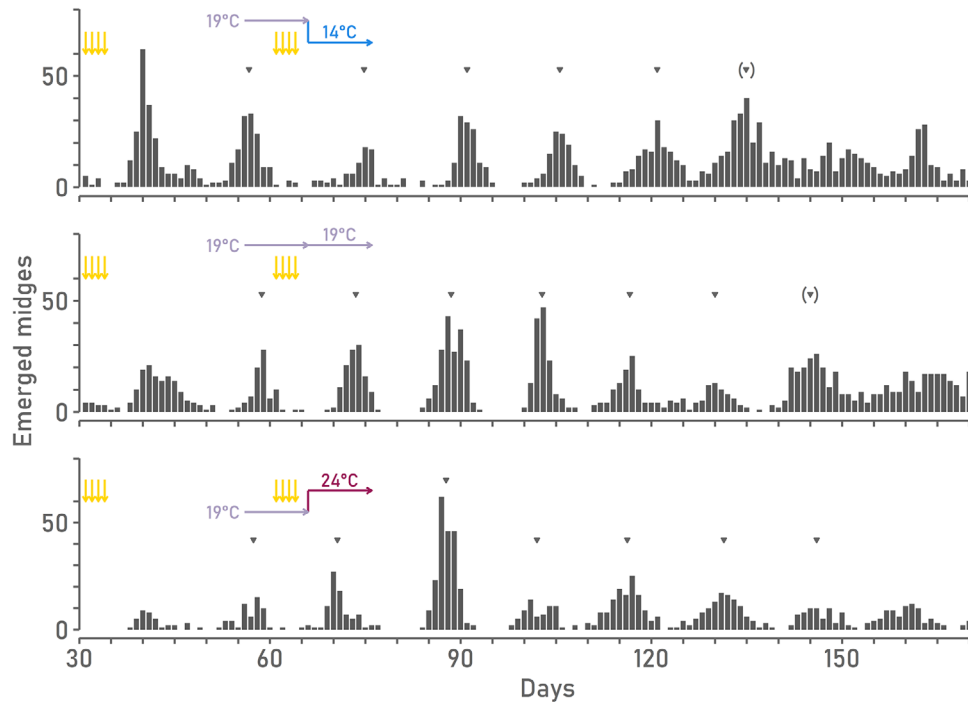


FIGURE 2 The circasemilunar rhythm of *Clunio tshimensis*. After entrainment by moonlight (four nights every 30 days; yellow arrows) the semilunar rhythm persists (*free-runs*) for several months with a period of 15 days. Arrowheads indicate the median of individual emergence peaks. The free-running period is the same under 14°C, 19°C and 24°C, that is, the rhythm is *temperature-compensated*. Figure re-drawn according to [21] with permission from Springer Nature.

clocks—usually control an organism's long-term investments, which are development, maturation and reproduction. Again, regulation of similar physiological processes may suggest the two clocks are related, an idea that we will discuss in more detail at the end of our essay.

In summary, biological clocks are experimentally confirmed endogenous time-keeping mechanisms. They time an organism's physiology and behavior to regular environmental cycles and can be distinguished based on their underlying period. Circalunar and circasemilunar clocks have long periods and usually control reproduction.

Free-run experiments indicate that circalunar clocks are wide-spread in marine organisms

Proof of the endogenous nature of a biological rhythm comes from *free-run experiments*. In such an experiment the organism is kept under constant conditions without exogenous cycles. If the behavioral or physiological rhythm continues, it must be controlled by a biological clock. In a free-run experiment the rhythm must fulfill three major criteria,^[12] which are illustrated in Figure 2.

First, the rhythm must free-run, that is, it must continue without any exogenous cues. Additionally, the *period of the free-running rhythm* (termed τ) must be close to the exogenous cycle that it is supposed to correspond to. The period of a free-running rhythm is rarely exactly the same as the period of the exogenous cycle and there is also individual variation within a species. For this reason free-running rhythms are called 'circa'-dian, 'circa'-lunar and so on. The deviation in period

from the exogenous cycle is crucial for the free-run experiment, as it rules out that the observed rhythm is controlled by exogenous cues that could not be excluded from the experiment (e.g., cycles in gravity or magnetic fields in the case of circalunar clocks).

Second, it must be possible to synchronize or reset the rhythm with specific environmental cues. Such specific cues are called *zeitgeber* (German; literally 'time-giver') and the process of synchronization is called *entrainment* of the clock. Entrainment ensures that the endogenous clock is always synchronized with the environmental cycle, despite the slight deviation of its free-running period.

Third, the free-running rhythm must be *temperature-compensated*, that is, the free-running period should be unaffected by ambient temperatures, in the range that is physiologically relevant to the organism. Usually, biochemical processes proceed 2–3 times faster if the temperature is raised by 10°C, which is expressed as them having Q_{10} values of 2 to 3. Biological clocks have Q_{10} values of close to 1 (compare Figure 2)—if they had not, they could not reliably tell time.

While lunar rhythms are reported for many organisms,^[3,13] most famously the palolo worm^[14] or the pacific grunion,^[15] experimental confirmation of an underlying circalunar clock is only available for a few (Table 1). First experimental proof of an endogenous circalunar clock came in 1960 for the marine annelid *Platynereis dumerilii*^[16] (confirmed by^[17]), followed by the brown alga *Dictyota dichotoma*^[18] and the marine midge *Clunio marinus*.^[19] To date endogenous circalunar or circasemilunar clocks have been demonstrated in about two dozen of species (Table 1). For most of these organisms only the

TABLE 1 Organisms with experimental proof of a circalunar or circasemilunar clock

Organism	Nature of the rhythm	Free-run with appropriate period (Crit 1)	Entrainment (crit 2)	Temperature compensation (crit 3)
<i>Algae</i>				
<i>Dictyota dichotoma</i>	Semilunar rhythm of egg release	[18]	ML ^[18]	? ^[18]
<i>Fucus vesiculosus</i>	Semilunar rhythm of gamete release	? (period is ~7days) ^[87]	–	–
<i>Ulva pseudocurvata</i>	Semilunar rhythm of gamete release	? (period is 7 days) ^[88]	ML? ^[88]	–
<i>Cnidarians</i>				
<i>Pocillopora damicornis</i>	Lunar rhythm of planula-larvae release	? ^[89]	ML? ^[89]	–
<i>Annelids</i>				
<i>Platynereis dumerilii</i>	Lunar rhythm in spawning	[16]	ML ^[16]	–
<i>Syllis prolifera</i> (previously <i>Typosyllis prolifera</i>)	Lunar rhythm in stolonization	[20,60]	ML ^[20]	[20]
<i>Molluscs</i>				
<i>Liolophura japonica</i> (previously <i>Acanthopleura japonica</i>)	Semilunar rhythm in spawning	[90]	Undetermined tidal factor ^[90]	–
<i>Haliotis asinina</i>	Semilunar rhythm in spawning	[91]	–	–
<i>Crustaceans</i>				
<i>Chiromantes haematocheir</i> (previously <i>Sesarma haematocheir</i>)	Semilunar rhythm of egg-laying and zoea-larvae release	[92]	ML ^[92]	–
<i>Leptuca pugilator</i> (previously <i>Uca pugilator</i>)	Semilunar rhythm of larval hatching	[93]	–	–
<i>Talitrus saltator</i>	Semilunar rhythm of locomotor activity and molt synchrony	[94]	–	–
<i>Eurydice pulchra</i>	Semilunar rhythm of swimming behavior	[95]	TTu ^[41,95,96]	–
<i>Excirrolana chiltoni</i>	Semilunar swimming activity	[97]	–	–
<i>Scyphax ornatus</i>	Semilunar activity rhythm	[98]	–	–
<i>Bathyporeia pilosa</i>	Semilunar periodicity in swimming activity	[99]	–	–
<i>Gammarus chevreuxi</i>	Semilunar periodicity in swimming activity	[100]	–	–
<i>Insects</i>				
<i>Clunio marinus</i>	Lunar or semilunar rhythm in adult emergence	[19]	ML ^[19] TTu ^[47] TTe ^[40]	–
<i>Clunio tsushimensis</i>	Semilunar rhythm in adult emergence	[21]	ML ^[21]	[21]
<i>Pontomyia oceana</i>	Semilunar rhythm in adult emergence	[101]	ML ^[101]	[101]
<i>Myrmeleon obscurus</i>	Lunar rhythm in pit size	? ^[102]	–	–
<i>Fish</i>				
<i>Fundulus grandis</i>	Semilunar spawning activity	[23,103–104]	–	[23]
<i>Fundulus heteroclitus</i>	Semilunar spawning activity	[103]	–	–
<i>Oncorhynchus kisutch</i>	Semilunar growth rate and feeding rhythm	[105]	ML? (switch from LL to LD) ^[106]	–
<i>Oncorhynchus mykiss</i> (previously <i>Salmo gairdneri</i>)	Semilunar growth rate and feeding rhythm	[107–108]	–	–

Abbreviations: ML = moonlight, TTu = tidal turbulence, TTe = tidal temperature

first two criteria—free-running nature and entrainment—have been tested. The remarkable phenomenon of temperature compensation in these long-term developmental or reproductive cycles has only been demonstrated in the annelid *Syllis prolifera* (previously *Typosyllis prolifera*),^[20] the midges *Clunio tsushimensis*^[21] (see Figure 2) and *Pontomyia oceana*,^[22] as well as the killifish *Fundulus grandis*.^[23]

Circalunar clocks are found in a broad variety of taxa, most of which are marine (Table 1). The prevalence of circalunar clocks in marine organisms may be due to their two-fold adaptive value. Many intertidal organisms use circalunar clocks to time the delicate events of reproduction to ecologically suitable tides (see Figure 1C; e.g., in crabs^[24–25] or *Clunio* midges^[26]). Additionally, circalunar clocks synchronize reproduction within a population, maximizing the chances of successful fertilization. This aspect is most important for broadcast spawners, which release their gametes into the open water (e.g., corals^[27] or annelids^[28]). Particularly in tropical oceans, with no seasonality to synchronize reproduction, lunar rhythms are very common. In line with this idea, lunar reproductive rhythms have also been reported for a few tropical freshwater insects.^[29–30]

Experimental tests for the nature of the circalunar oscillator suggest different mechanisms and multiple evolutionary origins of the circalunar clock

Simplistically, biological clocks are described to consist of three parts. At the core of the clock is the *oscillator*, which maintains the free-running endogenous rhythm. The *input pathways* convey zeitgeber information to the oscillator for entrainment of the clock. The *output pathways* serve to orchestrate physiology and behavior according to the endogenous clock. In the following sections, we integrate what we know about these stylized compartments for the circalunar clock and we derive hypotheses and experiments for future research.

While the core molecular clockwork maintaining circalunar rhythms is still unknown, there are three main hypotheses for its mechanism.^[18] The default assumption is that there is a circalunar oscillator with a 30-day period (or a 15-day period for circasemilunar clocks), based on a yet unknown physiological mechanism. Already in the 1960s Erwin Bünning developed the *beat hypothesis* as an alternative mechanism.^[18,31] Inspired by work on intertidal crabs, which show simultaneous circadian and circatidal rhythms (e.g.,^[32]), he proposed that lunar phase can be determined by superposition of a circadian and a circatidal clock. These two clocks would adopt the same phase relationship every 15 days and this ‘beat’ could serve to produce a circasemilunar rhythm (Figure 3A). By the same principle, superposition of a circadian with a circalunidian clock (‘lunar day clock’, 24.8 h) would produce a 30-day beat for a circalunar rhythm. As a third hypothesis, circalunar and circasemilunar clocks could rely on a counter mechanism counting 15 or 30 circadian cycles (also known as *frequency demultiplication hypothesis*, borrowing from circannual clock research^[33–35]). In a narrow sense, the terms ‘circalunar clock’ and ‘circasemilunar clock’ can be understood to only refer to circalunar or circasemilunar oscillator mechanisms. However, throughout this essay

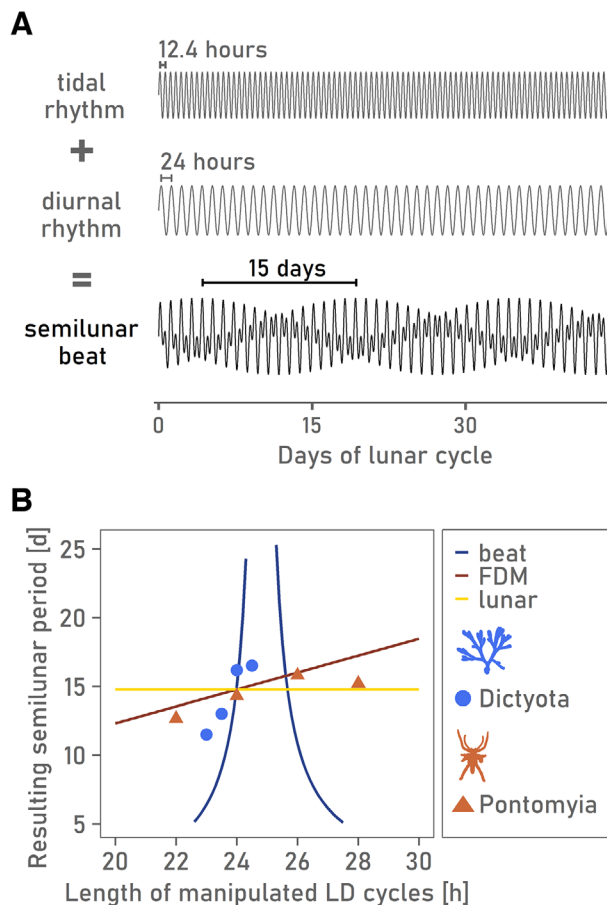


FIGURE 3 Three possible mechanisms for circa(semi)lunar clocks. (A) The beat hypothesis suggests that a semilunar clock relies on superposition of a tidal rhythm with a diurnal rhythm, which produces a semilunar beat. (B) Alternatives to the beat hypothesis (blue) are an independent semilunar oscillator with a period of about 15 days (yellow) or the counting of days (frequency demultiplication hypothesis, FDH; red). Manipulation of daylength affects these mechanisms differently and is predicted to lead to discriminative changes in the period of the free-running semilunar rhythm (colored lines). Experimental evidence (colored dots) suggests that different mechanisms underlie the semilunar rhythms in the alga *Dictyota dichotoma*^[18,36] and the midge *Pontomyia oceana*.^[35]

we use the terms in a broad sense, including all mechanisms capable of producing free-running circalunar or circasemilunar rhythms.

Bünning also proposed an experiment to discriminate between the proposed clock mechanisms.^[18,31] Circadian clocks usually synchronize to light. Driven by exogenous light-dark (LD) cycles, they can adopt periods slightly longer or shorter than 24 h. Circatidal clocks usually do not respond to light, as light is not a very good indicator of the tides. Thus, under a 23-h LD cycle, the circadian clock would assume a 23-h period whereas the circatidal clock would keep a 12.4-h period. This would change the beat dramatically in a non-linear way (Figure 3B). In contrast, a counter mechanism should change in a linear relationship to the change in the LD cycle, while a dedicated circalunar oscillator should be unaffected (Figure 3B).

TABLE 2 Different mechanisms for circalunar and circasemilunar clocks and their experimental support

Mechanism	Species	Publication
Circalunar oscillator	probably in <i>Platynereis dumerilii</i>	[16–17]
Beat hypothesis	<i>Dictyota dichotoma</i>	[18,36]
Counting/Frequency demultiplication hypothesis (FDH)	<i>Pontomyia oceana</i>	[35]

In the brown alga *Dictyota dichotoma* the circasemilunar rhythm may rely on a beat phenomenon, but the effect is not as strong as expected (Figure 3B; [18,36]). The crustacean *Scyphax ornatus* might also use a beat mechanism.^[37] However, these experiments were performed under permanent entrainment by both LD cycles and tidal vibration. Under these conditions the two exogenous zeitgebers already produce a beat themselves, which the organism may respond to directly. For testing the beat hypothesis, one of the two clocks must free-run, to make sure the beat is produced endogenously. In contrast, the lunar rhythm in the marine midge *Pontomyia oceana* relies on counting circadian cycles (Figure 3B,^[35]). Finally, evidence for a dedicated circalunar oscillator is available for the annelid *Platynereis dumerilii*. After early experiments with manipulated LD cycles,^[16] chemical inhibition of the circadian clock left the circalunar rhythm unaffected.^[17] As both the beat hypothesis and counting are expected to involve the circadian clock, *P. dumerilii* is probably using an independent circalunar oscillator.

In summary, the circalunar and circasemilunar clocks of different organisms are likely based on different mechanisms (Table 2) and evolved multiple times independently. While circadian clocks rely largely on feedback loops in transcription, translation and protein modification,^[5] circalunar and circasemilunar clocks may rely on other physiological processes, which may more readily resonate with long periods. As these processes may well differ between organisms, we must be very cautious when transferring experimental insights on circalunar clocks from one organism to another.

Zeitgeber perception likely involves a circadian clock

Early experiments on the zeitgebers entraining circalunar clocks were performed in *Platynereis dumerilii*, showing that artificial moonlight—that is, dim light presented for few nights—can synchronize lunar spawning behavior.^[16] Ablation of the worm's eyes did not alter periodic swarming behavior, suggesting that moonlight is perceived independent of the eyes.^[38] Entrainment of circalunar and circasemilunar clocks by moonlight was also demonstrated in various other taxa (Table 1), but the underlying photoreceptors and integration processes remain unclear. It was shown that circasemilunar rhythms can also be entrained by 12.4-h tidal cycles, which change over the 15-day spring-neap tide cycle. Semilunar rhythms in the marine midge *Clunio marinus* are synchronized by tidal turbulence cycles^[39] and tidal temperature cycles.^[40] Similarly, entrainment of semilunar rhythms in

some crustaceans depends on wave agitation^[41] (Table 1). Such tidal zeitgebers might be of particular importance at high latitudes, where moonlight cannot be reliably perceived, or in locations where tides are very strong.^[42]

Mechanistic insights into how zeitgebers are perceived mainly come from the marine midges *Clunio marinus* and *Clunio tsushimensis*. In a very elegant experiment,^[43] *C. tsushimensis* was subjected to constant darkness for four days once per lunar cycle. Constant darkness allowed for the presentation of artificial moonlight at different times of day. Moonlight only entrained the circalunar clock when it was presented around midnight, suggesting the moonlight receptor is insensitive during the day. Receptor sensitivity must be controlled by a circadian clock, as it is still regulated in constant darkness.^[43] Muting the moonlight receptor during the day not only solves the problem of discriminating moonlight from sunlight, but also serves to obtain information on lunar phase. The daily timing of moonlight changes throughout the lunar cycle and in the intertidal zone can be further narrowed by the tides.^[44] Therefore, a nocturnal light sensitivity window will only coincide with available moonlight during a few nights of the lunar cycle. Such a mechanism is called *coincidence detection*. It is closely related to the *external coincidence model* for photoperiodism,^[45] which we will return to in the last section of this essay.

Coincidence detection is also related to the beat hypothesis. But importantly, the beat hypothesis describes the oscillator. The beat is fully internal to the organism, based on coincidence between two endogenous rhythms, producing an internal mechanism to measure time. In contrast, coincidence detection refers to synchronizing the oscillator with the outside world. It combines a single endogenous rhythm in sensitivity (usually a circadian rhythm) with an external environmental cycle. This constitutes a fundamental conceptual difference, which must be carefully discriminated in any experimental setup.

Coincidence detection is also at the heart of entraining circalunar clocks with tidal turbulence and temperature pulses, as was shown in *C. marinus*.^[40,46–48] In the intertidal zone, water turbulence, water temperature and many other factors fluctuate over the tidal cycle of 12.4 h. As an example, strong water turbulence, which corresponds to rising tide,^[47] will due to its 12.4-h cycle gradually shift in daytime. Every 15 days it will occur at the same time of day. When a circadian clock tunes turbulence receptor sensitivity to a specific time of day, sensitivity and stimulus will only coincide every 15 days. In that way, coincidence detection can extract long-term information on lunar phase from two short-term cycles. Again, this mechanism can be experimentally assayed by manipulating the LD cycle. In *C. marinus* entrainment of the circalunar clock indeed changes with manipulations in the LD cycle^[48] and is no longer possible when the length of the LD cycle is outside the ranges of entrainment of the circadian clock.^[49] Experiments in the crustaceans *Eurydice pulchra*^[41] and *Scyphax ornatus*^[37] suggest they may possess similar mechanisms. Finally, a tidal oscillation of melatonin receptor gene expression is reported for the puffer fish *Takifugu niphobes*.^[50] The combination of tidally modulated melatonin sensitivity with daily changes in melatonin levels might produce a coincidence detection system that entrains the semilunar rhythm.^[50]

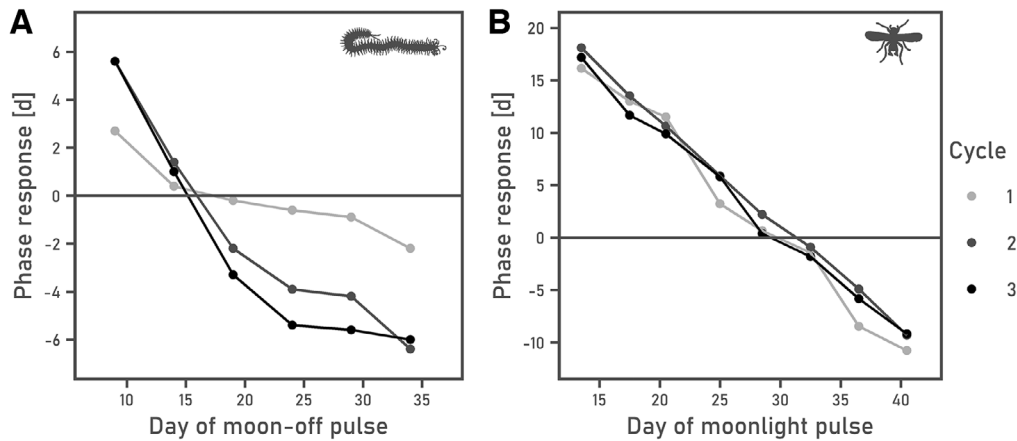


FIGURE 4 Phase response curves (PRCs) for moonlight entrainment of circalunar clocks. (A) The PRC for the circalunar clock of *Syllis prolifera* shows a non-linear response and transient cycles (data from [54]). (B) The PRC for the circasemilunar clock of *Clunio tsushimensis* shows a linear response and no transient cycles, consistent with an immediate resetting of the clock by moonlight (based on unpublished data by D. Neumann, Figure S1).

In conclusion, all three well-studied lunar zeitgebers—moonlight, as well as mechanical and temperature stimuli associated with the tides—may have similar detection mechanisms, which involve a circadian clock. Notably, involvement of a circadian clock in entrainment (*coincidence detection*) does not imply that the circadian clock is also involved in the oscillator (*beat hypothesis*). These two aspects need to be carefully discriminated when designing behavioral or molecular experiments. Identification of cellular or molecular integration points between putative lunar zeitgeber receptors and the circadian clock seems a valuable target for future research.

A phase response curve for the circalunar clock of *C. marinus*

The way a clock entrains to a zeitgeber usually depends on when that zeitgeber is presented. At some points of a clock's oscillation, zeitgebers can strongly shift the clock, at other points they have basically no effect. This is captured in a so-called *phase-response curve* (PRC). The now classic PRC for circadian entrainment in the fly *Drosophila pseudoobscura*[45] shows that light exposure during expected day barely shifts the clock, whereas light during expected darkness shifts the circadian clock to various extents. Light immediately resets the circadian oscillator,[51–53] but the observed behavioral rhythm usually requires a few days to gradually follow the adjustment, showing so-called *transient cycles*.[45]

A PRC for a circalunar clock has to date only been published for the annelid *Syllis prolifera*.[54] Similar to circadian PRCs, it shows a non-linear response to the zeitgeber, as well as transient cycles (Figure 4A). The late Dietrich Neumann obtained unpublished phase response data for the circasemilunar clock of the midge *C. tsushimensis*, which we analyzed and present here and which reveals notable differences to the PRC of *Syllis prolifera* (Figure 4B, raw data in Figure S1). For interpreting these results, it is important to know that in *Clunio* the time point of

adult emergence and thereby its circalunar rhythm is predetermined 15–20 days beforehand.[55] As a consequence, all emergence peaks that occur within a window of 15–20 days after the resetting moonlight stimulus are unchanged in phase (Figure S1). Emergence peaks occurring after that are at once shifted to the new phase. There are no transient cycles (Figure 4B). The shift in adult emergence is proportional to the shift in the zeitgeber, so that there is a linear phase response (Figure 4B). This can be interpreted as an immediate resetting of the circalunar rhythm within the limits imposed by the required predetermination of development. The contrasting observations made in *Syllis prolifera* and *Clunio tsushimensis* underscore that circalunar clocks diverge in their properties and may follow different mechanistic principles.

Output pathways are complex to assess and likely divergent between species

As circalunar clocks control development and maturation, it is generally assumed that their outputs must at some point converge with the hormonal systems underlying these two processes, be it steroid hormones, gonadotropic hormones or other neuropeptides (for a recent overview see[4]). The classic demonstration of hormonal control of the lunar rhythm comes from the annelid *Platynereis dumerilii*. Annelids' large and soft bodies enable transplant experiments and made them model organisms for development and regeneration.[56] When in *P. dumerilii* the head is removed, the animal immediately undergoes maturation and reproduces irrespective of lunar phase.[57–59] If the head is re-implanted anywhere in the body, maturation is inhibited again. Similar results were obtained for *Syllis prolifera*.[60] Likely, the release of the brain hormone inhibiting maturation is controlled by a circalunar clock. Recently the inhibitory brain hormone of *P. dumerilii* was identified to be methylfarnesoate.[61] Interestingly, this molecule also acts as a juvenile hormone or juvenile hormone precursor in

many crustaceans and insects. In the insect *C. marinus*, the levels of juvenile hormone's opponent ecdysone may fluctuate across the lunar cycle.^[55]

These findings do not necessarily imply evolutionary conservation of direct circalunar clock outputs. The conserved hormonal pathways regulating development and reproduction can be far downstream of the circalunar clock, with circalunar clock outputs merely converging on them. Recent -omics studies on two main circalunar clock models, *C. marinus* and *P. dumerilii*, underline this call for caution. In *P. dumerilii*, a combined transcriptomic and proteomic approach, identified a number of lunar marker transcripts/proteins, which are differentially abundant during two different lunar phases.^[62] But none of the corresponding genes is even present in the high-quality reference genome of *C. marinus*.^[63] Thus, while both organisms likely share the use of juvenile hormone in suppressing development, other circalunar clock outputs will likely differ.

Nevertheless, global screening approaches such as transcriptomics, proteomics and metabolomics are undoubtedly promising to detect circalunar clock outputs. Transcriptomic changes have also been assessed over the lunar cycle in *Acropora* corals^[64-66] and the limpet *Cellana rota*.^[67] In many other organisms, expression of circadian clock genes has been studied at different lunar phases (reviewed in^[2,4]). But these experiments are usually very hard to interpret. From the varying experimental setups it is often not clear if differences in gene expression are driven by exogenous factors and at best serve as an input for setting the circalunar clock, if they are part of the oscillator according to the beat hypothesis or a counting mechanism, or if they are outputs of the circalunar clock (discussed in detail in^[2,4]). Precisely recording experimental and environmental conditions would be a first step to reach better comparability.^[2] But for pinpointing circalunar clock effects we have to face the challenge of combining the experimental sophistication of classic behavioral studies with the standards set for circadian -omics experiments.^[68]

First, experiments should be performed under free-running conditions, so that influences from exogenous *zeitgeber* cycles can be excluded. This is already difficult, as many organisms with circalunar clocks cannot be bred in the laboratory. Second, several lunar cycles should be assessed, which makes experiments very long and prone to technical failure. Third, a sufficiently large number of time points per cycle need to be assessed. If a large number of cycles are recorded, theoretically two time points per cycle are sufficient to detect rhythmicity.^[69] However, in circadian time-series over few cycles the standard procedure of collecting six time points per cycle was already found to be statistically underpowered and 12 time points are recommended.^[68] Fourth, as there likely are interactions between circadian and circalunar clocks at some level, ideally for every circalunar time point several circadian time points are tested. Including the required biological replicates and possible experimental treatments quickly leads to enormous numbers of required samples. Fifth, in order to disentangle the usually overwhelming signals of development and maturation, which likely are merely circalunar clock outputs, from any signals of a circalunar oscillator, experiments would require testing a long-lived life stage that has a running circalunar clock, but does not

(yet) develop and mature. For most species such a life stage might not exist.

In summary, circalunar control of maturation and development can be expected to generally involve some hormonal regulation, but possibly far downstream the circalunar clock. Circalunar clock outputs are accessible to current-day large-scale screening techniques, but these experiments are logistically very challenging. Circalunar clock outputs are not necessarily conserved across species, again arguing for caution when trying to transfer findings across taxa.

A circalunar clock derived from photoperiodic diapause induction?

Photoperiodism is the ability of organisms to anticipate seasonal changes by using daylength as a predictive cue.^[70] Photoperiodism is not a circannual clock mechanism, as there is no yearly endogenous oscillation involved, but photoperiodism can serve to synchronize a circannual clock. The Bünning hypothesis,^[71] later refined by Pittendrigh and Minis to become the external coincidence model,^[45] suggests that detection of daylength depends on light sensitivity in the early or late night, regulated by a circadian clock. If light is perceived during that sensitivity window, days must be long. If there is no light, days must be short. As outlined above, this very mechanism has been experimentally shown to be at the core of moonlight entrainment of the circasemilunar clock of *Clunio tsushimensis*.^[72] It has also been discussed for lunar phase detection in other organisms.^[2,73] In line with these findings, moonlight has been found to affect photoperiodism or seasonal features of circadian timing in beans,^[74] hamsters,^[75] fruit flies^[76] and other insects.^[77-78] Thus, photoperiodism may not only be a way of entraining circannual clocks,^[79-80] but there are also hints that photoperiodism and circannual clocks might share mechanistic principles with circalunar clocks.

In marine midges of the genus *Clunio* there are additional lines of evidence, which combined make a compelling case that the circalunar clock of these insects might be directly derived from a photoperiodic diapause mechanism. Firstly, moonlight detection follows an external coincidence model as discussed above. Secondly, moonlight cues must be integrated over several nights,^[19] just like most insects must integrate daylength cues over many days before entering diapause.^[81] Thirdly, the larval development of *Clunio* is only synchronized with lunar phase in the last larval instar. Synchronization is achieved via a short arrest in development, reminiscent of a very short diapause.^[55] Even more, this circalunar developmental arrest happens at exactly the same larval stage as the regular photoperiodic diapause, that northern European *Clunio* populations undergo in winter.^[82-83]

Insects evolved on land, where seasonal rhythms are prominent, and the closest relatives of *Clunio* are terrestrial midges^[84] with no known lunar rhythms. We must assume that *Clunio* secondarily evolved a circalunar clock only after colonizing the sea. It seems highly plausible that an existing mechanism such as photoperiodic diapause induction was re-used and complemented with some form of resonance with the

lunar cycle. Involvement of the circadian clock in photoperiodism is well established,^[85] and therefore circadian clocks might also play a role in such a derived circalunar time-keeping mechanism. In contrast, taxa with an ancient marine origin, for example, algae or annelids, likely evolved their circalunar clocks earlier and on a different molecular background. This underscores the idea of multiple evolutionary origins of circalunar clocks and various circalunar clock mechanisms, as outlined above. But as life originated in the sea, circalunar clocks can be expected to be ancient timing mechanisms. Could it be that photoperiodism or circannual rhythms are derived from an ancient circalunar clock? Could *Clunio*'s circalunar clock actually be reusing a photoperiodic timer which was reusing a circalunar clock? Such a scenario would well befit the tinkering nature of evolution.^[86]

CONCLUSIONS

Organisms are adapted to regular environmental cycles through various time-keeping mechanisms (Figure 1). While the circadian clock is well understood, the molecular mechanisms underlying circalunar clocks and other long-term rhythms remain elusive. Classic behavioral experiments suggest that circalunar rhythms evolved several times based on distinct mechanisms, not all of which require a circalunar oscillator. Some circalunar time-keeping mechanisms may re-use existing molecular pathways involved in photoperiodic diapause or circannual, circadian and circatidal clocks. The next decade of investigations into the molecular basis of circalunar clocks, might reveal close mechanistic and evolutionary links between all of these time-keeping mechanisms.

NOTES

Tobias Kaiser holds an archive of Dietrich Neumann's published and unpublished data, which can be reviewed upon request. Jule Neumann is not a relative of Dietrich Neumann, but—*nomen est omen*—writes her PhD on diapause and circalunar clocks in *C. marinus*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data is available in publications or in the supplementary material.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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