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

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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

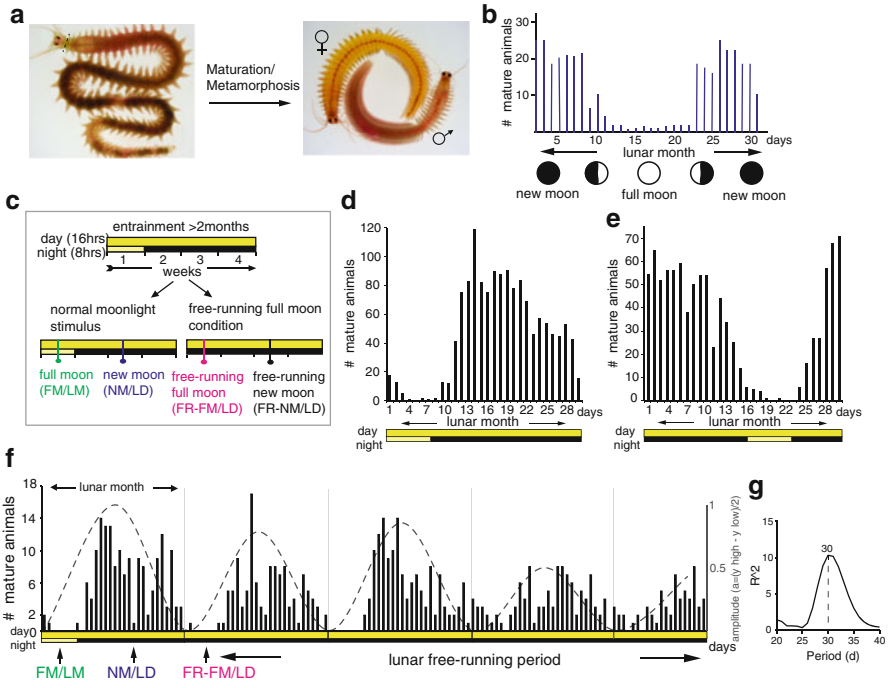


Fig. 8.1 *Platynereis* circalunar periodicity in gonadal maturation and reproductive behavior is entrained by nocturnal light and controlled by an endogenous clock. (**a**) *Left*: Adult worm before the start of sexual maturation. *Right*: Mature male and female at the time of spawning as counted for 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)

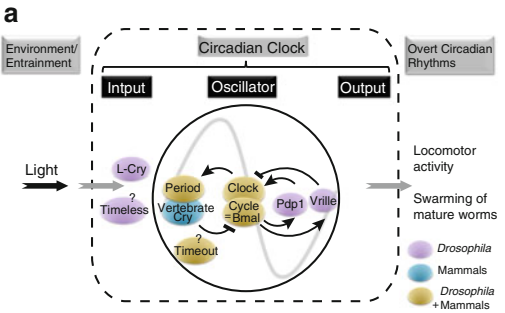
Thus, currently *Platynereis* presents itself as possibly the functionally most powerful system for the elucidation of the molecular and cellular mechanisms that underlie circalunar rhythms.

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



b

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

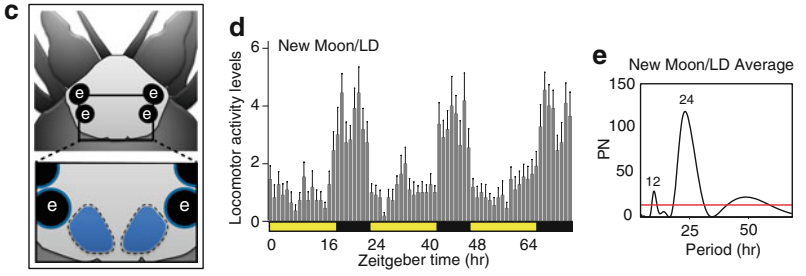


Fig. 8.2 Putative core transcriptional-translational circadian clock feedback loop of *Platynereis dumerilii*. (**a**) Tentative position of *Platynereis* circadian clock gene orthologues in the worm's transcriptional circadian oscillator. (**b**) Summary of *Platynereis* circadian clock gene regulation 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").

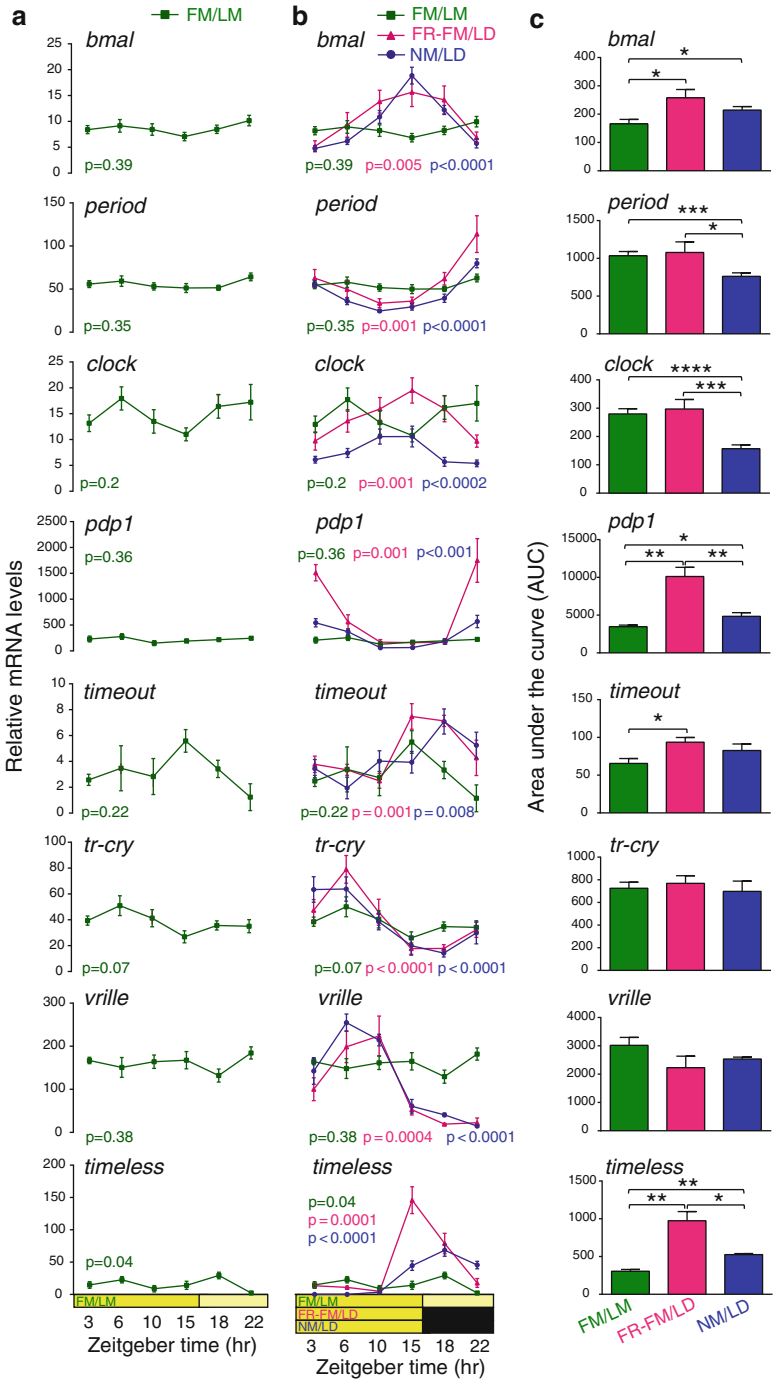


Fig. 8.3 Nocturnal illumination disrupts regular circadian transcript level oscillations. (**a**)Temporal profiles of clock gene RNA expression in *Platynereis* heads at the indicated *zeitgeber* time points sampled at full moon FM/LM (*LM* light–moonlight). Worms were entrained under a daily cycle

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 $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$)

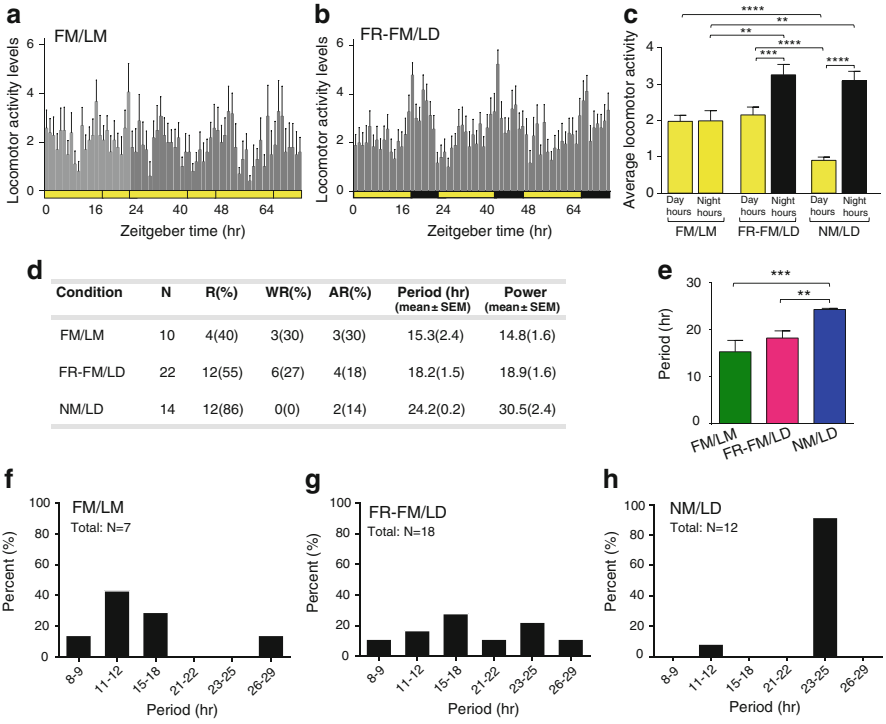
8.6 Regulation of Circadian Behavior

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.

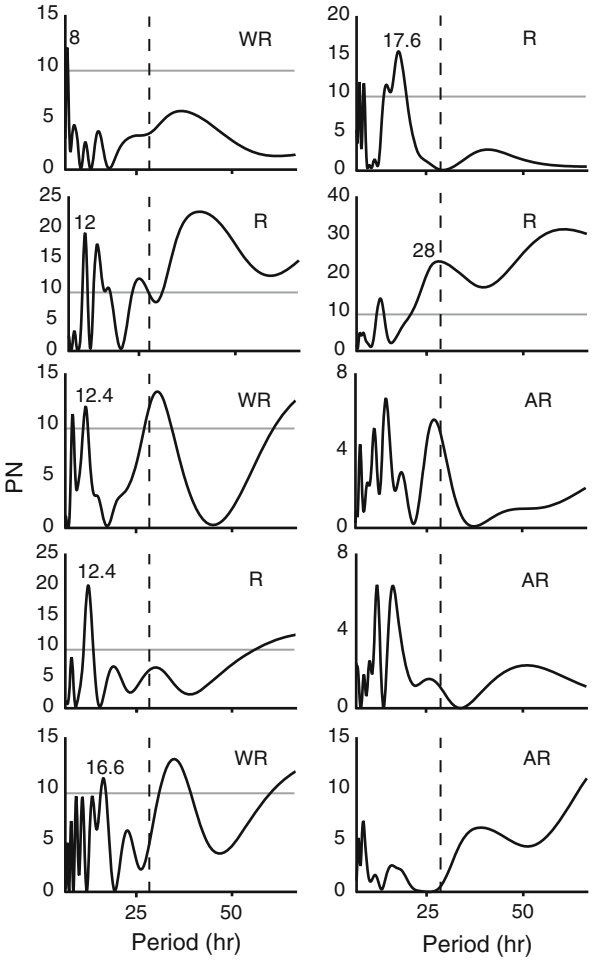


Fig. 8.5 Nocturnal light abolishes 24-h rhythms in locomotor activity in individual worms. Individual Lomb–Scargle periodograms at FM/LM arranged according to increased period length (from *top* to *bottom*). *Gray horizontal line* indicates single *p*-level = 0.05. Period lengths >29 were excluded from 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

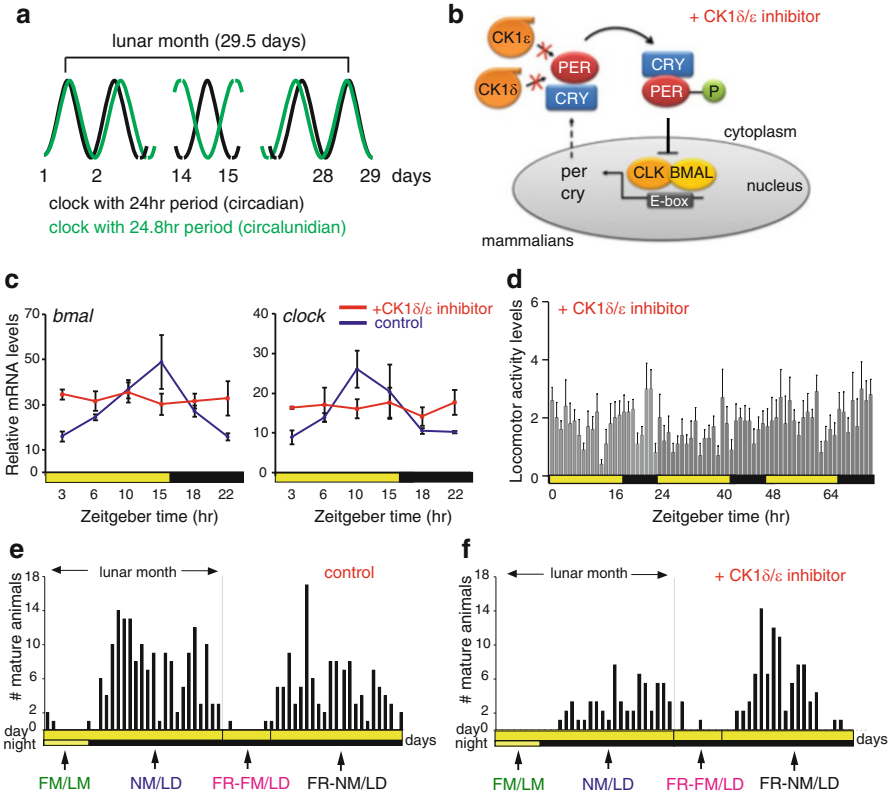


Fig. 8.6 Circalunar clock is independent of circadian clock gene oscillations. (**a**) Dual oscillator model that could explain circalunar rhythm generation. This model is based on a circadian oscillator (24 h, *green oscillations*) and a circalunidian oscillator (24.8 h, *black oscillations*), which 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*

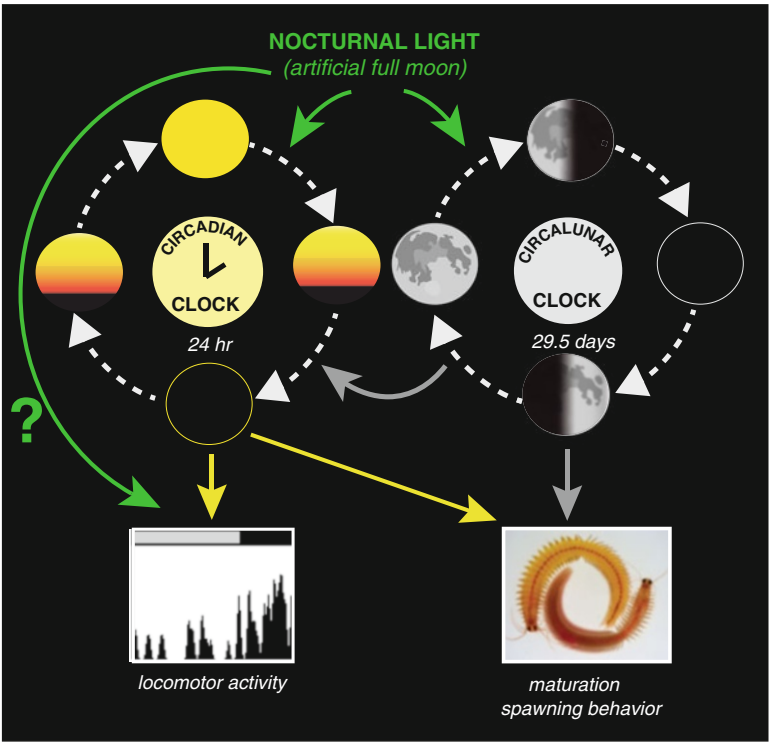


Fig. 8.7 Circadian and circalunar clocks and the impact of light in *Platynereis dumerilii*. As the oscillations of the circadian transcriptional-translational clock are not required for circalunar rhythm generation, it is most plausible that *Platynereis* possesses two distinct molecular clocks that 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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