

Chapter 7

Circadian Rhythm



Sakiko Shiga

Abstract Two-day rhythms, referred to as circa“bi”dian rhythms, were first reported in humans. In insects, a circadian rhythm has been reported in the flight activity of the cool-weather mosquito *Culiseta incidens* under constant darkness. In both humans and mosquitoes, the appearance of the circadian rhythm is labile under constant conditions, and the rhythm does not continue for a long time. In contrast, the black chafer *Holotrichia parallela* exhibited a rigid 2-day circadian rhythm under both field and laboratory conditions. Three characteristics of the biological rhythms, free-running, entrainment to the zeitgeber, and temperature compensation of the period, were observed in the circadian rhythm in *H. parallela*. Phase responses to light pulses suggest that the circadian clock mechanism is involved in the circadian rhythm. The results of the brain surgery experiments imply that the optic lobe-pars intercerebralis axis in the brain is involved in the circadian rhythm of *H. parallela*. Molecular phylogeny and behavioral observations suggest that after separation into *Pedinotrichia*, including *H. parallela* and *Holotrichia picea*, and *Nigrotrichia*, the circadian rhythm probably appeared once in the ancestral species of *Pedinotrichia*.

Keywords Circadian clock · *Holotrichia* · Optic lobe · Pars intercerebralis · Temperature compensation · Two-day rhythm

7.1 Introduction

It is widely considered that most organisms on Earth have circadian clocks with a period close to Earth’s rotation cycle. The circadian clock is a physiological mechanism that measures approximately 24 h to drive circadian rhythms in behavior and physiology (Dunlap et al. 2004; Patke et al. 2020; Chap. 2). Organisms use rhythms to prepare in advance for daily changes in the physical environment, such as

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Table 7.1 Two-day periodicity reported in animals

Species	Phylum	Class	Phenomenon	Detection under ^a	References
<i>Culiseta incidens</i>	Arthropod	Insecta	Flight activity	DD	Clopton (1984)
<i>Holotrichia parallela</i>	Arthropod	Insecta	Pheromone gland size	Natural conditions	Leal et al. (1993)
			Emergence on the ground	LD, DD, natural conditions	Yoshioka and Yamasaki (1983), Kawasaki et al. (2017)
<i>Holotrichia picea</i>	Arthropod	Insecta	Emergence on the ground	LD, DD	Shiga et al. (2022)
<i>Mercenaria mercenaria</i>	Mollusc	Bivalvia	Shell growth	Natural conditions	Pannella and Macclintock (1968)
<i>Comptopallium radula</i>	Mollusc	Bivalvia	Shell striae formation	Natural conditions	Thébault et al. (2006)

^aDD constant dark conditions, LD 12-h light/12h dark cycle conditions, LL constant light conditions

temperature, humidity, and illumination, and the resulting changes in the biological environment, such as food availability and predation risk (DeCoursey et al. 1997, 2000).

Periodicities with an integral multiple of ~24 h have been reported, although this period seems irrelevant to environmental cycles. A circaseptan rhythm with a period of approximately 7 days appears in unicellular marine algae: the growth rate of *Acetabularia mediterranea* (Chlorophyta) and the glow intensity of *Gonyaulax polyedra* (Dinoflagellata) (Cornelissen et al. 1986; Schweiger et al. 1986). In both cases, the amplitude of the diurnal rhythm changed over a period of approximately 7 days. However, their periodicity is mostly unclear, and it is debatable whether endogenous circaseptan rhythms actually exist (Piccione et al. 2004).

Another integral multiple of ~24 h is the 2-day periodicity (Table 7.1). When humans were isolated in a cave or underground bunker, a 2-day periodicity was observed in the sleep-wakefulness rhythm under constant dim light conditions, although the period of the body temperature rhythm was approximately 25 h (Aschoff et al. 1967; Colin et al. 1968). Endogenous 2-day periodicity is called circa“bi”dian rhythm, and in humans, different patterns were observed. For example, sleep and wakefulness times were approximately twice as long as normal; an extremely lengthened activity time interrupted by naps appeared with a relatively small increase in sleep time (Honma and Honma 1988; Wever 1979). These human circabidian rhythms were often unstable and subsequently returned to their circadian rhythm or became obscure. Circabidian rhythms are considered to result from internal desynchronization, in which uncoupling of multiple oscillators, such as an oscillator for activity and an oscillator for body temperature, occurs under constant conditions (Aschoff et al. 1967).

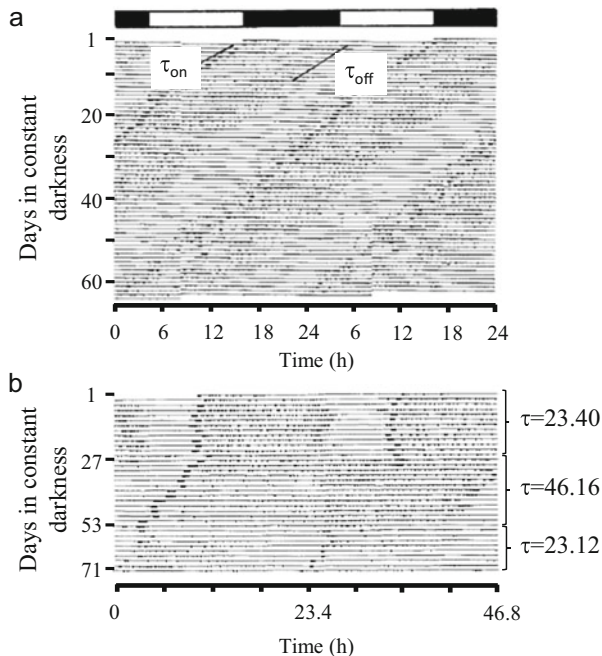
There are a few reports of biological rhythms with 2-day periodicity under natural conditions. In mollusks, shell pattern formation of the hard clam *Mercenaria mercenaria* and the tropical scallop *Comptopallium radula* exhibits 2-day periodicity (Table 7.1, Pannella and Macclintock 1968; Thébault et al. 2006). In *M. mercenaria*, daily shell layer growth commonly occurs in one thick increment, followed by a relatively thin one. Juvenile *C. radula* forms one stria every 2 days under natural daily environmental conditions. In the Pacific Ocean, some meteorological and oceanographic parameters have displayed 2-day variations (Kenyon 1996). For sea-level atmospheric pressure and wind velocity, the amplitude of the 2-day variations was larger than that of the diurnal variations. This may act as a zeitgeber to shell growth rhythm in these bivalves (Thébault et al. 2006). However, it remains unknown whether the 2-day periodicity is endogenous in these species.

Some insects also exhibit a 2-day periodicity or circadian rhythms (Yoshioka and Yamasaki 1983; Kawasaki et al. 2017; Shiga et al. 2022), although any 2-day variation in the physical parameters of the terrestrial environment has rarely been reported. This chapter introduces the insect circadian rhythm and discusses its mechanisms.

7.2 Circadian Rhythm in the Mosquito *Culiseta incidens*

In insects, the circadian rhythm was first reported in the flight activity of the cool-weather mosquito *Culiseta incidens* under constant darkness (DD) (Table 7.1, Clopton 1984). Adult *C. incidens* exhibits a circadian rhythm with activity in the subjective night of DD. Many individuals, but not all, displayed radical changes in the free-running period (τ) calculated from activity onset (τ_{on} , Fig. 7.1a, b). Circadian rhythmicity is occasionally found in prolonged DD, as a highly variable phenomenon (Clopton 1984, 1985). τ_{on} doubled abruptly, with little or no prior period lengthening (Fig. 7.1b). As τ_{off} , calculated from the activity offset, and τ_{on} change differently in many individuals, it has been considered that a circadian pacemaker controlling flight activities consists of two mutually coupled oscillators, E and M, which predominantly control the evening (τ_{on}) and morning (τ_{off}) flight rhythms, respectively, as in the common house mosquito *Culex pipiens* (Jones 1982; Clopton 1984, 1985). The circadian rhythm can be explained by the period of the E-oscillator lengthening to where it synchronizes with the M oscillator in two consecutive cycles of M: one cycle of the E mode (Clopton 1984). Clopton (1984) proposed that E and M oscillators may behave similarly to the human activity and temperature oscillators mentioned above (see Sect. 7.1). In contrast to the human model, circadian components were not observed during the circadian period of *C. incidens*. After the τ of the E oscillator lengthens to a period longer than 24 h, E predominates over the M oscillator (Clopton 1984).

Fig. 7.1 Representative flight activity rhythm of the mosquito *Culiseta incidens*. **(a, b)** Double-plotted actogram of the flight activities under constant darkness. Previous light and dark schedule bar is shown in **(a)**. τ_{on} and τ_{off} indicate free-running of the dusk (E) and dawn (M) activity rhythm, respectively (refer to the main text). **(b)** Under prolonged constant darkness, circadian rhythm with the periodicity of 46.16 h occasionally occurs. Adapted from Clopton (1984), with permission from Springer



7.3 Circadian Rhythm in *Holotrichia* Species

In both *C. incidens* and humans, the appearance of the circadian rhythm is labile under constant conditions, and the rhythm does not continue for a long time (Fig. 7.1b). In contrast, some *Holotrichia* species (Insecta: Coleoptera: Scarabaeidae) exhibited a rigid 2-day periodicity under both field and laboratory conditions (Table 7.1). Yoshioka and Yamasaki (1983) originally reported that *H. parallela* populations appear on the ground every 2 days. Furthermore, measurements of pheromone titers in the pheromone glands of field-collected females suggest a 2-day periodicity in *H. parallela* (Leal et al. 1993). The genus *Holotrichia* includes serious pests of agricultural crops, such as *H. loochooana loochooana* of sugar canes and *H. parallela* of potatoes and glass roots in East Asia. Their behavior has been examined in different aspects of pest control, but their chronobiology has been unknown until recently.

7.3.1 *Three Characteristics of Biological Rhythms in the Circadian Rhythm of the Ground Emergence Activity*

Under laboratory conditions, field-collected *H. parallela*, individually placed in a plastic container with soil, exhibited an approximately 2-day emergence rhythm on the ground. Under a 12 h light/12 h dark cycle (LD12:12) at 25 °C, male and female beetles appeared on the ground during the dark phase every 2 days (Fig. 7.2a). On the ground, beetles feed and walk around and remain underground for the rest of the time. Under LD12:12, the period of the ground emergence rhythm was 48.0 h in both males and females. Under DD, their emergence rhythm continued with a free-running period of slightly less than 48 h (Fig. 7.2a, Kawasaki et al. 2017). Thus, *H. parallela* exhibited a clear endogenous circadian rhythm with a period of approximately 48 h under DD, and this rhythm was entrained to two cycles of LD 12:12 (Fig. 7.2a).

The circadian rhythm might be due to developmental or physiological processes specific to *H. parallela*, such as gonadal development or the digestive system independent of the clock. Yoshioka and Yamasaki (1983) reported that *H. parallela* females deposit eggs in the daytime just before the night of adult emergence on the ground. A half-day feeding may require a long digestive time of 1.5 days. These physiological processes may suppress the behavioral output from the circadian clock every other day to produce a 2-day rhythmicity. If this is the case, the period of behavioral rhythm may become 24 h at higher temperatures because these physiological functions are temperature-dependent. Activity rhythms were recorded under DD conditions at different temperatures. Free-running periods did not differ at a range from 20.0 to 30.0 °C. The free-running period was 47.5 ± 0.5 h (mean \pm S.D., $N = 11$) at 20 °C, 47.7 ± 0.2 h ($N = 11$) at 25 °C, and 47.4 ± 0.2 h ($N = 11$) at 30 °C. The average calculated temperature coefficient Q_{10} was 1.00 (Nakagawa and Shiga, unpublished Fig. 7.2b). This contradicts the hypothesis that the mechanism underlying 2-day rhythmicity involves some temperature-dependent physiological or developmental processes but suggests that the timekeeping mechanism in the circadian rhythm solely involves a temperature-independent biological clock in *H. parallela*. This circadian rhythm is also found in another species, *H. picea*, with some variation (Fig 7.2c, Shiga et al. 2022). Details are discussed in a later section (Sect. 7.4).

7.3.2 *Phase Response of the Circadian Rhythm to Light Pulses*

There are two possibilities for the clock mechanism underlying circadian rhythm: the circadian rhythm is driven by the circadian clock or by the circadian clock (Fig. 7.3a). Circadian behavior may be driven by every two cycles of the circadian

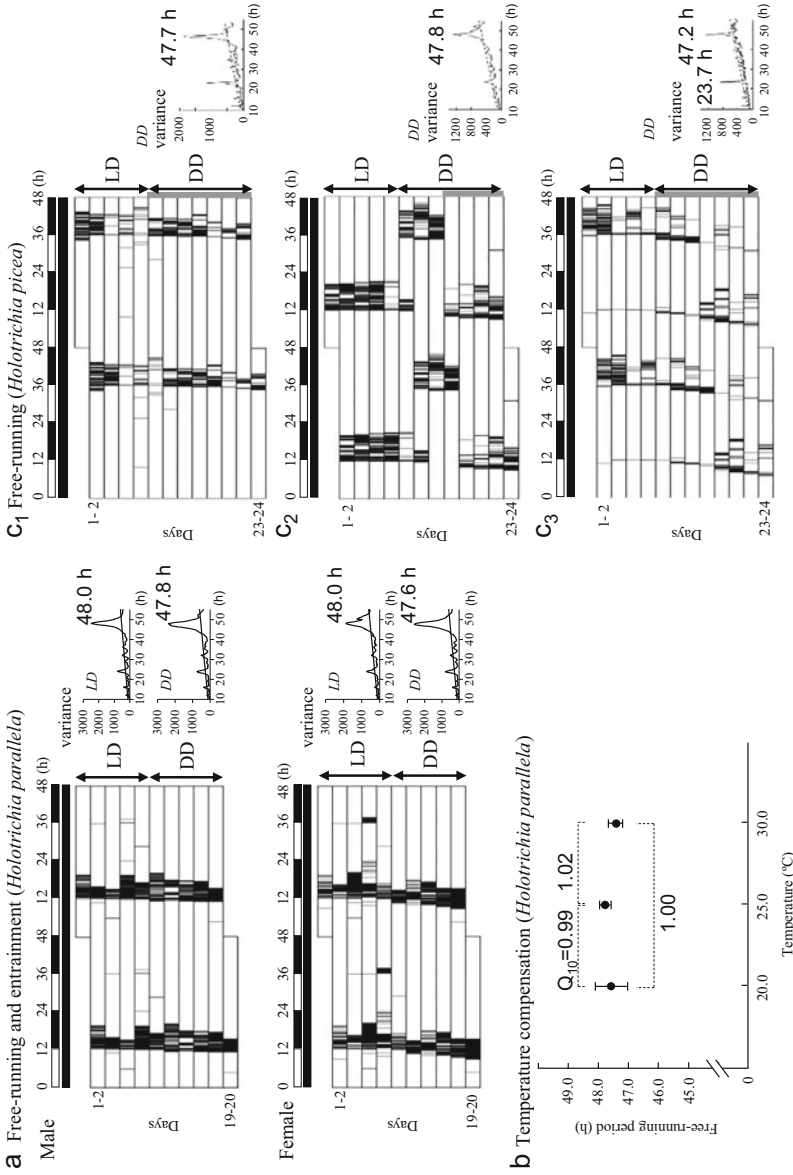


Fig. 7.2 Circadian activity rhythm in *Holotrichia* species. **(a)** Representative actograms and chi-square periodogram of *H. parallela* under 12 h:12 h light: dark (LD) cycles and constant darkness (DD). **(b)** Average and SD of free-running periods under DD at different temperatures in *H. parallela*. Three calculated Q_{10} values are shown in the graph. **(c)** Representative actograms and chi-square periodogram of *H. picea* under LD cycles and DD. **(c₁)** and **(c₃)**, female adults; **c₂**, sex undetermined. **(a)** Adapted from Kawasaki et al. (2017); **(b)** Issei Nakagawa and Sakiko Shiga (unpublished); **(c)** Adapted from Shiga et al. (2022), with permission from Zoological Science

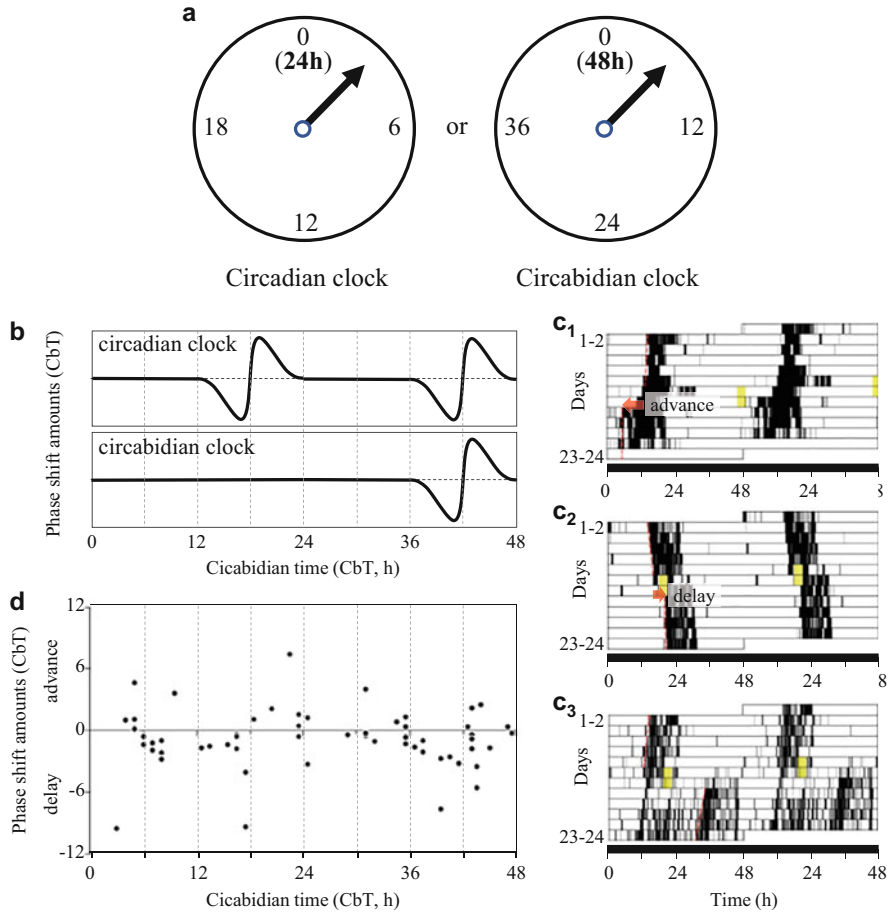


Fig. 7.3 Phase response of the circadian rhythm to light pulses in *Holotrichia parallela*. **(a)** Two clock models underlying the circadian rhythm. **(b)** Putative phase responses of the 24-h clock (upper) and of 48-h clock (lower). Advanced and delayed phase shift values are plotted as positive and negative values on the ordinate. **(c)** Representative actograms with light pulses (in yellow) twice. Phase changes are indicated by red arrows. **(d)** Phase responses to 3-h light pulses emitted at different circadian times under constant darkness. **(c and d)** Adapted from Kawasaki et al. (2017)

clock. In this case, a mechanism doubling the circadian clock cycle must be present in the brain to produce a 2-day period. Alternatively, circadian behavior might be driven by the cycle of the circadian clock. One way to estimate the cycle of the clock that drives a rhythm is to examine the phase responses of the rhythm to zeitgeber stimuli. It is well known that a phase advance or delay of a rhythm occurs in response to a zeitgeber stimulus depending on the clock phase, which is a unique characteristic of oscillator-type clocks (Pittendrigh 1960; Chap. 3). Phase-response curves have been drawn for different biological rhythms. In the circadian clock, the clock phases are divided into subjective day and night periods. Circadian rhythms

driven by the circadian clock exhibit little or no response to light pulses during the subjective day but a delay in the first half and an advance in the last half of the subjective night (Okada et al. 1991). Because the *H. parallela* circadian rhythm entrains to two LD 12:12 cycles, light must function as a zeitgeber. If the circadian rhythm is driven by “a circadian clock,” phase delay and phase advance would occur once in a circadian cycle. However, if the circadian rhythm is driven by “a circadian clock,” phase delay and advance would occur twice in the circadian cycle (Fig. 7.3b). Based on this assumption, Kawasaki et al. (2017) examined the phase responses to light pulses. After the light pulse was applied, the onset of the activity phase was advanced, delayed, or unchanged depending on the light pulse phase (Fig. 7.3c₁, c₂). Although the shape of the response curve was not very clear, two sets of the less-responsive period (circadian time, CbT 0–12, CbT 24–36) and more-responsive period (delay or advance CbT 12–24, CbT 36–48) appeared in one circadian cycle (Fig. 7.3d). This suggests that the circadian cycle is composed of two cycles of the circadian clock. Occasionally, circadian-like activity rhythms appeared after the light pulse (6.2%, $N = 65$, Fig. 7.3c₃, Kawasaki et al. 2017). This also suggested the presence of an oscillator with a period of approximately 24 h in *H. parallela*.

7.3.3 *The Optic Lobe-Pars Intercerebralis Axis in the Brain Is Involved in Circadian Rhythm of H. parallela*

To discuss circadian clock involvement in the circadian rhythm, brain regions necessary for the rhythm were examined. In insect brains, circadian clock cells are localized in the optic lobe of flies and cockroaches (King and Sehgal 2020; Shiga and Numata 2009; Reischig and Stengl 2003). Another brain region known to be involved in circadian rhythm output is a region called the pars intercerebralis (PI), where neurosecretory cells are concentrated. In *Drosophila melanogaster*, cells in the PI are connected to the circadian clock cells in the optic lobe through a polysynaptic circuit (Cavanaugh et al. 2014). Neuropeptides expressed in PI cells are required for locomotor activity rhythms (Cavanaugh et al. 2014; King and Sehgal 2020). The optic lobes and PI were examined to determine whether they are necessary for *H. parallela* circadian rhythms. Adult beetles collected in the field were kept in the laboratory and subjected to removal of the optic lobes or PI (Watanabe and Shiga 2020).

7.3.3.1 Roles of the Optic Lobe

In *H. parallela*, the brain with bilateral optic lobes is located posteriorly in the head (Fig. 7.4). To remove the optic lobes, small cuticular openings were made just medial to the compound eyes, and the optic lobes were removed (Fig. 7.4 left). Most intact and sham-operated control beetles showed a clear circadian rhythm,

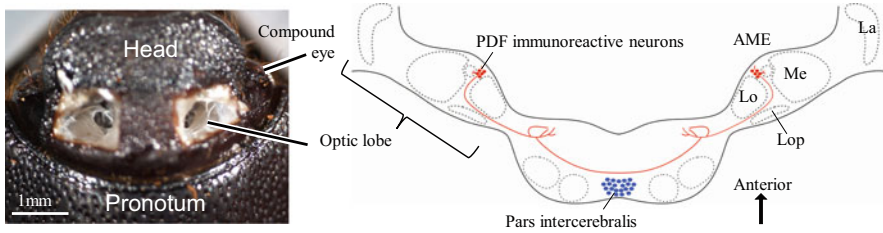


Fig. 7.4 Dorsal view of the head and brain of *Holotrichia parallela*. For optic lobe removal, bilateral small windows were opened (left). Distribution of pigment-dispersing factor (PDF) immunoreactive neurons and pars lateralis neurons (right). *La* lamina, *Lo* lobula, *Lop* lobula plate, *Me* medulla. Left, photo courtesy of Kohei Watanabe; right, redrawn from Hamanaka and Shiga et al. (2022)

although some stayed underground, probably because of winter dormancy in the late season of the experiment (Watanabe and Shiga 2020, Fig. 7.5a, b). When the bilateral optic lobes were removed, approximately half of the beetles exhibited arrhythmicity, and the other half never appeared on the ground. None of the beetles exhibited circadian rhythms (Fig. 7.5a, b₁). After recording, beetles were dug up and their survival was confirmed. All beetles underground were active, similar to those in the intact and sham-operated groups. This suggests that the optic lobe itself, or the connection between the compound eye and midbrain, is involved in the formation of the circadian rhythm and probably in emergence behavior on the ground. Clock protein PERIOD-immunoreactive cells have been found in the optic lobe of the beetle *Pachymorpha sexguttata* (Frisch et al. 1996). In *H. parallela*, a putative clock cell neuropeptide, pigment-dispersing factor, was observed in approximately 100 somata at the anterior base of the optic lobe medulla with medial fiber projections (Fig. 7.4 right, Hamanaka et al. 2022). Based on these findings, it is possible that circadian clock cells are located in the optic lobe of *H. parallela* and may be involved in the circadian rhythm.

Questions such as the following, are both optic lobes (possibly bilateral clock systems) necessary for the circadian rhythm and is it possible that an interaction between the two circadian clocks doubles the cycle of the 24-h clock?, still require exploration. If the two circadian clocks on the left and right sides are able to inhibit output signals from the contralateral clock to the premotor circuitry every 24 h and after the inhibition is released they become refractory to contralateral inhibition for 24 h, it might be possible to produce a 48-h cycle. This is called internal masking, in which the pacemaker output appears to be internally masked (Page 1989). Page (1989) introduced the circadian rhythm of *C. incidens* and cockroaches as an example of internal masking.

For internal masking, unilateral optic lobe removal was performed. However, circadian rhythm remained in all individuals (Fig. 7.5b₂), suggesting that the bilateral organization of the optic lobes, possibly containing the circadian clock, is not a prerequisite for circadian rhythm. Interestingly, about one-fifth of circadian beetles showed “day switching” (Fig. 7.5a, b₂). In day switching, beetles maintained

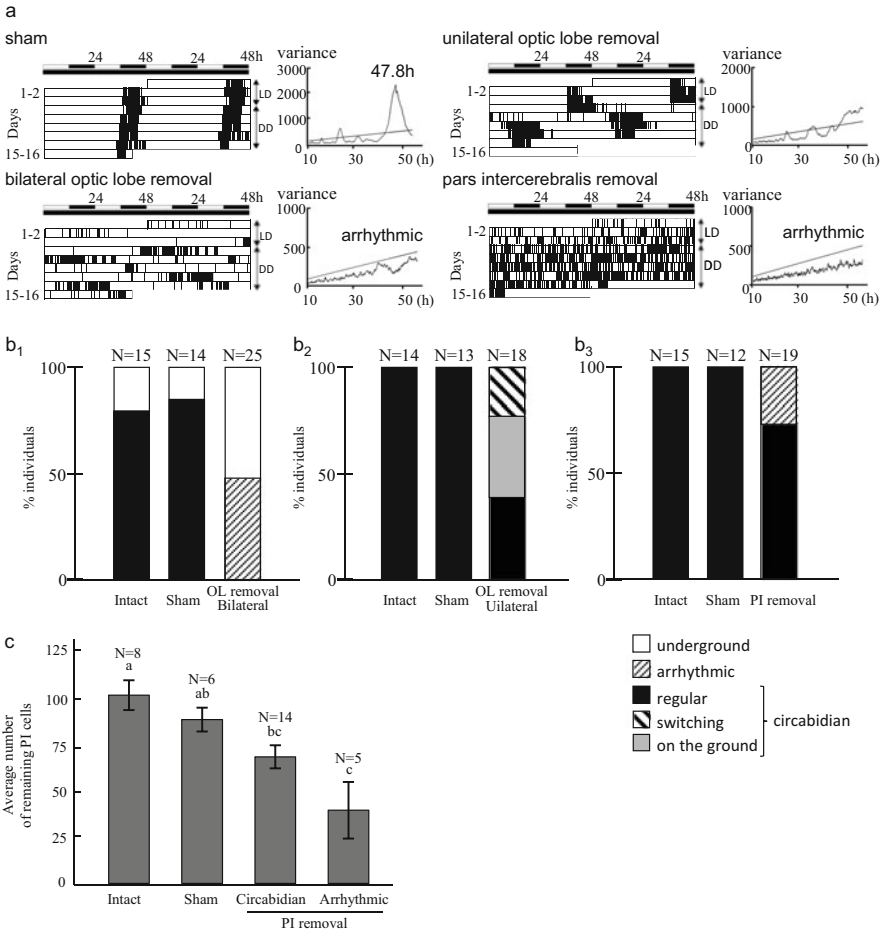


Fig. 7.5 Effects of brain surgery on the circadian rhythm in *Holotrichia parallela*. **(a)** Representative actograms under 12 h:12 h light/dark (LD) cycles and constant darkness (DD). Chi-square periodogram is shown for activities in DD. **(b)** Summary of effects of optic lobe (OL) removal (**b₁** and **b₂**) and pars intercerebralis (PI) removal (**b₃**). **(c)** The average number and SE of paraldehde fuchsin-stained PI cells left. Arrhythmic beetles in the PI removal group had significantly reduced cell numbers compared to those in the control groups ($P < 0.05$, Tukey test). Adapted from Watanabe and Shiga (2020), with permission from Springer

a 2-day periodicity but changed their appearance night from odd to even days or vice versa by emerging on or skipping two serial nights (Kawasaki et al. 2017). Day switching has occasionally been observed in the field in *H. parallela* (Kawasaki et al. 2017). It occurs after heavy rain, suggesting some physiological mechanisms to suppress emergence on the ground under adverse conditions. The appearance of day switching by a single optic lobe may indicate that bilateral coupling between two optic lobes is necessary to maintain a regular 2-day rhythm, and weakening connections or decoupling between the right and the left circadian clock in the optic lobe

may cause day switching. In the cockroach *Rhyparobia (Leucophaea) maderae* and the cricket *Gryllus bimaculatus*, the circadian clocks in left and right optic lobes possess neural communication to allow synchronization (Page 1989; Tomioka 1993). This indicates that coupling between the bilateral optic lobe circadian clocks is responsible for the 24-h clock oscillation, and in *H. parallela*, decoupling of bilateral clocks or a solitary clock may weaken 24-h oscillation to cause an irregular pattern of the circadian rhythm, that is, day switching. Clock coupling between the two optic lobes may be necessary for robustness of the 48-h rhythmic cycle.

In the unilateral optic lobe removal, another 40% of circadian beetles did not go underground and remained aboveground throughout the day (Fig. 7.5b₂). Even beetles restricted above ground exhibited circadian rhythms. Although underground behavior has not been shown, observation of their activities throughout the day on the ground raised the possibility that they are inactive underground and the total (above and below the ground) locomotor activity is circadian.

7.3.3.2 Roles of the Pars Intercerebralis

In the PI of *H. parallela*, approximately 100 cells were positive for paraldehyde fuchsin, which stains a certain type of neurosecretory cell (Watanabe and Shiga 2020). Brain surgery experiments targeting the PI cells were performed, similar to optic lobe removal (Fig. 7.4). The surgery caused a reduction in paraldehyde fuchsin-stained cells in the PI (Watanabe and Shiga 2020). After this surgery, approximately one-fourth of the beetles showed arrhythmicity (Fig. 7.5b₃). The number of paraldehyde fuchsin-stained PI cells in the arrhythmic group was significantly reduced compared with that in the intact and sham-operated groups (Fig. 7.5c). Arrhythmic beetles walked and ate leaves randomly throughout the day, and their activity levels were higher than those of the control groups and PI-removed circadian beetles (Watanabe and Shiga 2020). These results suggest that some paraldehyde fuchsin-stained PI cells are necessary for circadian rhythm and the suppression or regulation of locomotor activity.

The results of these surgical experiments suggest that cells of the optic lobe and part of the PI are necessary for circadian rhythms. The neural circuit of circadian clock cells in the optic lobe to the PI might be evolutionarily conserved for biological timing mechanisms in insects and may be involved in the generation of circadian rhythms in *H. parallela*.

7.3.4 Two-Day Rhythm of *H. parallela* in the Field

Field observations by Kawasaki et al. (2017) showed male and female adults of *H. parallela* mostly appeared above ground a few hours after sunset from June to October and visited trees such as the Chinese elm *Ulmus parvifolia*. *H. parallela* remained on the tree throughout the night, and their appearance and disappearance

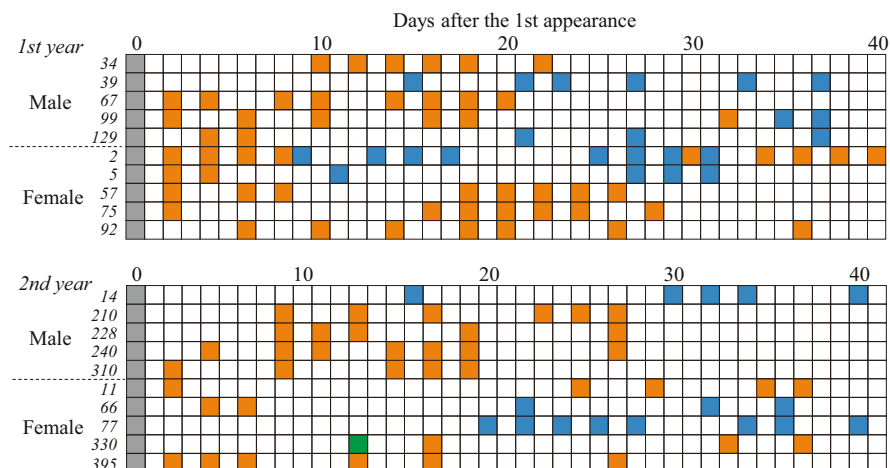


Fig. 7.6 Representative individual plots of *Holotrichia parallela* appearance in the field. The horizontal axis indicates the number of days from the first appearance. Orange cells and blue cells indicate an even and odd numbers of days, respectively, counted from the first appearance. Adapted from Kawasaki et al. (2017)

were mostly synchronized with sunset and sunrise, respectively (Kawasaki et al. 2017). At sunrise, they dug into the soil in an area of 15-m semidiameter around the tree where they stayed at night. A mark and recapture study showed that beetles repeatedly appeared on the same tree approximately every 2 nights (Kawasaki et al. 2017). However, the periodicity was not very rigid, and individuals often switched appearance days. Figure 7.6 shows individual plots of beetle appearance over 40 days in the field. Male no. 34 emerged on the same tree on days 10, 12, 14, 16, and 18 (every 2 days). In contrast, female no. 2 appeared on even days until day 8, but from day 9, she switched appearance day to odd days until day 29. From day 29, she appeared every day for 3 days and returned to an even-day appearance with 2-day periodicity (Fig. 7.6). Although a large amount of precipitation causes day switching in many beetles, day switching was sometimes observed without rain (Kawasaki et al. 2017).

The occurrence of day switching also supports the idea that the circadian clock drives circadian rhythm. If the circadian rhythm was created by a hypothetical circadian clock (Fig. 7.3a right), a phase shift of a half-cycle (~ 24 h) of the clock would have had to occur to switch the appearance days. For an oscillator-type clock, it is difficult to make a half-period phase shift at one time (Benstaali et al. 2001), and it usually requires a transient period to complete a full shift. If day switching was adaptive (e.g., facultative avoidance of aversive conditions or increased population size), the beetles would have had to develop a mechanism to shift the appearance day without transients. If the circadian rhythm is driven by the circadian clock system (Fig. 7.3a, left), an immediate switch in the appearance day may be possible. Circadian output might be activated or suppressed every two circadian oscillations

by unknown mechanisms, such as counting two circadian cycles. If so, some environmental stimuli may provide an input signal to the cycle counting mechanism to produce an output after one or three circadian oscillations, thus resulting in day switching.

Phase responses to the light pulse, involvement of the optic lobe-PI axis, and day-switching characteristics suggest that the circadian rhythm in *H. parallela* is driven by a ~ 24-h circadian clock. If this is the case, a novel function for the circadian clock that creates an integral multiple rhythm can be proposed. Circadian rhythms may be produced by the release of an output signal from the circadian clock every two cycles to produce a 2-day rhythm. In future experiments, molecular and neuronal bases of the involvement of the circadian clock in circadian rhythm should be elucidated.

7.4 Origin of the Circadian Rhythm in the Genus *Holotrichia*

To elucidate the ancestral state of the circadian rhythm in an evolutionary context, the activity rhythms of related species were examined (Shiga et al. 2022). *Holotrichia* (Coleoptera, Scarabaeidae, Melolonthinae, Rhizotrogina) is a large genus that includes heterogeneous species groups inhabiting Southeast and East Asia (Ward et al. 2002; Anitha et al. 2006; Matsumoto 2016). In addition to *H. parallela*, *Holotrichia picea* also exhibited a circadian rhythm (Fig. 7.2c). In *H. picea*, three types of rhythms, including the regular circadian pattern, circadian patterns with day switching, and a circadian activity-like pattern, were observed under laboratory conditions. In the day-switching pattern, *H. picea* switched appearance from odd to even days, or vice versa, as did *H. parallela* (Fig. 7.2c₂). In the circadian-like activity patterns, major whole-night activity and minor dusk activity appeared alternately (Fig. 7.2c₃). The switching and circadian-like behavioral patterns in *H. picea* also support the idea that circadian rhythms in *Holotrichia* species are driven by the circadian clock mechanism. *Holotrichia kiotonensis*, *Holotrichia convexopyga*, and *Holotrichia loochooana loochooana* exhibit a 24-h circadian rhythm (Shiga et al. 2022).

Two distinct clades were recognized in the phylogenetic trees – constructed using histone H3, cytochrome c oxidase subunit 1, and 16S ribosomal RNA – of the *Holotrichia* species (Shiga et al. 2022). This phylogenetic separation was in accordance with the subgeneric classification based on external morphology by Matsumoto (2015a, 2015b, 2016) and behavioral rhythms (Fig. 7.7a, b, Shiga et al. 2022). One clade included *Nigrotrichia* group members, *H. kiotonensis*, *H. convexopyga*, and *H. loochooana loochooana*, showing circadian rhythms, while the other clade included *Pedinotrichia* group members, *H. parallela* and *H. picea*, showing circadian rhythms (Fig. 7.7). This suggests that after separation into *Nigrotrichia* and *Pedinotrichia* groups, the circadian rhythm probably appeared once in the ancestral species of *Pedinotrichia*.

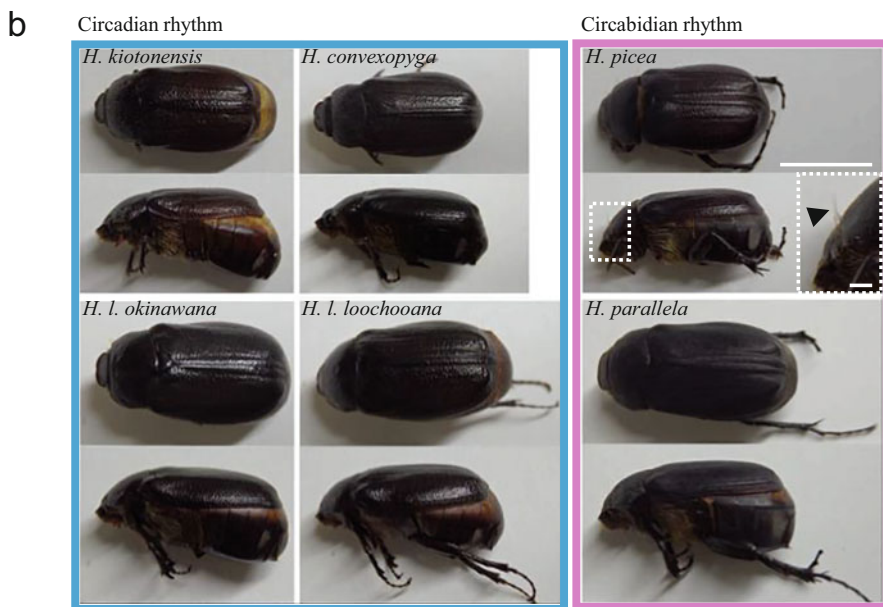
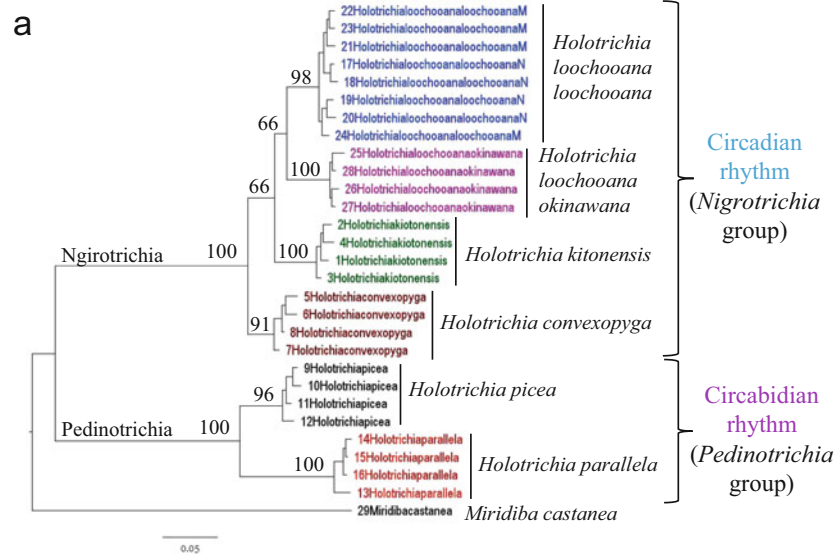


Fig. 7.7 Molecular phylogeny and behavioral analysis in *Holotrichia* species inhabiting Japan. (a) Histone H3, cytochrome c oxidase subunit 1, and 16S ribosomal RNA phylogenetic trees revealed two distinct clades: one clade (Nigrotrichia) including *Holotrichia kitonensis*, *Holotrichia convexopyga*, and *Holotrichia loochooana loochooana* exhibiting a circadian rhythm, and the other clade (Pedinotrichia) including *H. parallela* and *Holotrichia picea* exhibiting circadian rhythm. (b) Dorsal and lateral views of *Holotrichia* species (males). Adapted from Shiga et al. (2022), with permission from Zoological Science

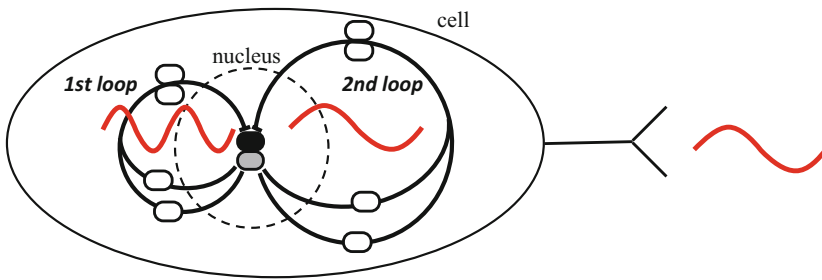
7.5 Mechanism for Doubling the Circadian Clock Cycle

The occurrence of circadian-like activity patterns and day-switching patterns in *H. parallela* and *H. picea* suggests circadian clock involvement in the circadian behavioral rhythm (Figs. 7.2c₂, c₃, 7.3c₃, and 7.5a). The phase-response patterns to light pulses (Fig. 7.3d) and the necessity of the optic lobes in *H. parallela* (Fig. 7.5b₁) also support this hypothesis. If this is the case, there must be some mechanism doubling the circadian clock cycle to achieve a 2-day periodicity. If *H. parallela* and *H. picea* possess circadian clock cells with a conventional transcriptional-translational negative feedback loop of clock genes, such as in *D. melanogaster* (Chap. 4), clock cycle doubling might occur in the clock cells intracellularly or intercellularly (Fig. 7.8).

Kinases may play an important role in intracellular mechanisms. In cultured mammalian cells, perturbation of phosphorylation by casein kinase 1 ϵ (CK1 ϵ) or CK1 δ changes the period of clock gene expression rhythm from circadian (24 h) to circadian (48 h) (Isojima et al. 2009). Kinases may be involved in the rate control of circadian oscillations. In clock cells of the circadian species, some negative feedback loops (here, we say the first loop) of clock genes may exhibit a 24-h rhythm in their expression, and another loop (the second loop) may produce a 48-h rhythm by certain phosphorylation processes activated every two cycles of the first loop (Fig. 7.8a). In a single clock cell, clock-controlled genes for output signals that are under the control of the second loop may be able to produce a 2-day periodicity. The blind cavefish *Phreatichthys andruzzii*, which lives in perpetual darkness, does not show a clear circadian rhythm, and the clock gene expression of their cultured cells exhibits circadian oscillation (Cavallari et al. 2011). These results suggested that circadian clock genes may be able to oscillate in a double period under certain circumstances.

Another possibility is that a 2-day periodicity is produced in the neuron network (Fig. 7.8b). Two-day periodicity can be produced by alternate suppression and activation of circadian clock cell output. Flip-flops and counters are sequential circuits with alternating on and off outputs. In moth olfactory processing systems, flip-flopping interneurons have been reported, and the flip-flop signal is thought to underlie locomotion occurring during pheromone-triggered orientation behavior (Olberg 1983; Namiki and Kanzaki 2016). This type of neuron switches back and forth between long-lasting high- and low-firing rates in response to repeated stimuli (Olberg 1983). A similar type of flip-flop interneuron might be incorporated into the clock network in *H. parallela* and *H. picea*. These neurons receive daily input from 24-h clock cells to alternate turning on and off of the postsynaptic neurons to produce 2-day cycles (Fig. 7.8b left). Another candidate is a network that contains counter neurons. Counter neurons count the number of circadian cycles; they do not activate (turn off) postsynaptic neurons when counting one cycle from the circadian clock but do (turn on) when signals for 2 days accumulate in them (Fig. 7.8b right). With this counter circuit, it is possible to produce other integral multiples of 24 h, such as 3- or 7-day rhythms.

a Intracellular mechanism



b Intercellular mechanism

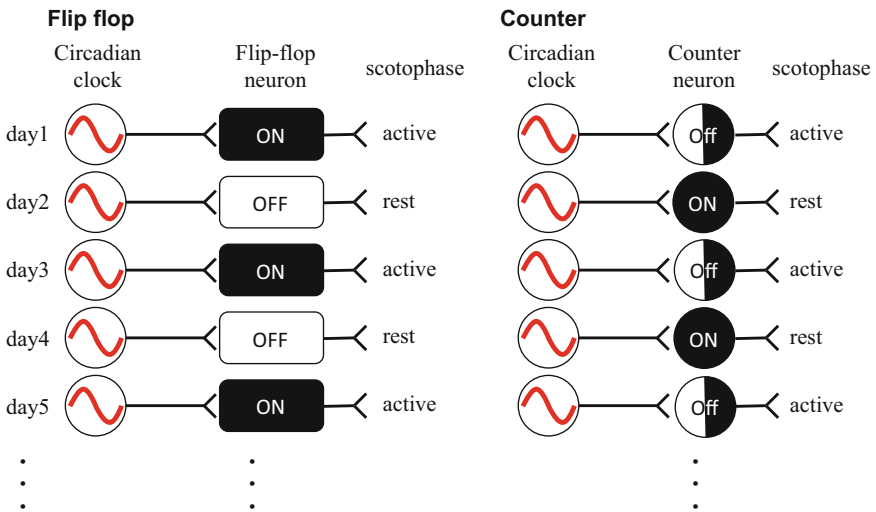


Fig. 7.8 Hypothetical mechanisms for doubling the circadian clock cycle. (a) In an intracellular mechanism, the first transcriptional-translational feedback loop produces conventional 24-h oscillation, and the second loop oscillates every two cycles of the first loop. Ovals indicate different types of clock proteins. (b) In intercellular mechanisms, neuronal circuitry containing flip-flop neurons or counter neurons may double the circadian clock cycle

When female bedbugs *Cimex lectularius* were fed on a 7-day cycle during juvenile development and allowed to feed and mate every 7 days after eclosion, the lysozyme-like activity gradually increased for the antibacterial immune response in the blood lymph in anticipation of mating in the next 7 days (Siva-Jothy et al. 2019). The male bedbug traumatically inseminates a freshly fed female, so it makes sense for the female to increase lysozyme activity prior to mating in order to prepare an immune response to bacteria entering the female's body during insemination. This report suggests that bedbugs learn the 7-day cycle of feeding and mating to produce a 7-day cycle of immune activity. This may involve some mechanism to learn an integral multiple of days and might support the presence of the clock-counter system.

7.6 Concluding Remarks

Several physiological data suggest the involvement of the circadian clock in circadian rhythm. However, molecular evidence is missing. In the near future, oscillation patterns of circadian clock genes should be clarified, and their function in circadian rhythm should be examined. Furthermore, the mechanism underlying clock cycle multiplication is fascinating. Using neuroanatomy and molecular genetics, it should be clarified whether the doubling of the circadian clock cycle occurs intracellularly or intercellularly in the brain.

Another important question is the ecological significance of the circadian rhythm. In general, a reduction in the number of appearance days is unfavorable for feeding and mating opportunities. If predation pressure is high, it may make sense to reduce the number of emergence days. However, *H. parallela* remains on tree leaves during the night, and no active predators are known against *H. parallela* and *H. picea*. Therefore, it is unclear why these species exhibit circadian rhythms. It is possible that some radical environmental changes have caused unfavorable conditions in a restricted region or era, and this may have benefited beetles in the *Pedinotrichia* group to emerge every 2 days, resulting in the appearance of the circadian rhythm. However, if a few individuals return to the circadian rhythm when unfavorable environmental conditions disappear, beetles with circadian rhythms may have lower reproductive fitness than those with circadian rhythms. Subsequently, the circadian rhythm is lost. However, this rhythm has persisted in certain species, suggesting that the circadian rhythm may have advantages that we are unaware of.

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