Locomotor activity rhythms in laboratory-reared adults of the mangrove cricket, *Apteronemobius asahinai*

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Abstract

Adults of the mangrove cricket, *Apteronemobius asahinai*, collected in the field continuously show a circatidal rhythm in their locomotor activity. However, it remained unclear whether adult crickets reared from eggs without cyclic tidal influence in the laboratory show the circatidal rhythm. We show that under constant red dim light, slightly less than half of crickets showed a circatidal rhythm continuously for more than ten days. On the other hand, some others showed a unimodal daily rhythm either before or after the tenth day in the activity recording. Thus, some environmental factors other than the laboratory conditions should be required in order for all crickets to express a clear circatidal rhythm. Even under constant light, unimodal daily rhythm appeared. The unimodal daily rhythm was entrained to light-dark cycles in some crickets, but not in the others. Therefore, we cannot conclude whether the unimodal daily rhythm is a circadian rhythm or a rhythm derived from a circatidal rhythm with its period doubled.

Key words: circadian rhythm, circatidal rhythm, entrainment, light-dark cycles

INTRODUCTION

Circatidal rhythm is a biological rhythm with a period of approximately 12.4 h, and is usually shown by organisms affected by tidal ebbing and flooding (Neumann 1981; Naylor 2010). The mangrove cricket, *Apteronemobius asahinai* Yamasaki, inhabits the floor of mangrove forests, and is affected by tidal cycles (Satoh *et al.* 2008). Under constant darkness, mangrove crickets collected in the field show circatidal and circadian rhythms in their locomotor activity: the circatidal rhythm produces active and rest phases, and the circadian rhythm modifies the activity levels (Satoh *et al.* 2008).

Only a few authors have examined whether the expression of a circatidal rhythm requires periodic environmental cues during development. Laboratory-reared juveniles of the European shore crab, *Carcinus maenas* (Linnaeus), did not show a circatidal rhythm but a unimodal daily activity rhythm under constant dim light (Williams & Naylor 1967). In contrast, zoeae of *C. maenas* that had never experienced tidal influences showed a circatidal rhythm in their vertical migration (Zeng & Naylor 1996). In the horseshoe crab *Limulus polyphemus* (Linnaeus), larvae that had experienced periodic tidal stimuli during the embryonic stage showed a circatidal rhythm, whereas larvae that had not experienced such tidal stimuli showed arrhythmicity (Ehlinger & Tankersley 2006).

In the previous studies of the circatidal rhythm in *A. asahinai*, only adults collected from the field were used (Satoh *et al.* 2008, 2009; Takekata *et al.* 2012, 2014ab, 2018, 2020; Sakura & Numata 2017; Satoh 2017; Satoh & Terai 2019). The purpose of the present study was to clarify whether adults of *A. asahinai* reared continuously from eggs without any tidal experience in the laboratory show a circatidal rhythm or not.

MATERIALS AND METHODS

Insects

Adults of *A. asahinai* were collected from mangrove swamps in Ginoza, Okinawa

Prefecture, Japan (26°30′N, 127°59′E). Their progeny were successively reared under 16 h light and 8 h darkness (LD 16:8) at 25.0 ± 1.0°C on insect pellets (Oriental Yeast, Tokyo, Japan) and water. The photophase was provided by a fluorescent lamp (FL15D; Toshiba, Tokyo, Japan) with approximately 1.4 W/m^2. Adults of the first and second generations in the laboratory stock culture were used for the locomotor activity recording 7–13 days after emergence.

Recording of locomotor activity

Activity was recorded according to Sakura and Numata (2017). Crickets were individually put in plastic petri dishes with sufficient food and wet cotton protruding from a water source. The food consisted of insect pellets (Oriental Yeast), agar and propionic acid. The petri dishes were placed in an incubator maintained at 25.0 ± 1.0°C. Infrared beams (EE-SPW321, Omron, Kyoto, Japan) were set across the petri dishes and the number of interruptions of the beam was recorded at 6 min intervals.

In Experiment 1, locomotor activities were recorded under constant dim red light (615 nm) at approximately 14.6 mW/m^2 with an LED (HLMP-EH22, Avago Technologies, San Jose, California, USA). In the following experiments, the photophase was provided by a fluorescent lamp (FL15D, FL15EX-N-Z, or FL15EX-D-Z, Toshiba, Tokyo, Japan) at approximately 1.5 W/m^2. Experiments 2 and 3 were performed under constant light and constant LD 12:12, respectively. Furthermore, activities were recorded under LD 12:12, and on the eighth or ninth day of recording, the photophase was advanced and delayed by 6 h, in Experiments 4 and 5, respectively.

Data analysis

Locomotor activities successfully recorded for more than 17 days were used for the analysis. Rhythmicity of a locomotor activity was detected by a chi-square periodogram (Sokolove & Bushell 1978). Considering the multiplicity of chi-square tests, the significant level for each chi-square test was fixed to 0.005. In the present study, a unimodal daily rhythm was defined as a rhythm of which the free-running period was between 16 h and 32 h; and a bimodal daily rhythm was defined as a rhythm of which the free-running period was between 10 h and 16 h. When no significant period was detected between 10 h and 32 h, we defined the locomotor activity as arrhythmic. The phase of the rhythm was detected by the cycle-by-cycle adaptation of the HFS (cHFS) method, devised by Sakura and Yasugi (2019). The cHFS method requires an input of an approximate expected period of a biological rhythm. In the present study, the expected period was calculated using the chi-square periodogram. The cHFS method assumes the approximate stationarity of a biological rhythm. Therefore, when an actogram showed considerable change of the period in the rhythm, or a disappearing rhythm, the cHFS sometimes failed to detect the phase. In such cases, we detected the onsets by eyes. Under light-dark cycles, when the period of a locomotor activity rhythm was between 23 h and 25 h and the onsets of active phases lined up at the beginning of the scotophase, we defined the activity rhythm as being entrained to light-dark cycles.

RESULTS

Because there was no apparent difference in the proportions of rhythm patterns between males and females in any of the experiments, we show the pooled data of the two sexes. Because many individuals apparently showed a change in rhythm patterns around the tenth day, we divided the actogram into two periods: earlier ten days and later ten days, and independently analyzed the activities in these periods.

Experiment 1: Locomotor activity under constant red dim light

In some crickets, a bimodal daily rhythm continued for more than ten days (Table 1(A); Figure 1(A)), although the contrast between the intensities of active and rest phases appeared to be smaller than that of the circatidal rhythm in crickets collected in the field (Sakura & Numata 2017). Some other crickets showed a unimodal daily rhythm either before or after the tenth day (Table 1(A)), and Figure 1(B) shows an example in which a bimodal daily rhythm changed into a unimodal daily rhythm. A few crickets showed arrhythmic activities (Table 1(A)), and Figure 1(C) shows an example in which a bimodal daily rhythm became arrhythmic.

Experiment 2: Locomotor activity under constant light

There are two possibilities to explain the unimodal daily rhythm observed in Experiment 1: One is that the unimodal daily rhythm is a circadian rhythm, and the other is that the unimodal daily rhythm is a rhythm originating from a circatidal rhythm of which the period is doubled. In the following experiments, we examined whether the unimodal daily rhythm is a circadian rhythm. Because the circadian rhythm modifying the activity levels in *A. asahinai* is disrupted under constant light (Satoh, 2017), we examined whether the unimodal daily rhythm observed under constant red dim light disappears under constant light. Some crickets showed a unimodal daily rhythm before and/or after the tenth day (Table 1(B)). Figure 2 shows an example in which a bimodal daily rhythm changed into a unimodal daily rhythm. There was no significant difference in the proportions of crickets showing unimodal daily rhythms between constant red dim light (12/27) and constant light (8/16) ($P > 0.05$, Fisher's exact test). Thus, we conclude that the unimodal daily rhythm does not disappear under constant light.

Experiment 3: Locomotor activity under light-dark cycles

In the following experiments, we examined whether the unimodal daily rhythms observed under constant red dim light entrained to light-dark cycles. Hereafter, we call a unimodal daily rhythm that is entrained to light-dark cycles "an entrained unimodal rhythm", and a unimodal daily rhythm that is not entrained to light-dark cycles "a non-entrained unimodal rhythm". Some crickets showed a non-entrained unimodal rhythm before and/or after the tenth day, whereas some others showed entrained unimodal rhythms before and/or after the tenth day (Table 2). Figure 3(A) shows an example in which a bimodal daily rhythm changed into a non-entrained unimodal rhythm, and Figure 3(B) shows an example in which an entrained unimodal rhythm continued throughout the recording. These results show that there is individual variation in the entrainability of the unimodal daily rhythm.

Experiment 4: Locomotor activity with phase advance under light-dark cycles

To further examine the entrainability of the unimodal daily rhythm, we exposed crickets to phase advance of the light-dark cycles. After the photophase was advanced by 6 h, some crickets showed a unimodal daily rhythm, and in all of them the rhythm was entrained to the new light-dark cycles (Table 3; Figure 4). Thus, the unimodal daily rhythm can be reset in response to a phase advance.

Experiment 5: Locomotor activity with phase delay under light-dark cycles

Finally, we exposed crickets to phase delay of the light-dark cycles. After the photophase was delayed by 6 h, some crickets showed a unimodal daily rhythm, and half of them showed a non-entrained unimodal rhythm (Table 3; Figure 5(A)); while the others showed an entrained unimodal rhythm (Figure 5(B)). These results show individual variation in the ability of the unimodal daily rhythm to be reset in response to a phase delay.

DISCUSSION

The present results indicate three differences between *A. asahinai* adults reared from eggs in the laboratory and those collected in the field: (1) Less than half of the laboratory-reared crickets showed a bimodal daily rhythm for more than ten days, whereas most field-collected crickets showed it (Sakura & Numata 2017). (2) The bimodal daily rhythm of laboratory-reared crickets was not as clear as that of field-collected crickets (Sakura & Numata 2017). (3) Some laboratory-reared crickets showed a unimodal daily rhythm, whereas no field-collected crickets showed it except for one individual (Satoh *et al.* 2008; Sakura & Numata 2017; Takekata *et al.* 2018). Thus, approximately half of *A. asahinai* show a bimodal daily rhythm even without experience of field conditions.

On the other hand, some crickets did not show a bimodal daily rhythm. It is probable that an environmental factor related to the tides is required to induce all crickets to express a clear bimodal daily rhythm. Similar results were reported in *L. polyphemus* (Ehlinger & Tankersley 2006). In *A. asahinai*, rearing with periodic water inundations, salt water, or soil collected in the field did not significantly increase the proportion of crickets showing a bimodal daily rhythm (K. Sakura and H. Numata, unpublished data). It is necessary to examine whether other external factors or combinations of the above factors produce the locomotor activity rhythm patterns shown by field-collected crickets.

In *A. asahinai*, field-collected crickets show circatidal and circadian rhythms simultaneously (Satoh *et al.* 2008; Takekata *et al.* 2012, 2014b). Goto and Takekata (2015) regarded this as evidence that the circatidal clock is independent of the circadian clock. In the present study, a unimodal daily rhythm was observed under constant red lim right by laboratory-reared crickets. Here we propose two alternative hypotheses to explain the unimodal daily rhythm: (1) The unimodal daily rhythm is a circadian rhythm. (2) The unimodal daily rhythm is a rhythm originating from a circatidal rhythm of which the period is doubled.

In the present study, we examined whether the unimodal daily rhythm disappeared under constant light. Satoh (2017) showed that the circadian modification of activity levels is disrupted in field-collected *A. asahinai* under constant light, of which the intensity was similar to that of the present study. There was no significant difference between the proportion of crickets showing a unimodal daily rhythm under constant red dim light and that under constant light. More than 60 years ago, Bruce (1960) pointed out that the circadian rhythm disappears under constant light in many organisms. In fact, circadian rhythms in locomotor activities are not observed under constant light in some insects, e.g., flies *Drosophila melanogaster* Meigen and *Calliphora vicina* Robineau-

Desvoidy, and crickets *Gryllodes sigillatus* (Walker) and *Gryllus bimaculatus* De Geer (Tomioka & Chiba 1982; Hong & Saunders 1994; Abe *et al.* 1997; Tomioka *et al.* 1998). If the circadian rhythm generally disappears under constant light, we can conclude that the unimodal daily rhythm is not a circadian rhythm. However, circadian rhythms in locomotor activities do not disappear even under constant light in some other insects, e.g., the Japanese honeybee, *Apis cerana japonica* Radoszkowski, the bed bug, *Cimex lectularius* Linnaeus, and the blow fly *Protophormia terraenovae* Robineau-Desvoidy (Fuchikawa & Shimizu 2007; Romero *et al.* 2010; Hamasaka *et al.* 2011). Moreover, it is possible that the constant light of which the intensity is similar to that of the experiment in Satoh (2017) does not disrupt the circadian rhythm producing rest and active phases but instead disrupts the circadian modification of activities under the circatidal rhythm in *A. asahinai*. If so, even if the unimodal daily rhythm were a circadian rhythm, this unimodal daily rhythm would not be disrupted under constant light. Therefore, we cannot conclude that the unimodal daily rhythm is not a circadian rhythm.

Then, we examined whether the unimodal daily rhythm is entrained to light-dark cycles because entrainment to light-dark cycles is a fundamental feature of circadian rhythms. Whether the unimodal daily rhythm was entrained to light-dark cycles differed among individuals, although the unimodal daily rhythm was reset in response to phase advance, supporting the first hypothesis. Therefore, we neither verified nor ruled out the first hypothesis that the unimodal daily rhythm is a circadian rhythm. Some other intertidal organisms show both bimodal and unimodal daily rhythms (Rudloe 1979; Akiyama 1997; Kim *et al.* 2003; Darnell *et al.* 2010; Anderson *et al.* 2017). Akiyama (1997) concluded that the unimodal daily rhythm in the cumacean *Dimorphostylis asiatica* Zimmer is a circadian rhythm because light-dark cycles function as the Zeitgeber for the rhythm. Kim *et al.* (2003) suggested that the unimodal daily rhythm in the Washington clam, *Saxidomus purpuratus* (Sowerby II), is governed by the circadian clock. In *A. asahinai*, although the clock mechanism of the unimodal daily rhythm is still unclear, it is possible that a unimodal daily rhythm is a circadian rhythm that has higher entrainability after phase advance than after phase delay.

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DATA AVAILABILITY

The data that support the findings of this study are openly available in Zenodo at http://doi.org/10.5281/zenodo.4555927.

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Table 1 The number of adults in *Apteronemobius asahinai* showing different rhythm patterns in the locomotor activity during earlier and later periods under constant red dim light (A, $n = 27$) and constant light (B, $n = 16$).

(A)

		Rhythm patterns during later ten days		
		Bimodal	Unimodal	Arrhythmic
Activity patterns during earlier ten days	Bimodal	12	9	2
	Unimodal	3	0	0
	Arrhythmic	0	1	0

(B)

		Rhythm patterns during later ten days		
		Bimodal	Unimodal	Arrhythmic
Rhythm patterns during earlier ten days	Bimodal	7	3	0
	Unimodal	2	3	0
	Arrhythmic	1	0	0

Table 2 The number of *Apteronemobius asahinai* showing different rhythm patterns in locomotor activities in earlier and later periods under light-dark cycles (*n* = 16).

			Rhythm patterns during later ten days		
			Bimodal	Unimodal	
				Entrained	Non-entrained
Rhythm patterns during earlier ten days	Bimodal		4	2	4
	Unimodal	Entrained	2	1	1
		Non-entrained	1	0	1

Table 3 Rhythm patterns and entrainment of adults in *Apteronemobius asahinai* after phase advance or delay by six hours under light dark cycles.

		Rhythm patterns			
		Bimodal	Unimodal		Arrhythmic
	n		Entrained	Non-entrained	
Phase advance	15	6	9	0	0
Phase delay	15	6	4	4	1

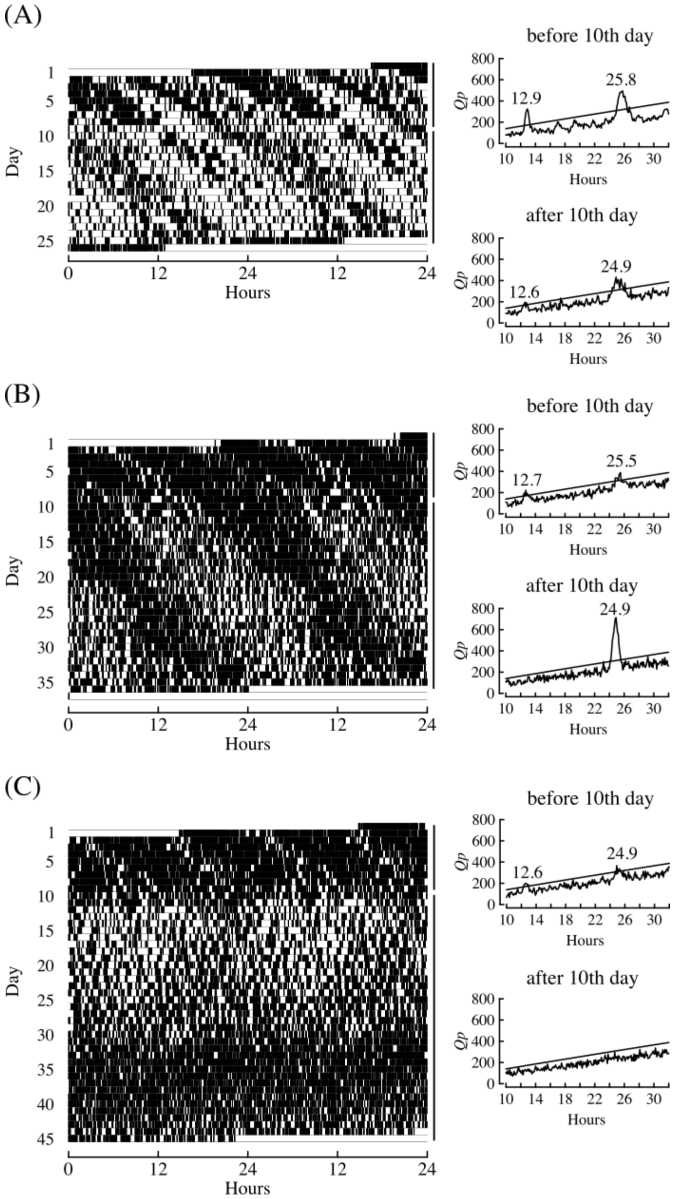

Figure 1 Representative locomotor activity rhythms under constant red dim light in laboratory-reared *Apteronemobius asahinai*. (A) A bimodal daily rhythm continued for more than ten days. (B) A bimodal daily rhythm was observed before the tenth day and changed into a unimodal daily rhythm after the tenth day. (C) A bimodal daily rhythm before the tenth day became arrhythmic after the tenth day. The chi-square periodograms of activities before and after the tenth day are shown on the right. The oblique line in the periodogram corresponds to the significance level of $\alpha = 0.005$ of each chi-square test. The peak values above the oblique line were determined to be significant.

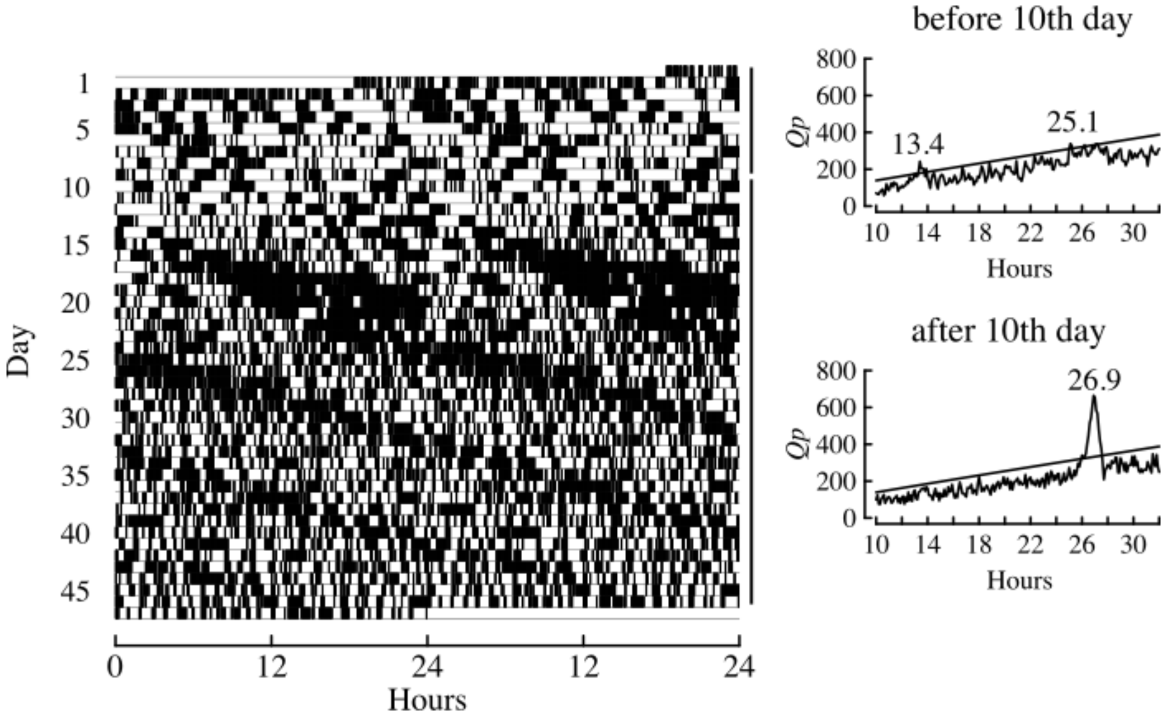

Figure 2 Representative locomotor activity rhythm under constant light in laboratory-reared *Apteronemobius asahinai*. A bimodal daily rhythm was observed before the tenth day and changed into a unimodal daily rhythm after the tenth day. The chi-square periodograms of activities before and after the tenth day are shown on the right. The oblique line in the periodogram corresponds to the significance level of α = 0.005 of each chi-square test. The peak values above the oblique line were determined to be significant.

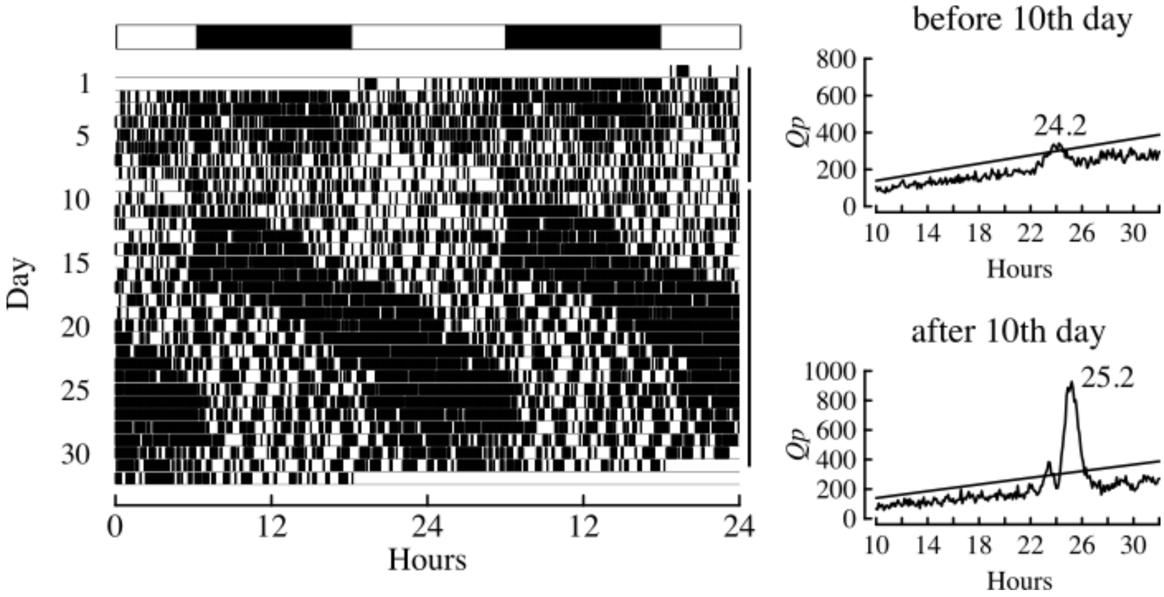

Figure 3 Representative locomotor activity rhythms in laboratory-reared *Apteronemobius asahinai* under constant light-dark cycles. (A) A bimodal daily rhythm was observed before the tenth day and changed into a unimodal daily rhythm that was not entrained to the light-dark cycles after the tenth day. (B) A unimodal daily rhythm that was entrained to the light-dark cycles continued more than ten days. Triangles on the actogram show the onsets of active phase detected by the cycle-by-cycle adaptation of the HFS method (Sakura & Yasugi 2019). The chi-square periodograms of activities before and after the tenth day are shown on the right of the actogram. The oblique line in the periodogram corresponds to the significance level of $\alpha = 0.005$ of each chi-square test. The peak values above the oblique line were determined to be significant.

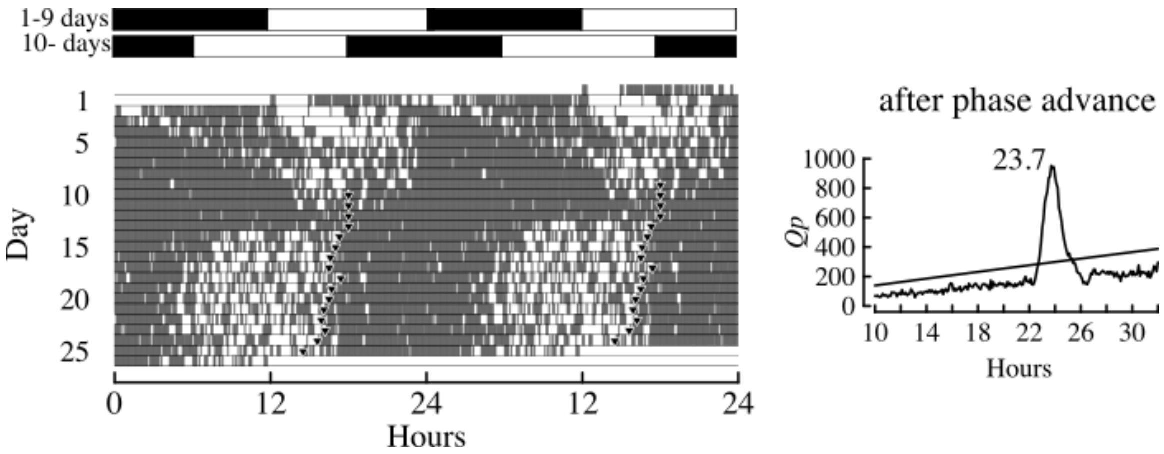

Figure 4 Representative locomotor activity rhythm in laboratory-reared *Apteronemobius asahinai* under light-dark cycles with a 6 h phase advance. A unimodal daily rhythm was entrained to the light-dark cycles after the phase advance. Black bars and white bars indicate the scotophases and photophases, respectively. Triangles on the actogram show the onsets of active phase detected by the cycle-by-cycle adaptation of the HFS method (Sakura & Yasugi 2019). The chi-square periodogram of activities after the phase advance is shown on the right. The oblique line in the periodogram corresponds to the significance level of α = 0.005 of each chi-square test. The peak values above the oblique line were determined to be significant.

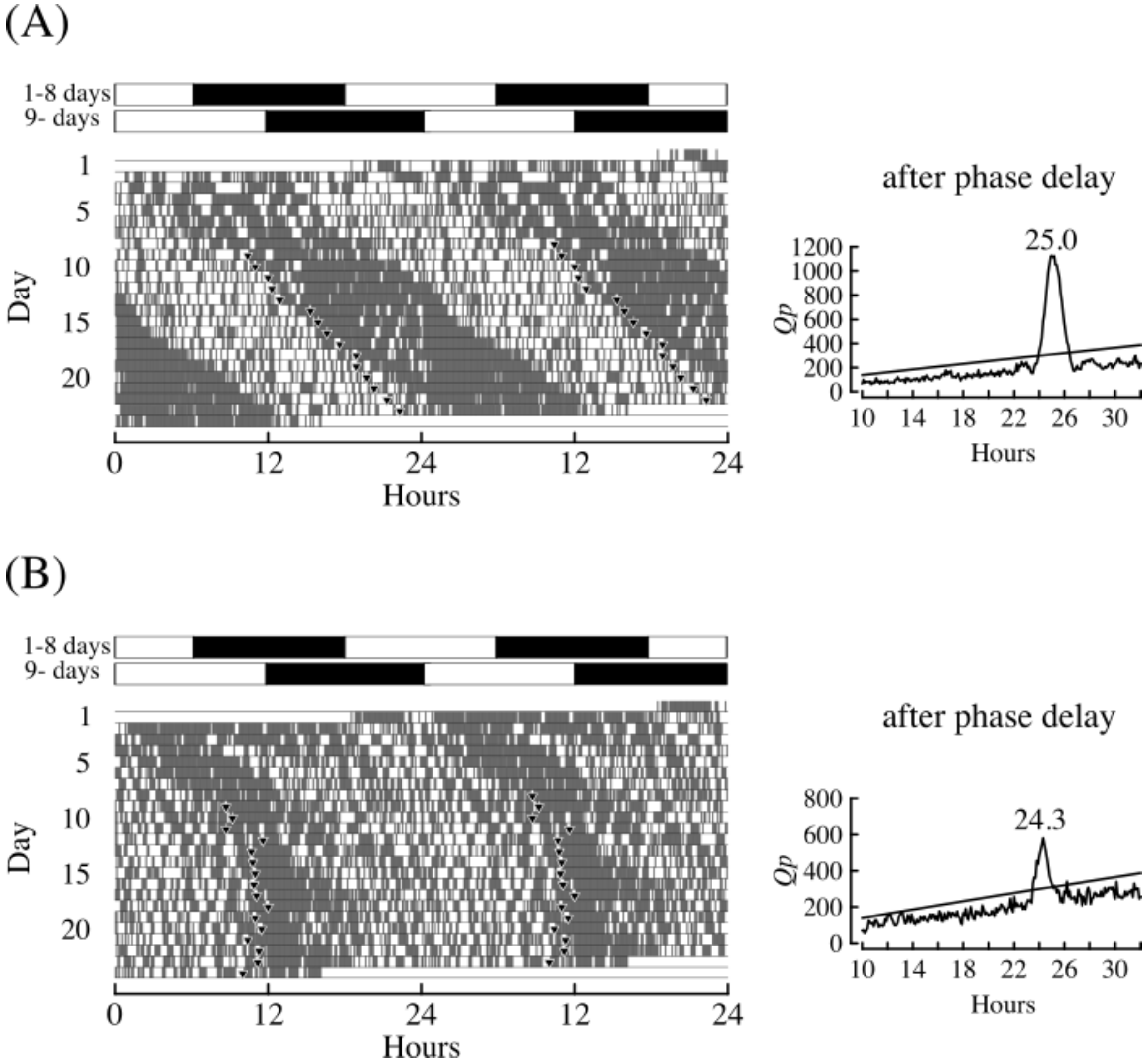

Figure 5 Representative locomotor activity rhythms in laboratory-reared *Apteronemobius asahinai* under light-dark cycles with a 6 h phase delay. (A) A unimodal daily rhythm was not entrained to the light-dark cycles after the phase delay. (B) A unimodal daily rhythm was entrained to the light-dark cycles after the phase delay. Black bars and white bars indicate the scotophases and photophases, respectively. Triangles on the actograms show the onsets of active phase detected by the cycle-by-cycle adaptation of the HFS method (Sakura & Yasugi 2019). The chi-square periodograms of activities after the phase delay are shown on the right. The oblique line in the periodogram corresponds to the significance level of $\alpha = 0.005$ of each chi-square test. The peak values above the oblique line were determined to be significant.