Activity Rhythm of the Tri-Spine Horseshoe Crab *Tachypleus tridentatus* in the Seto Inland Sea, Western Japan, Monitored with Acceleration Data-Loggers

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1 Introduction

All lives evolved on our planet are exposed to periods of day and night. Thus, endogenous *circadian* rhythms (24-h) can be found everywhere in bacteria, plants, and animals (Foster and Kreitzman 2004). Intertidal zone is the coastal region which alternately submerged and then exposed by the sea twice each lunar day (24.8-h). Organisms living there often show a tidal rhythm (12.4-h) in their behavior and physiology (for reviews, see Palmer 1976; Naylor 2010). Activity rhythms of the intertidal animals have been most studied in crustaceans. Fiddler crabs have clear, endogenous, *circatidal* rhythms that free-run in constant conditions (the rhythms persist in constant conditions with periods close to 12.4-hours) (Bennett et al. 1957; Barnwell 1966). The green shore crab, *Carcinus maenas* also exhibits circatidal locomotive activity rhythms, with peaks at the time of high tide (Naylor 1958). For another model species, activity rhythm of the American horseshoe crab, *Limulus polyphemus* has recently been well-documented. Horseshoe crabs (the class Merostomata) are an ancient group of marine arthropods which has had little change in external morphology since the Cretaceous (Botton et al. 1996). The four extant species are assigned to three genera in two families (Sekiguchi and Shuster 2009). *Limulus polyphemus*, only an extant species in the family Limulidae, migrates into the intertidal zone along the eastern coast of North America in the late spring to early summer for breeding. Their breeding activity is synchronized to high tides.

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Adult pairs appear 1–2 h before high tide and return to deep water about 2 h after high tide (Barlow et al. 1986; Penn and Broc kmann 1994).

Activity rhythms of adult *Limulus polyphemus* had been monitored in laboratory works exposed to artificial light/dark (24.0-h) and tidal (12.4-h) cycles (Chabot et al. 2004, 2007, 2008). Most individuals clearly synchronized their activity to the imposed artificial tidal cycles, some of which showed clear evidence of entrainment after the artificial tides were terminated. These results demonstrate that the endogenous tidal (12.4-h) clock that influences locomotion in *Limulus* that can be entrained by imposed artificial tides. However, many individuals also exhibit a daily pattern of activity, either to be more active during the day or night (Chabot et al. 2004, 2007). In the recent works, these activity patterns could result from light influencing the expression of one of the two coupled circalunidian clocks (24.8 h) that are 180° out of phase with each other (Chabot and Watson 2010, 2014).

In comparison to *Limulus*, activity rhythms of other species in Merostomata are poorly studied. The three Indo-Pacific species of horseshoe crabs (*Tachypleus tridentatus*, *Tachypleus gigas*, and *Carcinoscorpius rotundicauda*) are distantly related to *L. polyphemus* (Sekiguchi and Shuster 2009). The divergence between the Asiatic and American lineages probably occurred during the Jurassic period. The tri-spine horseshoe crab, *Tachypleus tridentatus* is the largest species in its body size with the widest distribution ranging from coastal Southeast Asia to western Japan. The behaviors separated by at least 100 million years of isolation between the two groups of horseshoe crabs (Botton et al. 1996).

Living organisms have adapted to the temporal structure of their environment in different ways with the overall periodicity of the environment. The biotic factors, such as food supply, competition for food, predator pressure, vary on a daily or lunar basis (Erkert 1982). Biological rhythms of various intertidal animals have been studied mainly in the physiological and biochemical aspects clarifying the mechanisms (e.g. Naylor 2010; Chabot and Watson 2014). However, their ecological importance and evolutionary significance are poorly discussed. The main purpose of our study is to provide information for the activity rhythms of *T. tridentatus* and to compare the results with those of *Limulus* to understand the evolution of biological clocks in Merostomata. Our interests are not only for examining proximate factors influencing the biological clocks of *T. tridentatus* but also for examining evolutionary ecological functions of the activity rhythms expressed by this species in its environments.

In this study, activity of *T. tridentatus* was monitored with an animal-borne datalogger. The use of animal-borne data-loggers (a.k.a. bio-logging) to study the physiology and behavior of various species has increased over the past decades. As a result, we are beginning to gain new insights into the behavior of these animals in their natural habitats (for reviews, see Cooke 2008; Ropert-Coudert et al. 2009; Rutz and Hays 2009; Bograd et al. 2010). Accelerometer tags are particularly useful to measure physical movements of animals. Their use in animal behavior studies has increased considerably during the recent decades (e.g., Yoda et al. 2001; Wilson et al. 2006; Watanabe et al. 2005, 2012; Moreau et al. 2009). In the preset study, we employed an accelerometer to monitor activity rhythm of *T. tridentatus* in

unrestrained conditions. Thus, we expect that activity of horseshoe crabs in our study to be expressed more naturally, and therefore providing more reliable information to understand the ecological functions of its activity rhythm.

2 Materials and Methods

2.1 Experimental Conditions

Fifteen adult male (mean ± s.d. =1164 ± 113 g, range = 880–1300 g) and 11 female (2473 ± 439 g, 1660–3100 g) *T. tridentatus* were used for our experiments. The animals were caught using fixed fishing nets or trawling as bycatch around Kasaoka Bay (34.46°N, 133.54°E) in the Seto Inland Sea, western Japan, and brought to the Kasaoka Horseshoe Crab Museum, Okayama prefecture, Japan (http://www.city. kasaoka.okayama.jp/site/kabutogani/).

Activity of *T. tridentatus* was investigated in two experimental conditions. The animals were exposed to photoperiodic (light/dark) cycles in laboratory experiments and to both photoperiod and tidal cycles in field manipulated experiments. These experiments were conducted during the spawning season of this species (July and August; Shuster and Sekiguchi 2009) from 2012 to 2014.

In the laboratory experiments, 10 individual horseshoe crabs (5 males and 5 females) were placed in a recirculating aquarium (300 cm × 100 cm, 80 cm deep). The bottom was covered with 20 cm thick layer of fine-grained sand where animals dug deep enough to cover themselves. The water temperature was controlled between 22 and 26 °C. Lighting was provided by four 100-W fluorescent bulbs suspended above the aquarium, which were switched on at 08:00 and off at 18:00 every day.

In the field manipulated experiments exposed to natural photoperiod and tidal cycles, 19 individuals of horseshoe crabs (11 males and 8 females) were placed in an experimental pond (12 m × 18 m) in an exterior area of the museum. The experimental pond is connected to Kasaoka Bay by a canal. The water level changes between 0.5 and 3.0 m depending on ebbing and rising tides synchronizing with natural tidal cycles in Kasaoka Bay (SioMieYell, http://sio.mieyell.jp/). The pond floor was deposited with ca. 1.0 m thick layer of fine-grained sand and mud where the animals feed freely and burrow for resting and spawning. One to four individuals were placed in the pond during the same recording period. Water temperature changes seasonally between 18 °C and 33 °C with a small daily change ±1.6 °C. *Tachypleus tridentatus* can be active in water temperature higher than 18 °C (Nishii 1975; Lee and Morton 2009; Watanabe et al. unpublished data). Change of water depth in the experimental pond was recorded by a water level logger (HOBO U20–001-02-Ti, Onset Corporation, Pocasset, MA) set on the bottom of the pond. Squid fillets and polychaetes were fed once every 3 days during the day in both experimental conditions.

2.2 Activity Recording

The locomotion activity of a horseshoe crab was recorded using an acceleration data-logger (HOBO Pendant G data-logger, 58 × 33 × 23 mm, 18 g, Onset Corporation, Pocasset, MA). The device is considerably adequate in size for adult horseshoe crabs (approx. 0.6–2.0% of body weight of the animals in our experiments) to monitor their activities. The device records acceleration and inclination through measurement of an analogue signal in each of its three axes (Moreau et al. 2009). These signals are converted to gravity units ranging from −3 g to +3 g (1 g = 9.8 m s^{-2}). The logger's memory of 64 kB permits to record 65,400 data points at user-determined intervals of 1 s to 18 h. In our experiments, each devise was set to record along only longitudinal axis (Fig. 1a) at 1-min interval to record up to 45 days.

A data-logger fixed on meshed PVC sheet (4 cm × 6 cm) with two plastic cable ties was attached using two-component epoxy glue (Quick 5, Konishi Co., Ltd) on the dorsal carapace of each animal (Fig. 1a). Before glue curing, the animals were kept in a plastic container with ca. 10 cm depth water at least for 30 min before released to the pond.

2.3 Data Analysis

The acceleration loggers were retrieved from recaptured animals or when they became naturally unglued and floated to the water surface. Data were readout through USB data transfer to PC by HOBO ware Pro (Onset Corporation, Pocasset, MA) and saved in ASCII format for further processing. Activity data were analyzed using customized macro programs in IGOR Pro ver. 6 (Wave Metrics, Inc., Lake Oswego, OR).

Acceleration signals are reflected by both accelerations related to changes in the movements of animals, that is, dynamic accelerations, and gravitational acceleration resulting from changes in the body posture (Yoda et al. 2001; Watanabe et al. 2005, 2012). The resolution of acceleration sensor: 0.025 g (0.245 m s^{-2}) is sufficient to determine whether an animal was being active or inactive. Amounts of change between consecutive data points (*delta XG*) were calculated; the value 0.1 g (0.98 m s^{-2}) was used as a criterion for active or inactive because the distribution of amounts of changes was bimodal with higher values (>0.1 g) corresponding to walking or digging on floor of the pond (Fig. 1b). If the *delta XG* in *p* and *p-1* was >0.1 g, the animal was determined active at the point (*p*). Number of active points was summed into 10-min bins for actograms double-plotted (refer to be described in Chabot et al. 2004, 2007, 2008, for more details) to improve visualization of the patterns for the rhythmicity of activity (see the results in Figs. 2, 3, and 4; upper left panels).
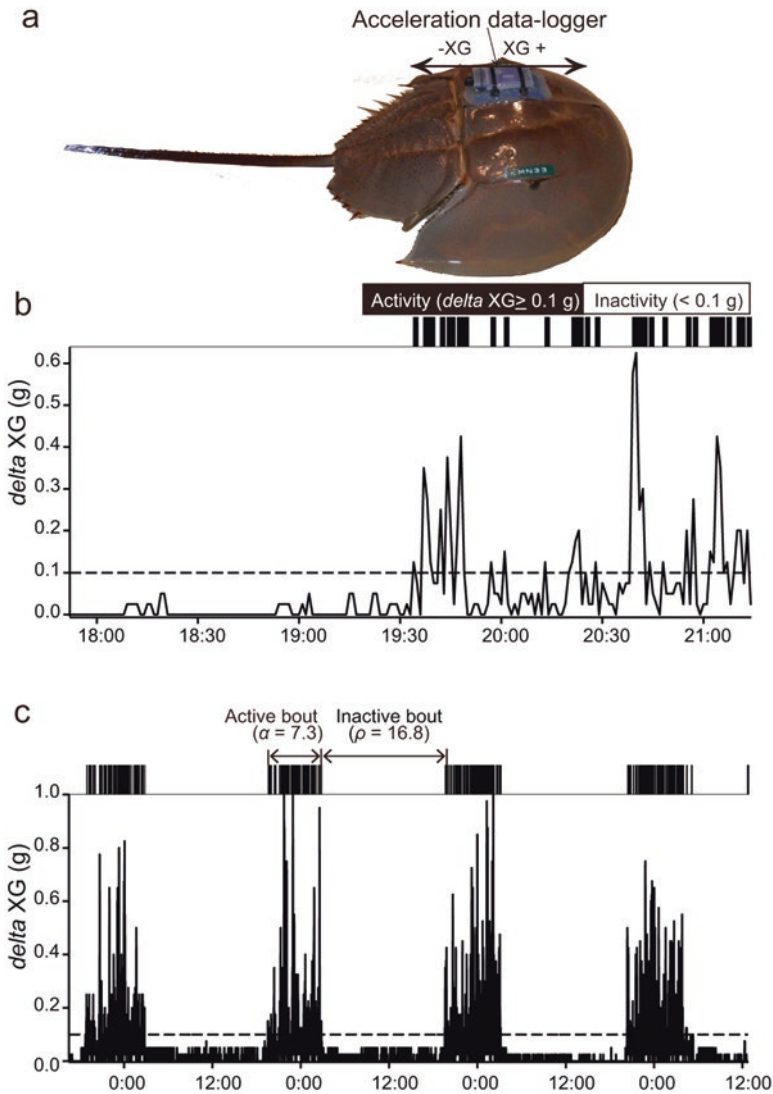
Fig. 1 Examples of activity data of tri-spine horseshoe crab, *Tachypleus tridentatus*. (**a**) An acceleration logger glued on the dorsal carapace of a female horseshoe crab to record its acceleration along the longitudinal axis (XG) at 1-min interval. (**b**) An extended record for 3.5 h in (**c**) approx. 3.5-days excerpt. Amount of change between consecutive data points (*delta* XG) was calculated. The dashed lines in (**b**) and (**c**) indicate the criterion with higher values (*delta* XG >0.1 g) corresponding to be active. Lengths of active bouts (α) and inactive bouts (ρ) are measured (**c**)

To determine a preference for activity during the daytime (06:00–18:00, *diurnality*) or night-time (18:00–06:00, *nocturnality*), cumulated number of active points during each of daytime and night-time was calculated (see the results in Figs. 2, 3,
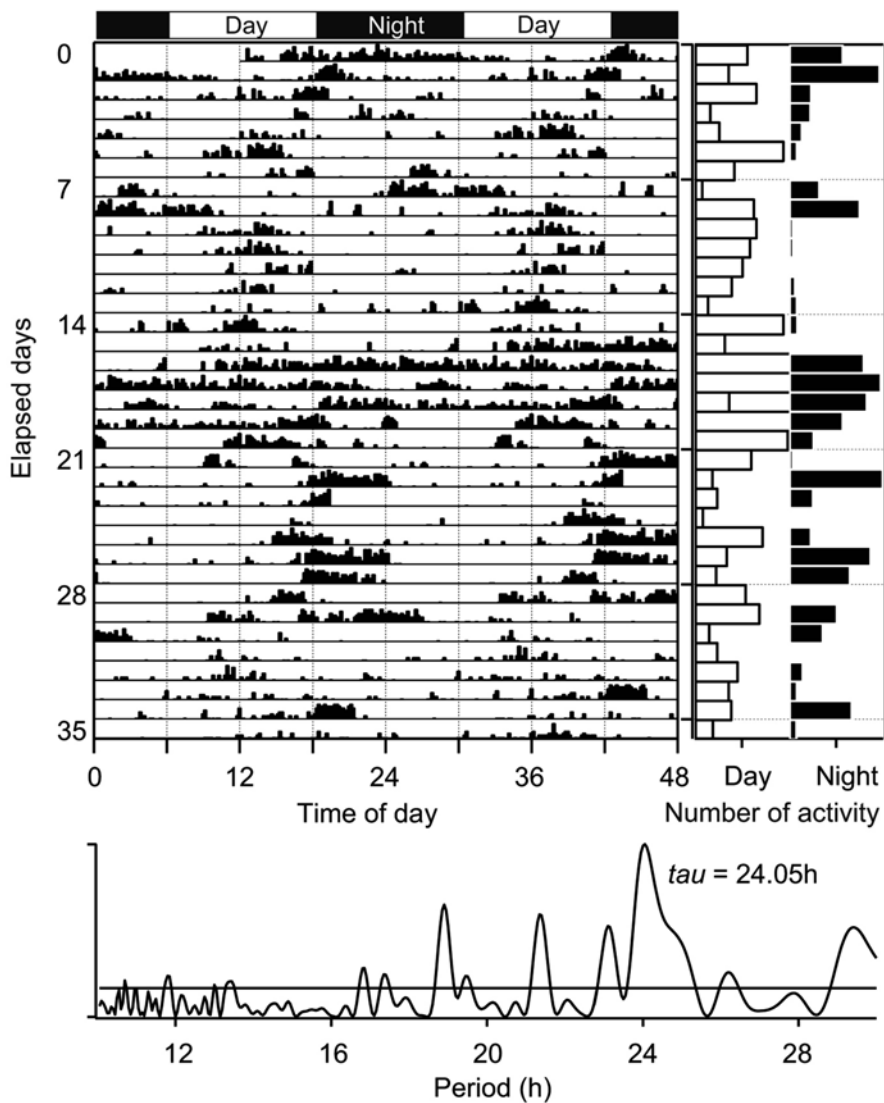
Fig. 2 An example of *circadian* rhythm expressed by *Tachypleus tridentatus* exposed to only light/dark cycles in the laboratory experiment. Upper left panel: actograms double-plotted to improve visualization of the patterns. Black/white bars at the top indicate daytime (06:00–18:00) and night-time (18:00–06:00). Upper right panel: cumulated number of activity points during each of daytime and night-time. Lower panel: Lomb–Scargle periodogram analyses of the actogram; vertical scale is relative Q (p). Largest peak value above horizontal line of significance ($P < 0.01$) indicated by numerical value (*tau*)
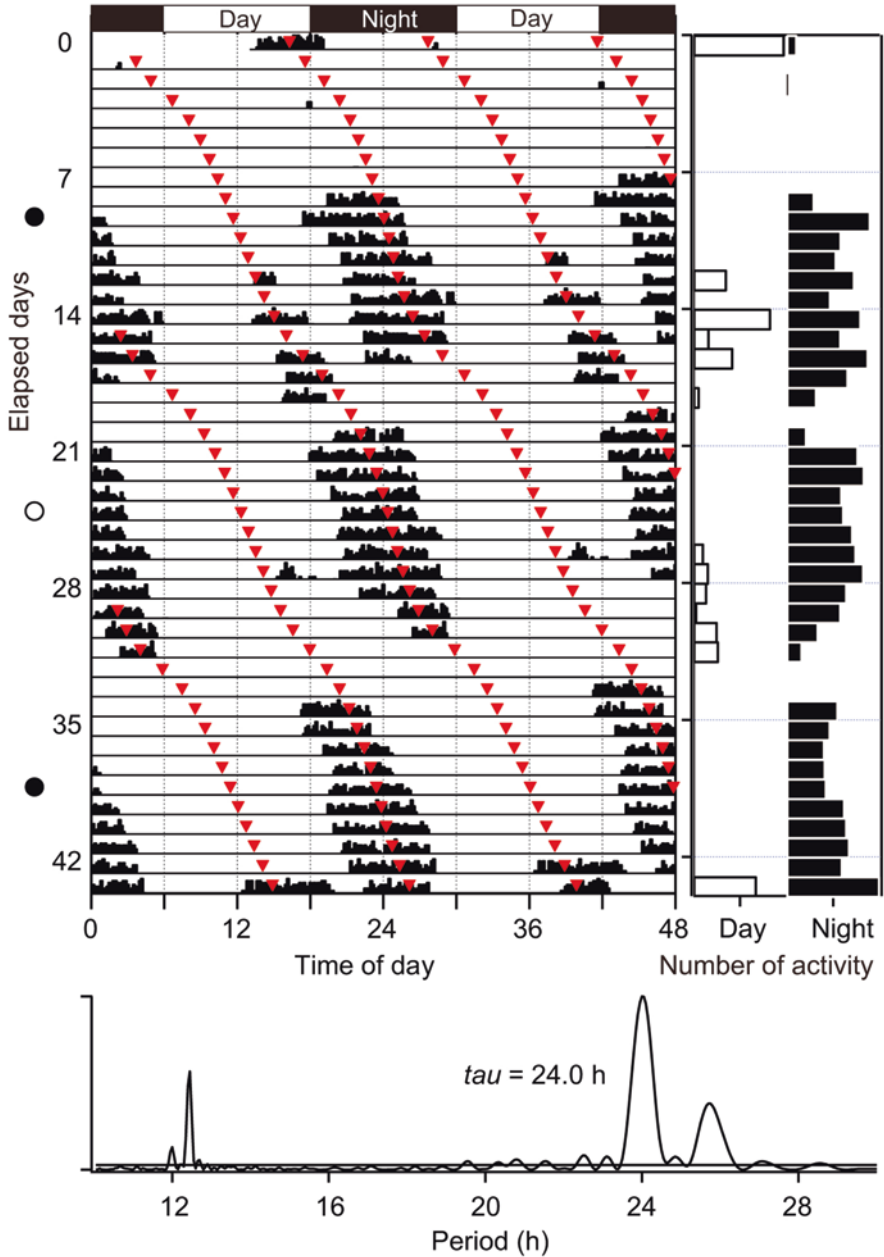
Fig. 3 An example of *circadian* rhythm expressed by *Tachypleus tridentatus* exposed to natural photoperiod and tidal cycles in the field experiment. Upper left panel: actograms double-plotted to improve visualization of the patterns. Black/white bars at the top indicate daytime (06:00–18:00) and night-time (18:00–06:00). Timing of high tides is indicated by triangles in the figure. Black/white circles on the left indicate new moon and full moon. Upper right panel: cumulated number of activity points during each of daytime and night-time. Lower panel: Lomb–Scargle periodogram analyses of the actogram; vertical scale is relative Q (*p*). Largest peak value above horizontal line of significance (*P* < 0.01) indicated by numerical value (*tau*)
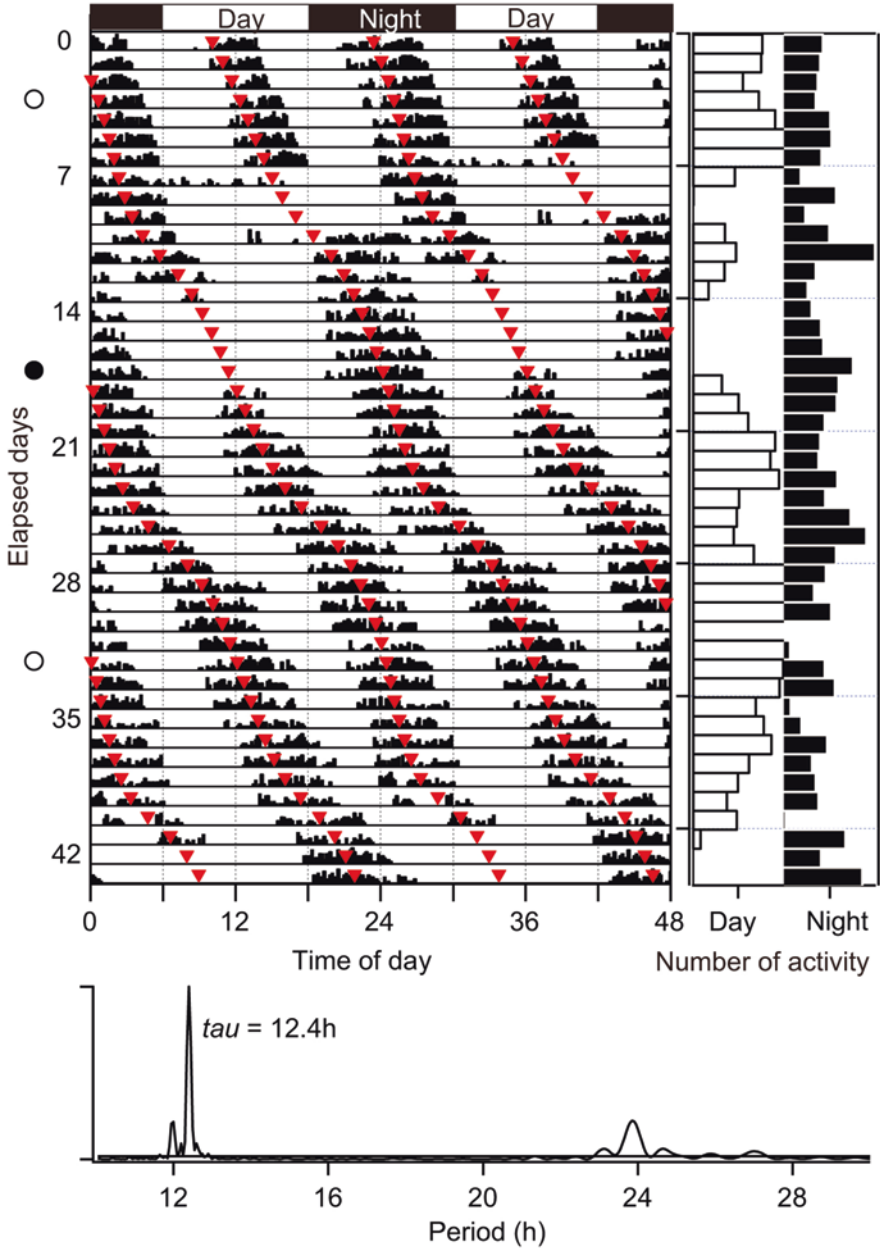
Fig. 4 An example of *circatidal* rhythm expressed by *Tachypleus tridentatus* exposed to photoperiod and tidal cycles in the field experiment. See legend in Fig. 3 for more detail

and 4; upper right panels). To determine statistical significance ($P < 0.05$) between the means during the daytime and night-time, Wilcoxon-signed rank test, a non-parametric statistical hypothesis test to compare two dependent samples, was used (Zar 2010). Percentage of active points for night-time (nocturnal activity rate) in each experimental trial was also calculated as an index of degree of nocturnality.

Activity of horseshoe crabs represented periodicity with active bouts after inactivity bouts at least 3 hours (Fig. 1c). The time lengths (h) of active bouts (α) and inactive bouts (ρ) were measured (Fig. 1c). To determine statistical significance ($P < 0.05$) for the means of α and ρ between the laboratory and field experimental conditions, Mann-Whitney U test, a non-parametric statistical hypothesis test to compare two independent samples, was used (Zar 2010).

In addition to calculate duration of each activity and inactivity bout, rhythmicity of activity throughout each experimental trial was examined by Lomb–Scargle periodogram analysis (Lomb 1976) as described in Chabot et al. (2008). Cumulated number of active points was summed every hour, and the maximal value of primary component of rhythmicity (*tau*, $P < 0.01$) was determined by the periodogram (see the results in Figs. 2, 3, and 4; lower panels). Rhythmicity of activity was determined as the *circadian* with *tau* nearly equal to 24-h or the *circatidal* with *tau* nearly equal to 12.4-h (see the results in Figs. 3 and 4; upper left panels as typical examples) for each trial. Visualization by actograms double-plotted and periodogram analysis were performed using custom-build macro programs written in IGOR Pro.

3   Results

A total of 487 and 569 days of activity data was obtained from 14 and 28 experimental trials in the laboratory and field experiments, respectively. In the laboratory experiments exposed to LD cycles, the animals exhibited rhythmicity in their activity but with large variations among trials with active bouts ($\alpha = 9.8 \pm 8.7$ h, *mean ± s.d.*) and inactive bouts ($\rho = 20.3 \pm 31.7$ h).

In the field experiments exposed to LD and tidal cycles, the animals exhibited clear rhythmicity of their activity with active bouts ($\alpha = 8.3 \pm 12.7$ h) and inactive bouts ($\rho = 12.1 \pm 3.8$ h). Means of α and ρ were not significantly different between the laboratory and field experimental conditions (α: $U = 221$, $P = 0.5131$, ρ: $U = 194$, $P = 0.9574$).

Lomb–Scargle periodogram analyses indicated that significant activity rhythms in the 24-h range (23.1–24.1 h) were found in all laboratory experimental trials exposed to only LD cycles. However, the timing of onset and offset of active bouts were not synchronized to LD cycles (see an example in Fig. 2: upper left panel). Preference for activity during each of daytime or night-time in a day (i.e., diurnality or nocturnality) was different among the individuals and trials. Out of a total of 14 trials, the animals exhibited diurnality in eight trials (nocturnal activity rate: 8.2%–39.2%, $P < 0.05$) and nocturnality in one trial (80.5%, $P < 0.01$); in the remaining five trials. The activity levels were not statistically different between daytime and night-time (37.2%–56.5%, $P > 0.05$).

Twelve of 19 animals exposed to both LD and tidal cycles expressed significant circadian rhythms in the 24-h range (23.8–24.4 h, $P < 0.01$) in 16 of 28 (57%) trials (see Fig. 3: lower panel as a typical example). Eight animals expressed significant circatidal rhythms in the 12.4-h range (12.3–12.5 h, $P < 0.01$) in 12 (43%) trials (see Fig. 4: lower panel as a typical example). Three animals expressed both circadian and circatidal rhythms in different trials. Eighteen of 19 animals expressed nocturnality in 26 (93%) trials (nocturnal activity rate: 66.2%–99.0%, $P < 0.05$), while no animal expressed diurnality. In the remaining two trials, the activity levels were not statistically different between daytime and night-time ($P > 0.05$).

In the actograms double-plotted for the animals with 24-h rhythms (as shown in Fig. 3: upper left panel), activity bouts were more likely to be shown around high tides during the night. These animals generally began to move about 2.5 h before high tides during the night. On the other hand, active bouts were shown around high tides during both day and night in those for animals with 12.4-h rhythms (as shown in Fig. 4: upper left panel).

4 Discussion

4.1 Activity Rhythm of Tachypleus tridentatus

Here we present the first report of the activity rhythm in the tri-spine horseshoe crab, *T. tridentatus*. The individuals exposed to both light/dark (LD) and tidal cycles exhibited 24-h (i.e., circadian) rhythms and nocturnality in more than half (57%) experimental trials. Their activity rhythms synchronized to high tide during the night. In the remaining experiments (43%), the animals exhibited 12.4-h (i.e., circatidal) rhythms synchronized to high tide during both day and night.

Activity rhythm of the American horseshoe crab, *L. polyphemus,* has been well-studied in laboratory works (e.g., Chabot et al. 2004, 2007, 2008; Chabot and Watson 2010, 2014). It is likely that there are some similarities and differences in the activity rhythms and their possible external synchronizing cues (*Zeitgebers*) between these species. In the experiments of *Limulus* exposed to LD and artificial tidal cycles, most adult *Limulus* exhibited clear circatidal (12.4-h) rhythms. Their activity synchronizes to high tide during both light and dark periods (Chabot et al. 2007, 2008). After a period with water-level fluctuation, their activity rhythms entrain in constant water level, which indicate that *Limulus* possess an endogenous circatidal (12.4-h) clock that drive its locomotion activity (Chabot et al. 2008).

In our study, *T. tridentatus* exposed to LD cycles in the constant water level exhibited circadian rhythms out of synchronization with LD cycles, that is, their activity was free-running with 24-h cycles. No animals expressed 12.4-h rhythms of their activity in the constant water level. It is, thus, more likely that *T. tridentatus* possess an endogenous circadian (24-h) clock, but LD cycles do not act solely as a

Zeitgeber in its activity. In contrast, it was reported that LD cycles can solely affect locomotion activity of *Limulus*. Many individuals in the present study expressed daily rhythms of activity when exposed to LD cycles in constant water level. Nearly half exhibited a preference for diurnal movements, while the others exhibited no preference. However, in *Limulus*, these daily patterns are much less apparent when tidal cycles are present (Chabot et al. 2008). Thus, the authors suggested that the tidal cues override the influence of LD cycles, which produce circatidal rhythms of their activity of *Limulus*.

In this study, we did not evaluate the solo effect of tidal cycles that *T. tridentatus* exposed to tidal cycles under LL or DD conditions. Thus, the effects of LD and tidal cycles cannot be separated. However, we presume that tidal cues combined with LD cycles regulate activity rhythms in *T. tridentatus*. It is similar to that suggested in *Limulus* (described as above, Chabot et al. 2008). It is, however, likely that the effects of LD cycles differ between the two species. LD cycles combined with tidal cues give stronger negative effects in locomotion activity of *T. tridentatus* compared with *Limulus*. In the experiments on *Limulus* exposed to artificial tidal cycles under constant light conditions (LL), the animals exhibit circatidal rhythms synchronizing with tidal cycles.

Contrary to this assumption, several individuals of *T. tridentatus* expressed 12.4-h rhythms or both 24-h and 12.4-h in different trials. In the studies on *Limulus*, most individuals exhibited circatidal rhythms, but many also exhibit a daily pattern of activity, either to be more active during the day or night (Chabot et al. 2004, 2007). The variations in activity rhythms in *Limulus* had been presumed as resulted from congenital traits such as individual and geographical differences, or from problems of monitoring techniques and experimental conditions. In their more recent works, it is hypothesized that a combination of daily and tidal activity patterns could result from light influencing the expression of one of the two coupled circalunidian clocks (24.8 h), resulting in one activity bout being diminished or suppressed relative to the other (Chabot and Watson 2010, 2014).

In our results, some individuals expressed both circadian and circatidal rhythms in different trials. All animals in this study were obtained within the narrow geographic range (approximately 20 km × 30 km) in the eastern part of Seto Inland Sea. In addition, genetic variation of *T. tridentatus* in the Seto Inland Sea is considerably much lower than those of other populations (Nishida and Koike 2009; Nishida et al. 2015). Thus, it is unlikely that different activity patterns are resulted from such congenital traits. Moreover, our recent works suggest that tidal level fluctuations due to lunar cycles influence whether *T. tridentatus* express circadian or circatidal rhythms of its activity (Watanabe et al. unpublished data) that support the two coupled circalunidian clocks hypothesis supposed to be in *Limulus*. Additional experiments are needed to identify entraining agents of activity rhythms of *T. tridentatus* and to understand the mechanisms of its biological clock.

4.2   Ecological Functions of the Activity Rhythm in the Seto Inland Sea

Our second purpose of this study is to understand the evolutionary ecological functions of the activity rhythms of *T. tridentatus*. Locomotion activity of *Limulus* had been monitored with experimental activity boxes and running wheels in restrained conditions in Chabot et al. (2004, 2007, 2008). Range of water level is limited with small capacity of water tanks (normally depth less than 50 cm) in the laboratory experiments while sea surface level fluctuates largely depending on lunar cycles in natural environments. In this study, we employed an accelerometer to monitor activity rhythm of *T. tridentatus* in unrestrained conditions. Thus, we expect that activity of horseshoe crabs in our study to be expressed more naturally, providing reliable information to understand the ecological functions of its activity rhythm.

Interspecific differences of chronotypes, that is, diurnality or nocturnality, have been investigated in various taxonomic groups to understand the ecological niches in its ecosystems. Our results suggest that *T. tridentatus* is primarily nocturnal, while most *Limulus* is active both during the day and night. Both diurnality and nocturnality are found among taxonomically similar species (Kronfeld-Schor et al. 2001), or even in a same species (Yokota and Oishi 1991). The choice of temporal ecological niche may be important to determine either diurnality or nocturnality. It may depend on complex interactions with coexisting species of their prey, predators, and competitors. Both *T. tridentatus* and *Limulus* probably adapted to similar environments and ecological niches in these coastal ecosystems. However, it is likely that local differences of their main prey and predators produce different chronotypes. *Limulus* is opportunistic foragers that can take advantage of a wide range of locally available prey (Botton and Shuster 2003), including bivalves, polychaetes, crustaceans, and gastropods (Botton 2009). It is likely that *T. tridentatus* is also an opportunistic forager, but its diet in the Seto Inland Sea is more highly consisted of benthic polychaetes (Souji et al. 2005, 2007, 2008). Although species identification of polychaetes in the diet samples was insufficient, most polychaete species in the diet are presumably nocturnal such as other large polychaete *Nereis virens* (Last and Olive 1999, 2004). If so, *T. tridentatus* may become more active feeding on nocturnal polychaetes crawling out of tidal flat sediments during the high tides at night. A similar example was reported in shorebirds *Charadrius alexandrines* as a predator for polychaetes. The shorebirds increase nocturnal feeding rates corresponding to nocturnal activity in polychaetes (Kuwae 2007). Thus, there is the possibility that *T. tridentatus* in the Seto Inland Sea evolved to synchronize their activity rhythms with those of their main prey.

For another possible reason, there is little quantitative information about adult mortality due to predation on horseshoe crabs. However, it is more likely that potential predators are less for *T. tridentatus* than for *Limulus*. It was reported that *Limulus* is eaten by sharks, large crustaceans, sea birds, and sea turtles (Botton and Shuster 2003). There is little information that large sharks and sea turtles migrate to the Seto Inland Sea (Shigeta 2008; Ishihara et al. 2014). They seem to be transient visitors

from the Pacific Ocean. Therefore, it is unlikely that *T. tridentatus* tends to be nocturnality due to avoiding from their potential predators.

Horseshoe crabs should be considered as keystone species in the coastal ecosystem, that is, an ecologically important species in the environment (reviewed by Botton 2009). Thus, identifying its activity is important to understand the coastal ecosystem. In addition, we believe that our bio-logging techniques will be a powerful tool for monitoring biological rhythms of keystone species in coastal ecosystems.

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