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Circadian Locomotor Activity Under Artificial Light in the Freshwater Crab *Pseudothelphusa americana*

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Abstract

Long-term recordings of locomotor activity were obtained from intact freshwater crabs, *Pseudothelphusa americana* in constant darkness (DD), constant light (LL) and different light-dark (LD) protocols. Bimodal rhythms were typically observed in this crab when subjected to DD or LD, with bouts of activity anticipating lights-on and lights-off, respectively. Freerunning circadian rhythms were expressed in both DD and LL for longer than 30 days. In DD, we observed that some animals presented different period lengths for each activity component. During LL, activity was primarily unimodal, however spontaneous splitting of the rhythms were observed in some animals. When activity was recorded under artificial long days, the morning bouts maintained their phase relationship but the evening bouts changed their phase relationship with the Zeitgeber. Our results indicate that, bimodal locomotor activity rhythm in the crab *Pseudothelphusa americana* is variable among organisms. The characteristics of phase relationship with LD and responses to LL for morning and evening bouts, suggest that, locomotor activity could be driven by multiple oscillators, and that coupling between these oscillators may be regulated by light.

Keywords: Circadian rhythm, freshwater crab, locomotor activity, *Pseudothelphusa*.

Introduction

Freshwater and marine decapods have been studied in order to understand the circadian organization of crustaceans (for review, see, Warner, 1977; DeCoursey, 1982; Brown, 1983; Aréchiga et al., 1993; Fuentes-Pardo & Hernández-Falcón, 1993;

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Fuentes-Pardo et al., 1996; Larimer & Smith, 1980). These studies point to a multi-oscillatory system comprised of components located in the eyestalks and supraesophageal ganglion. Although autonomous oscillators have been observed in other neuronal structures, such as the ganglion chain (Aréchiga & Rodríguez-Sosa, 1977; Prieto-Sagredo & Fanjul-Moles, 2001) the circadian pacemaker responsible for driving the locomotor activity rhythm has not yet been definitively identified. The nature of rhythm regulation in crabs also needs to be discussed in the context of the presence of circadian or circatidal activity. Some crabs exposed to tides display both circadian and circatidal rhythms (Naylor, 1996) also interpreted as circalunidian rhythms (Palmer, 2000). Studies in marine crabs demonstrate that free-running circatidal rhythms exhibit at least two intervals of activity that sometimes show different periodicities, indicating that activity in these species may be controlled by two oscillators with circalunidian periods weakly coupled to each other (Palmer, 2000). Data collected from species of crabs found in both saline and freshwater habitats, such as *Uca subcylindrica*, indicate that circadian rhythms are more accurate in species located far from tidal influence (Thurman, 1998). In this species, locomotor activity rhythm is bimodal when kept in DD and unimodal when kept in LL (Thurman & Broghammer, 2001). When subjected to variable photoperiod, the crabs change their activity according with day length, suggesting that seasonality in locomotor activity is regulated by two clocks with circadian properties more than circatidal.

Pseudothelphusa americana is a freshwater crab which inhabits shallow waters along the shores of rivers and lakes from Southern Mexico to Central and tropical South America (Álvarez & Villalobos, 1997). *Pseudothelphusa americana* exhibits a bimodal rhythm of locomotor activity when kept in light-dark cycles (LD). One bout begins shortly before lights-on, also named in this work as morning bout, and a second, larger peak in activity is observed a few hours before lights-off, also named as evening bout (Miranda-Anaya et al., 2003).

The present work was carried out to study whether the morning and evening bouts of activity in the fresh water crab *Pseudothelphusa americana*, are similar to the reported for marine crabs in long term conditions of constant darkness and constant light, and its synchronization to different photoperiodic intervals.

Materials and Methods

Animal maintenance

Freshwater crabs *Pseudothelphusa americana* were collected from freshwater ponds and wetlands in Mixquiahuala Hidalgo, Mexico, and transferred to glass aquaria containing tap water where they were maintained as published previously (Miranda-Anaya et al., 2003). Animals were held in natural light cycles for at least one week before the recording of locomotor activity commenced. Thirty-three crabs weighing 13.8 ± 1.26 g were used.

Experimental protocols

In all the protocols used, water was changed and animals were fed vegetables and meat weekly. In constant darkness (DD), twelve crabs were held in for approximately 35 days to observe the persistence of circadian rhythm in locomotor activity in this species, since has been noted in some marine crabs, that circadian locomotor activity may be confusing in long term recordings (DeCoursey, 1983). A second group of 9 crabs was subjected to Light-Dark cycles for one week (LD 12:12, photophase 0700–1900 h) to observe the phase reference of morning and evening peak regarding artificial photoperiod. After, recordings were obtained by at least 30 days in constant bright light (LL, 150 Lx), to observe period changes as well as if the bimodal circadian rhythm persists under constant artificial illumination. To study entrainment of the morning and evening bouts to different photoperiods, as well as phase relationship (ψ) regarding the artificial photoperiod, two groups of 6 crabs were subjected either to a long or short photoperiod (LD 16:8, 0700–2300 h or 8:16, 0700–1500 h respectively), for a minimum of 7 days. All the animals tested in long and short photoperiods were set after in DD for one more week to evaluate masking.

Activity recordings

Locomotor activity was recorded as previously described (Miranda-Anaya et al., 2003; Miranda-Anaya & Fanjul-Moles, 1997). Briefly, crabs were kept individually in an aquarium partially filled with tap water. Each aquarium was equipped with infrared light crossings to detect locomotor activity. Data were summarized in 10-min bins and stored on a PC by means of a data acquisition board (NAFRI, DF Mexico). Aquaria were kept in light-tight wooden boxes maintained at 23 ± 2°C in climate controlled rooms. A fluorescent lamp controlled by a timer provided the desired light conditions (150 lx).

Data analysis

Data were plotted in conventional actograms and the respective circadian periods were calculated using X^2 periodograms at 0–30 h intervals (Sokolove & Bushell, 1978), using the software program Tau (Mini Mitter Co., Inc., USA). Period values with spikes above the confidence interval ($P < 0.05$, indicated with a diagonal line in each periodogram) were considered significant. In LD, the phase reference of the activity rhythm with the Zeitgeber (ψ) was considered to be the point when activity during each interval, reached the mean value in an average waveform with respect to time of day. Results on circadian period lengths (τ) of free-running activity bouts under different protocols were analyzed using a non-paired students' t-test, and the differences in ψ for each morning and evening bouts, were analyzed using the ANOVA function of the software program Statistica (Stat-software, Jandel Scientific, San Rafael, CA. USA). Significant differences were considered when $P < 0.05$. All values are presented as means ± SE. Average waveforms were obtained from at least three days when entrained rhythms were observed.

Results

Locomotor activity in constant darkness

Of the 12 animals recorded in DD, eleven showed typical bimodal rhythms with one peak presented to the projected lights-on and the second peak to the projected lights off. Both circadian ($\tau = 25 \pm 0.49$ h) and ultradian rhythms ($\tau = 12.76 \pm 0.5$ h) were observed and consistent with previous observations (Miranda-Anaya et al., 2003); only one animal of this group showed arrhythmic behavior. Free running circadian periods of locomotor activity in DD are variable between the animals, however a bimodal circadian rhythm was clearly seen overt noisy recordings. Five of eleven animals used, displayed bimodal rhythms in which two significant different periods, for each bout of activity was observed. The second circadian period, usually was shorter in Qp amplitude, an larger in τ value.

Figure 1 presents an example of a 33-day recording of locomotor activity from a typical animal in DD. During the first 15 days, each bouts of activity presented different period ($\tau = 23.8$ h and $\tau = 25.6$ h respectively). During days 17–19, the locomotor activity rhythm became noisy, however no changes in the experimental conditions were noted. By day 24 the circadian rhythm returned and maintained the projected phase of the first bout. Lower periodogram indicates that from days 24 to 32 the second circadian peak reduces its Qp amplitude, being non-significant.

Locomotor activity in constant light

All crabs subjected to LL displayed circadian rhythms with larger variability in free running period ($\tau = 25.96 \pm 1.39$ h) than in DD, however, no significant differences in average τ were found between these experimental conditions. Four animals in LL showed only unimodal rhythms throughout the 30 days of recording. Five other animals switched their activity patterns between unimodal to bimodal. When unimodal activity was present, it lasted, on average, 14.53 ± 1.96 h; when activity was bimodal, the lights-on associated peak lasted 5.2 ± 3.8 h and the lights-off associated peak was 8.7 ± 3.5 h. Figure 2 shows an actogram of a crab held in LD (12:12) during the first 7 days. Two peaks are seen associated with lights-on and lights-off respectively; when switched to LL, the rhythm became unimodal (days 11–17, $\tau = 25.4$ h), however by day 22 a bimodal activity pattern reappeared with a notable shortening of circadian period. The corresponding periodogram (lower right) was obtained over days 23–28 and possesses two significant periods ($\tau = 12$ and 24.2 h). Bimodal activity persisted until day 30, when a crossing of each interval of activity was observed.

Locomotor activity patterns for different photoperiods

Table 1 presents the phase relationship (ψ) values for the observed locomotor activity from animals subjected to different LD conditions. All animals tested were able to entrain. The ψ of morning bouts was not different between groups, but ψ of the evening bouts was different between the LD 8:16 and the LD 16:8 group ($P < 0.05$, ANOVA). Figure 3 shows three average waveforms over three consecutive days of

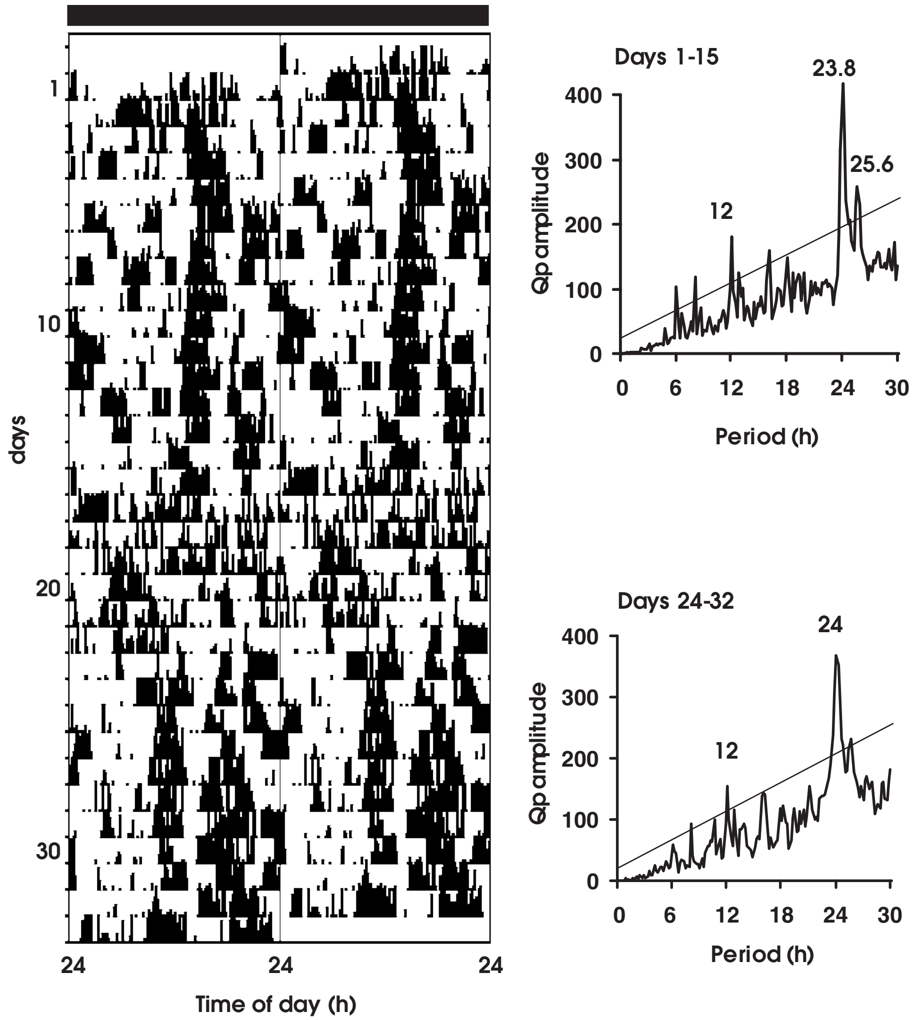

Figure 1. Double-plotted actogram showing locomotor activity rhythm from an intact crab in DD. During the first half of recording, a shorter interval displays a different freerunning period than the main bout. On the right, corresponding periodogram indicate $\tau = 23.8$ and 25.6 h of days 1–15. Lower periodogram corresponds of days 24–33.

the steady state rhythm, from different animals subjected to LD 16:8 (A), 8:16 (B) and 12:12 (C), respectively. The morning bout, maintain a similar phase relationship in all photoperiods tested. The evening bout does not change its ψ in short photoperiods compared with the LD 12:12 group; however under long photoperiods this bout became significantly delayed ($P < 0.05$, ANOVA). Each bout of activity freerun for at least two days in DD from their previous phase observed in LD (Fig. 3D).

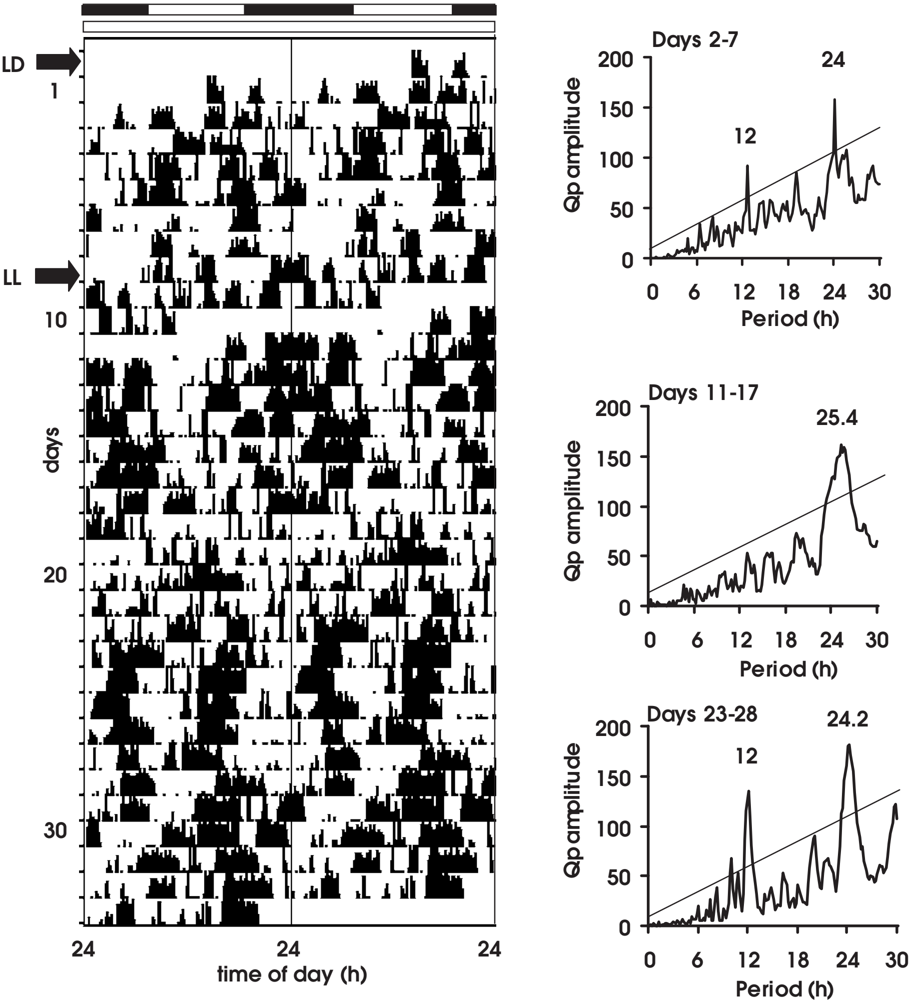

Figure 2. Actogram and periodograms from an animal held in LD 12:12 for one week and then placed in LL. Bimodality in LD conditions is also observed in this animal. When subjected to LL (second arrow in actogram), the rhythm becomes unimodal with a period length of 25.4 h. On day 20 the length of the freerunning period spontaneously shortens ($\tau = 24.2$ h) and bimodality reappears. Upper bars represent LD conditions; arrows represent a change in the experimental protocol.

Discussion

Circadian rhythms allow organisms to adapt their physiological and behavioral responses to specific times of the day. The bimodal rhythm of locomotor activity in *Pseudothelphusa americana* is circadian in nature. It synchronizes to LD cycles of

Table 1. Time of day when morning and evening bouts were observed. Each value represents the mean (±SE) of six animals. Phase references were calculated when the animal's rhythm was in a steady state of entrainment. Asterisks denote significant differences between groups $P < 0.05$.

Photoperiod	12:12	8:16	16:8
Lights-on bout (ψ) h	7.33 ± 0.49	7.0 ± 0.71	6.58 ± 0.17
Lights-off bout (ψ) h	14.66 ± 0.51	16.16 ± 0.98	18.88 ± 1.3*

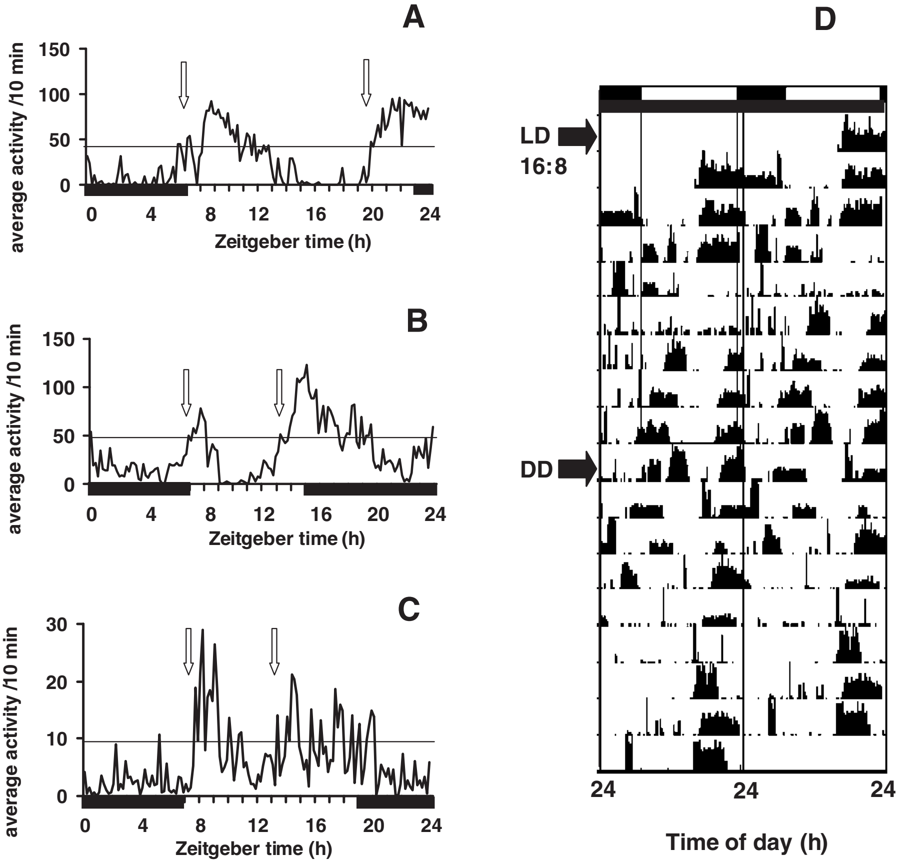

Figure 3. Waveforms from three days corresponding to the entrained steady state rhythms from LD 16:8 (A), 8:16 (B) and 12:12 (C). The lights-on associated bout of activity does not change its ψ with the Zeitgeber, however the lights-off associated peak does change in long photoperiods. Waveforms show the mean value (horizontal line) and white arrows represent ψ; black bars represent scotophase. D is a representative actogram from an animal held in LD 16:8 for 8 days and then released into DD.

different photic lengths. Of particular importance in this study is the observation that the bimodal rhythm is present even after more than 30 days in DD. The LL produced variable responses, and differences in free running period were not observed between DD and LL as denoted by Aschoff's rule (Aschoff, 1981). Lack of differences between these two conditions may be due, because of the high variability in period values observed, as noted previously (Miranda-Anaya et al., 2003). An interesting observation was that some animals subjected to LL switched between unimodal and bimodal rhythms (Fig. 2). Recordings obtained under long photoperiods showed that the phase relationship between the Zeitgeber and the lights-off associated peak is different from that observed in short photoperiods.

When taken together with the fact that these different intervals of activity often freerun with different periods in DD (Fig. 1), these results suggest that more than one endogenous circadian oscillator is controlling the locomotor activity rhythm in this species, as has been suggested for other marine crabs (Thurman, 1998), or freshwater crayfish (Fuetes Pardo et al., 1996). In LD, one bout synchronized to lights-on and another to lights-off, and resembles the description of the morning and evening oscillators that have been proposed to regulate circadian rhythmicity in animals (Daan et al., 2001; Kost'al et al., 2000; Helfrich-Förster, 2000; Jagota et al., 2000; Pittendrigh & Daan, 1976).

Multi-oscillatory systems of circadian organization have been demonstrated to be present in many invertebrates (Page, 2001; Helfrich-Förster et al., 1998) and vertebrates (Yamazaki et al., 2000; Menaker et al., 1997) examined to date.

Studies on the locomotor activity rhythm in the crayfish, *Procambarus clarkii*, have shown that it is possible to observe intervals of activity with different periods, with one interval responding to food and the other to light Zeitgebers (Fernández de Miguel & Aréchiga, 1990). Behavior arising from paired pacemakers have been frequently described in invertebrates, such as insects or mollusks, but the circadian system in the individual may, however, be composed of multiple autonomous localized in distinct tissues (Page, 2001).

The day length is the main environmental factor that modulates the circadian behavior in animals. Freshwater decapods have evolved adaptations that may be related to reproductive fitness in seasonal environments. Ovarian maturation in crayfish responds to photoperiodic induction experiments (Fanjul-Moles et al., 2001); and juvenile crayfish are able to entrain to skeleton photoperiods of different length (Fanjul-Moles et al., 1998).

The circadian rhythms of locomotor activity in other freshwater decapods, such as the crayfish, seem to be regulated by central nervous structures, most likely within the supraesophogeal ganglion (Page & Larimer, 1975), and are synchronized to light by extraretinal photoreceptors (Page & Larimer, 1972; Miranda-Anaya & Fanjul-Moles, 1997; Sandeman et al., 1990). In the freshwater crab *Pseudothelphusa americana*, eyestalk ablation reduces the ability to entrain to both LD cycles and skeleton photoperiods (Miranda-Anaya et al., 2003; Ramírez-Lomelí et al., 2002). The ability to change the locomotor activity patterns according with the day length indicates that *Pseudothelphusa* might have also physiological responses influenced by the season. The ability to change the activity patterns of crabs, regarding the photoperiod indicates a circadian rhythm strongly influenced by daylight along the year.

The variability in the period and phase relationship between the two oscillators may be a mechanism by which animals could measure seasonal changes in day length. These oscillators differ in spontaneous period, and their sensitivities to light seem to be different (Carmona-Alcocer & Miranda-Anaya, unpublished observations), as observed in nocturnal rodents (Pittendrigh & Daan, 1976). Preliminary results from our group indicate that light pulses given at specific circadian times produce phase shifts of different magnitudes for each peak, indicating the individual oscillators may be regulated differently by light.

Marine crabs like *Uca subscylindrica*, collected from freshwater environments or unexposed to tidal changes show only circadian rhythms (Thurman, 1998; Thurman & Brohammer, 2001), as do exclusively freshwater decapods (Page & Larimer, 1975; Miranda-Anaya & Fanjul-Moles, 1997, and present study). With increasing the distance from the seashore, decapods activity rhythms are predominantly based on light-dark intervals rather than changes in water level (Palmer, 1973). Many species are nocturnal or crepuscular, taking advantage of lower temperatures and increased ground moisture in the evening, night or dawn (Powers & Bliss, 1983).

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