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Circadian Locomotor Activity Rhythm in the Freshwater Crab *Pseudothelphusa americana* (De Saussure, 1857): Effect of Eyestalk Ablation

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Abstract

The circadian rhythm of locomotor activity in the freshwater crab, *Pseudothelphusa americana*, was studied in aquaria using infrared crossing sensors. Individuals with ablated eyestalks were compared with intact individuals in constant darkness (DD) and in light-dark cycles (LD). Our results showed that intact animals in DD displayed bimodal rhythms. In LD conditions the two peaks were associated with lights on and lights off, respectively. A significant difference in the free running periods before and after LD was observed in all intact animals. After eyestalk ablation (ES-X), the circadian rhythm of locomotor activity disappeared immediately, but reappeared several days later. Diurnal activity was seen in some ES-X animals when exposed to LD. Our results indicate that locomotor activity rhythm in *P. americana* is driven primarily by oscillators located outside the eyestalks, and that extraretinal photoreceptors mediate either entrainment or masking effects.

Keywords: Circadian rhythm, *Pseudothelphusa americana*, locomotor activity, eyestalk, extraretinal photoreceptors.

Introduction

Circadian locomotor activity rhythms are observed in almost every animal taxa. Changes in locomotor activity in fact reflect fundamental changes whithin the central nervous system (Brady, 1981). Particularly among decapods, the effect of eyestalk removal has contributed to understanding of the circadian organization that drives locomotor activity (Edwards, 1950; Bliss, 1962; Powell, 1965; Naylor & Williams, 1968; Page & Larimer, 1975).

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It has been suggested, for example, that circadian locomotor activity in crayfish is a consequence of coupling among different oscillators that are believed to be located in the eyestalks structures (Fuentes-Pardo & Inclan-Rubio, 1981). Juvenile crayfish with ablated retina and lamina ganglionaris display circadian rhythmicity and are able to entrain to non-parametric monochromatic light protocols (Miranda-Anaya & Fanjul-Moles, 1997). Eyestalk ablation in this crustacean also produces a significant change in the amount of activity (Aréchiga & Huberman, 1980), but some animals still show circadian rhythmicity (Page & Larimer, 1975). Further studies using juvenile crayfish showed that lesioning the protocerebral area of the supraesophagic ganglion produces arrhythmia (Fanjul-Moles, 1998), supporting previous observations that rhythmic locomotor activity in crayfish is driven by a pacemaker located in this ganglion (Page & Larimer, 1972, 1975).

Comparative studies have been useful in elucidating the circadian organization of crabs that belong to similar taxa (Honegger, 1976). *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). In this work we describe the circadian rhythm of locomotor activity in the fresh water crab *Pseudothelphusa americana*, as well as the effect of eyestalk ablation on this rhythm.

Material and Methods

Animals

Crabs weighting between 3.5 and 7 g were collected in the river shores and wet areas of Mixquiahuala, Estado de Hidalgo, and transferred to artificial ponds. These animals were allowed to reproduce for the purposes of establishing a colony before being used in the experiments. After transferring to the laboratory, 38 animals were kept in aquaria, fed with meat and vegetables weekly and exposed to natural LD cycles until commencement of activity recording. To perform Eyestalk ablation (ES-X), crabs were first cooled for 10–12 min at 2°C and their eyestalks removed with sharp fine scissors.

Activity recording

During the experiment, crabs were kept individually in an aquarium partially filled with tap water. Each aquarium was equipped with infrared light crossings to detect locomotor activity. Movements were counted in 10-min bins and data stored on a PC via data acquisition board (NAFRI disp. México D.F.). Aquaria were kept in light-tight wooden boxes, maintained at 23 ± 2°C. A fluorescent lamp controlled by a timer provided the light conditions (150 lx, LD 12:12, lights on 07:00 h, lights off 19:00 h).

Experimental protocol

The experiment used two groups of animals: A control group (n = 16), was subjected to constant darkness (DD) for at least 10 days, followed by LD during the next 10 days before being released again into DD. In the second group (n = 22), locomotor activity was recorded in DD for a minimum of 1 week, then ES-X was performed. Locomotor activity was recorded in DD for at least 1 week after lesioning. Afterwards, LD cycles were given for one more week before being released into DD for 7–10 days.

Data analysis

Locomotor activity data were double plotted in conventional actograms using the TAU software program (Minimitter Co., Inc., USA). Periodicity was calculated by means of X^2 periodograms at 0–30-h intervals. Periodograms with values of the ratio (Qp) from the estimate variance of data array (see Sokolove & Bushell, 1978), above a confidence interval ($p < 0.05$) were considered rhythmic. During LD, the average waveform of activity from at least 5 days was used to obtain the maximum activity peaks. In order to evaluate aftereffects, the free running periods obtained from intact and lesioned animals were compared before and after the LD protocols. The average period differences (± SE) from animals either with and without lesions were compared using a paired students t-test, significant differences were considered when $p < 0.05$.

Results

Locomotor activity in *P. americana* displayed bimodal activity with significant circadian periodicity in DD conditions. Figure 1 shows a typical record from an adult crab representing the most characteristic rhythm expressed by animals of this group; corresponding periodograms for each condition are shown. In Figure 1 the rhythm measured by the first peak had a free running period of $\tau = 24.8$ h, and a secondary peak occurred 9.4 h later. The average circadian period (± SE) of 16 animals held in this condition, was $\tau = 23.77 \pm 0.56$ h and an average ultradian period of 13.43 ± 0.78 h was observed. During LD, locomotor activity entrained after 2 cycles of transients. In most of the animals tested a burst of activity appears near lights-on at 7.30 ± 0.3 h, and a second peak was associated with lights-off at $17{:}10 \pm 1.5$ h. A third burst of activity was observed around midnight ($21{:}30 \pm 2.2$ h) in 7 of the animals.

When crabs were released into DD, a phase lock between the peaks associated with the previous lights on and lights off, respectively, was observed. The average free running period obtained in DD before and after the LD conditions was different in all of the intact animals tested; the average ultradian and circadian periodicities observed are shown in Figure 2. Ultradian periodicity becomes more robust in Qp ($\tau = 16.21 \pm 0.87$ h) and the average free running circadian period was longer than that observed prior to placement in LD ($\tau = 25.9 \pm 0.59$ h). Significant differences ($p < 0.05$) are denoted with a star.

Circadian locomotor activity after eyestalk ablation

Figure 3 shows an actogram of a crab, where a clear circadian rhythm is seen in intact conditions (days 1–6) in DD. On the 7th day, when the eyestalks were removed

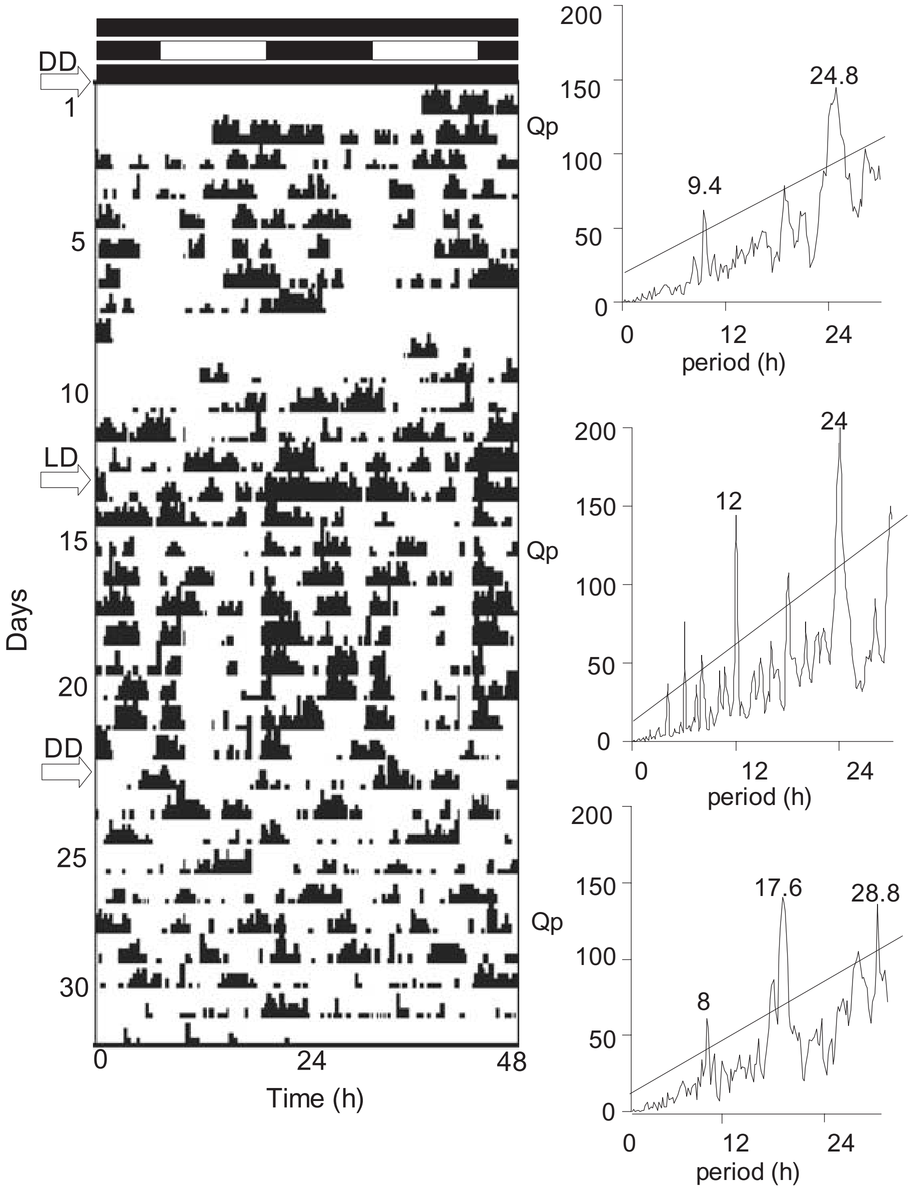

Figure 1. An actogram with a representative example of locomotor activity from *Pseudothelphusa americana* is shown. Upper bars represent the light conditions in which the animal was held, on the right are the corresponding periodograms. Note how the power (Qp) of the ultradian period increases after prior exposure to LD cycles and the circadian period becomes longer.

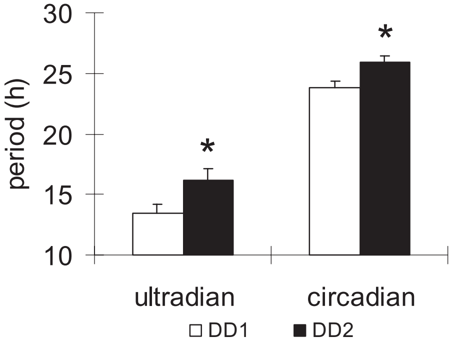

Figure 2. Differences in the average ultradian and circadian period before (white bar) and after (black bar) treatment with LD cycles. Stars denote significant differences within conditions ($p < 0.05$, *t* test).

(ES-X) the circadian rhythm disappears and the amount of locomotor activity notably increases. During exposure to LD cycles a daily rhythm of activity was observed, with a peak in activity observed near noon ($\tau = 24.6$ h). Finally, when the ES-X animal was released into DD conditions (Days 23–28), a statistically significant circadian rhythm is observed ($\tau = 23.4$ h). However, no phase-locking between the rhythm and the previous LD cycle was observed, indicating that masking was occurring during LD.

After eyestalk ablation, only 11 out of 22 animals survived, and entrainment was observed in only 5 of the remaining crabs. Free running rhythms were observed during LD in 4 animals, while 2 displayed arrhythmic behavior. Figure 4 shows an actogram with its corresponding periodogram from a crab without eyestalks. No entrainment was observed during the ten days in LD and two main bouts of activity displayed different free ruinning rhythms ($\tau = 21$, and 27.6 h). After the 11th day, when DD condition started (second arrow) a free running rhythm with $\tau = 27.4$ h was observed. A circadian rhythm of locomotor activity reappeared in the surviving animals approximately three weeks after eyestalk ablation.

The average free-running period of locomotor activity from the ES-X animals was also significantly different ($p < 0.05$) before ($\tau = 24.3 \pm 0.53$ h) and after eyestalk ablation ($\tau = 27.0 \pm 0.75$ h). Ultradian periodicities in ES-X animals, when present ($n = 5$), were not different from intact animals with locomotor activity in ES-X.

Discussion

The freshwater crab *Pseudothelphusa americana* displays a bimodal circadian rhythm of locomotor activity in DD, with free running periods ranging from 23 to 25 h. The large range of τ may be a consequence of the diversity in weight and age of the animals used in this study. Age and body condition have been demonstrated to affect the expression of overt circadian rhythms (Aschoff, 1981). In this work, the bimodal

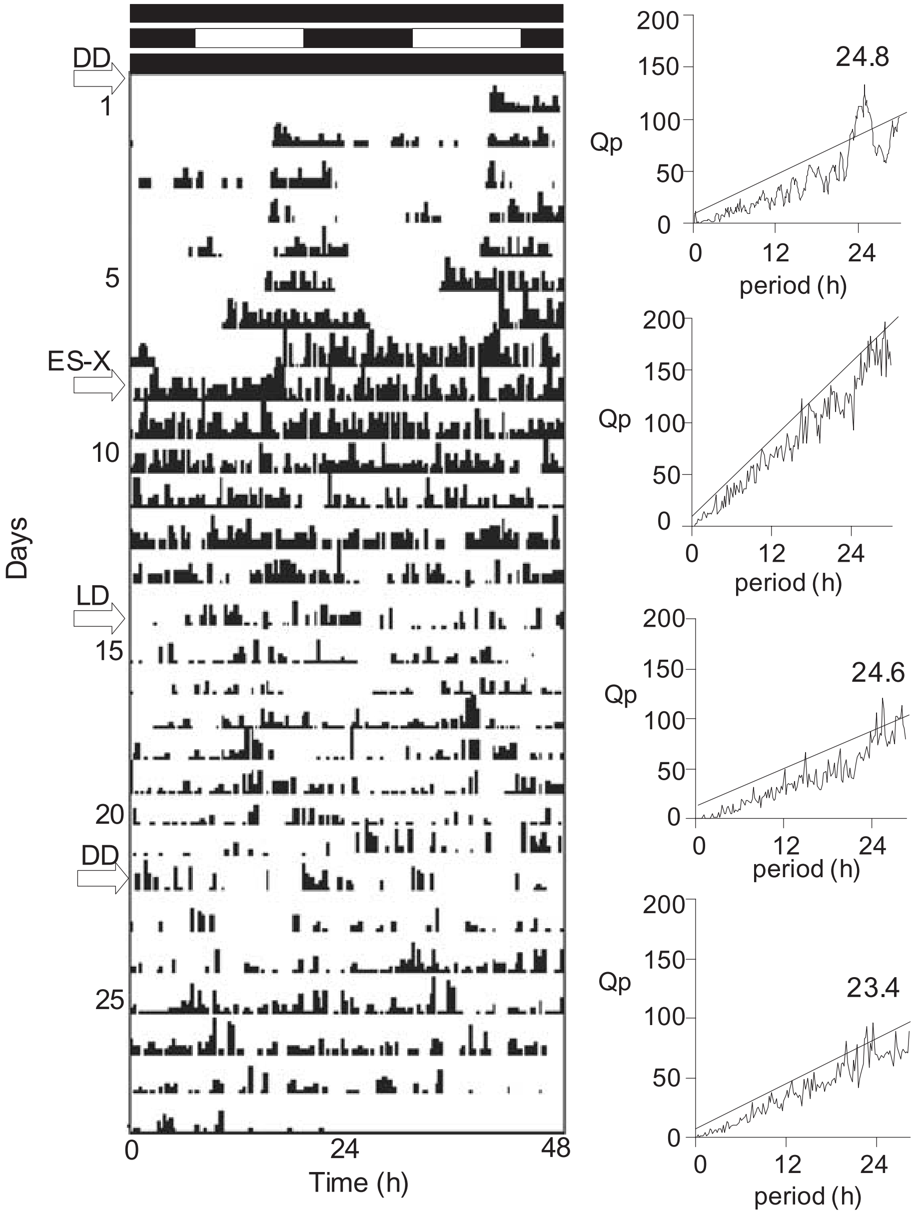

Figure 3. Representative actogram from a crab while intact, and after eyestalk ablation (first arrow, ES-X). After ablation, a circadian rhythm is not observed. LD conditions (second arrow) induced a free running diurnal rhythm (τ = 24.6 h). After placement into DD (third arrow), a weak circadian rhythm was observed (τ = 23.4 h). On the right are the corresponding periodograms for each experimental condition.

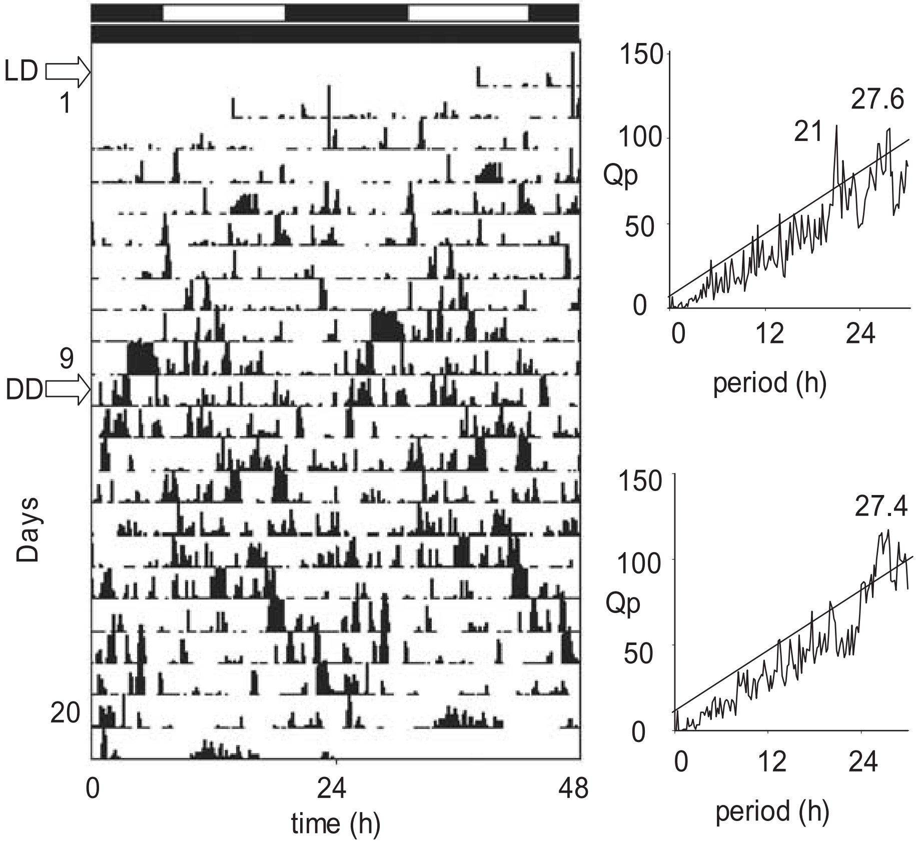

Figure 4. Locomotor activity from a crab without eyestalks. Black bars at the top of the figure represent the darkness and white bars represent the light regime. During LD conditions, a free running rhythm can be seen and two main peaks are present in the periodogram ($\tau = 21$ and 27.6 h), in DD a circadian rhythm is displayed. Periodogram analysis indicates only one main peak $\tau = 27.4$ h).

activity we observed was not consistent with a circatidal rhythm, as has been observed in marine crabs (Honegger, 1976; Neuman, 1981; Palmer, 2000). *Pseudothelphusa americana* is a fresh water crab, and the length of its ultradian periods (13.43 ± 0.78 h) are not typical of a free running tidal rhythm (normally 12.4 h), and these activity peaks can entrain to LD cycles. These bursts of activity were also phase-locked when the animals were released into DD, indicating they are not likely to be masking produced by lights on, as has been observed in crayfish (Page & Larimer, 1975). Moreover, this indicates that activity of *Pseudothelphusa* in the wild would be crepuscular; although no field observations on daily activity have been reported.

In some animals under constant conditions we observed separate bouts of activity with different free running periods (Fig. 1, and additional data not shown). This observation has also been made in other crabs such as *Uca subcilindrica* (Thurman & Broghammer, 2001) and *Helice crassa* (Palmer, 2000) and indicates that the locomotor activity rhythms in some crabs may arise from the output of two or more interacting oscillators. Previous works have discussed whether the oscillators that drive the locomotor rhythms in crabs are circamareal-circalunidean or circadian (Naylor, 1997; Palmer, 1997). In *Pseudothelphusa americana*, a non-tidal animal, there is still a bimodal rhythm, however our data suggest that the oscillator is circadian in nature.

The oscillators responsible for these locomotor rhythms may be strongly influenced by light through one or more circadian photoreceptors. An interesting observation is the difference in the free running period before and after LD conditions, indicating aftereffects from LD. This could arise from lights effects on oscillator coupling, resulting in inconsistent phase relationships when the overt rhythm is released into DD (Pittendrigh & Daan, 1976).

In *Uca subcilindrica*, the appearance of circadian rhythms were not clearly seen when animals were left free running in DD after prior exposure to artificial LD cycles. However after prior exposure to natural LD cycles, most animals displayed overt rhythmicity (Thurman & Broghammer, 2001).

In all of the animals tested, locomotor activity was arrhythmic immediately following ES-X; however, a circadian rhythm reappeared approximately three weeks after lesioning in 11 animals. This indicates that the arrhythmic behavior was likely a consequence of the trauma from surgery rather than the elimination of the oscillator responsible for driving the rhythm in activity. The lack of a circadian rhythm after ES-X might also indicate that the oscillators participating in the circadian regulation of locomotor activity could be mediated by hormonal mechanisms of the eyestalks as suggested in *Carcinus* (Naylor & Williams, 1968). The possible effects of a neurodepressing hormone (Aréchiga & Huberman, 1980) could be coordinated with an oscillator thereby regulating its release and affecting the circadian locomotor activity.

In the present work, we observed animals with circadian rhythm after eyestalk ablation, indicating that the oscillator driving circadian locomotor activity in *P. americana* is located outside the eyestalk, perhaps in the supraesophagic ganglion as has been suggested in other freshwater crabs (Page & Larimer, 1975; Fanjul-Moles, 1998).

The lack of entrainment by some ES-X animals suggests that the primary circadian photoreceptors are located in the retina, and the strength of entrainment by extraretinal photoreceptors is variable. The phase relationship of bimodal activity peaks during LD cycles is different between ES-X and intact animals, indicating that these two components might be the consequence of oscillators located elsewhere, possibly in the supraesophagic ganglion (Barrera-Mera & Block, 1990). The daily activity observed in some ES-X animals is also evidence for the existence of extraretinal photoreceptors mediating entrainment or masking effects. Among the extraretinal photoreceptors described in crustaceans, the most studied are the caudal photoreceptors (Page & Larimer, 1972; for review see Wilkens, 1988) and brain photoreceptors (Sandeman et al., 1990). It would not be surprising that similar structures mediate the entrainment in *Pseudothelphusa americana*.

Although the findings in this work are in agreement with those observed in many other crustaceans, an interesting new observation in *P. americana* is the possible coupling among oscillators controlling the circadian rhythm of locomotor activity. The circadian organization in this crab may be an interesting biological model in which experiments concerning multiple oscillators involved in the regulation of a behavioral circadian expression could be designed.

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