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Shifting phase of circadian locomotor activity by light in the freshwater crab *Pseudothelphusa americana*

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Abstract
The locomotor activity of the crab *Pseudothelphusa americana* displays a circadian rhythm consisting of two main bouts, a morning (lights-on related) and an evening (lights-off related) bout. The aim of the present work is to elucidate whether each bout of activity displays similar phase response shifts when exposed to a 2 h light pulse (150 lux). Phase shifts were evaluated in the free running rhythm under constant darkness from animals previously entrained to a light – dark regimen. Results obtained show that phase shifts are observed for both morning and evening peaks during the subjective day, while during the subjective night, only the evening peak of activity displays significant phase shifts. The aforementioned indicates that a multi-oscillatory system may drive the locomotor activity circadian rhythm in the fresh water crab *Pseudothelphusa americana*, and the differences in PRC shapes for evening and morning peaks could represent the light sensitivity of two different oscillators.

Keywords: *Phase shift, locomotor activity, circadian rhythm, freshwater crab*

Introduction

Entrainment of a circadian rhythm requires resetting the phase of a free running circadian pacemaker, by correcting the difference between the period of the time cue and that of the pacemaker (Pittendrigh, 1981; Johnson, 1992). The phase response of the circadian system allows understanding of how light – dark cycles (as well as other zeitgebers) entrain animals' circadian pacemakers. The phase response curve (PRC) has become an important tool for analyzing circadian organization in animals, since it allows a study of the properties of pacemakers independently of animal's overt rhythms (Moore-Ede et al., 1982).

Few studies of PCR on the locomotor activity of crustacean decapods have been made to date (Viccon-Pale & Fuentes-Pardo, 1994). The freshwater crab *Pseudothelphusa americana* displays a bimodal circadian locomotor activity rhythm. A morning related bout (M) and an evening related bout (E) are observable when animals are held under artificial LD cycles (Miranda-Anaya et al., 2003a). Both components of activity may free run with a

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different period when observed in constant conditions of darkness (DD) and show unstable phase relationships when exposed to constant light. Each bout also responds with a different phase relationship regarding long and short artificial days (Miranda-Anaya et al., 2003b), indicating that M and E bouts might be driven by different circadian oscillators. In order to investigate whether two oscillators drive the M and E bouts with differential sensitivity to light, we explore the phase response to light pulses on the peak of activity for both M and E bouts of the freshwater crab *Pseudothelphusa americana.*

Methods

Animal maintenance and activity recordings

Animals were obtained from Mixquiahuala, Hgo, México and maintained as indicated before (Miranda-Anaya et al., 2003a). After acclimation, crabs were individually held in a glass aquarium equipped with infrared light crossings. Locomotor activity was considered as the number of beam crossings each 10 minutes. Recordings were maintained at 23 ± 2 °C in a controlled environmental room of the Facultad de Ciencias. Light was provided by using a lamp (Phillips, 150 lux) and controlled by means of a domestic timer.

Data were collected onto a PC by means of a data acquisition board (NAFRI S.A. México) and stored until further analysis. Data were analyzed using double-plotted actograms, X^2 periodograms (Sokolove & Bushell, 1978) and average waveforms by means of DISPAC Software (Aguilar-Roblero et al., 1997).

Experimental protocol

Data of locomotor activity from animals held in LD for at least 5 days (lights on 0700 h, lights off 1900 h) were obtained, and a χ^2 periodogram was used to test the rhythm's period. When the best-fitting period was in the range 24 h ± 0.2, recordings could be used to define the peak of activity as a phase reference by using the average waveform (Figure 1 B and C).

A 2 h light pulse (150 lx) was applied during a free-running rhythm shortly after release from entraining conditions, a protocol suggested by Aschoff (1965) and discussed by Johnson (1992). A light pulse was given at different circadian times (CT) during the first day in DD, considering CT12 as the projected time of lights-off from the previous day in LD. A control group consisted of six animals, which did not receive pulses. Phase shifts were estimated by projecting the peak of activity of the M and E bouts, from at least the fourth day after the pulse to the correspondent phase observed in LD (Figure 1A).

Data analysis

Data were plotted separately for M and E peaks. Phase response curves (PRC) were obtained when phase shifts in response to single pulses were plotted as a function of the phase of the circadian system at the time of the pulse (Daan & Aschoff, 2001). Phase transition curves (PTC) were plotted using the new phase (ϕn) after the pulse as a function of the old phase (ϕ) at which the pulse was given ($\phi n = \phi - \Delta\phi$, Daan & Aschoff, 2001; Roenneberg et al., 2003). Phase shifts at different circadian times were compared using Student's *t*-test for independent samples (software Statistica: Jandel Scientific, San Rafael, CA, USA). Significant results were considered when $P < 0.05$.

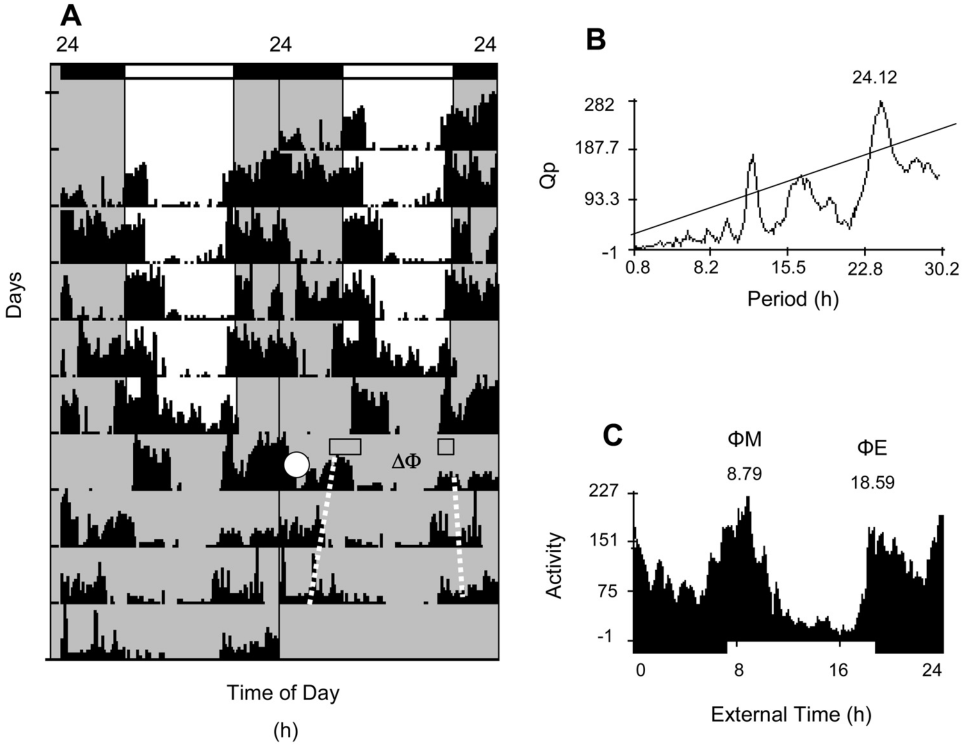

Figure 1. Double-plotted locomotor activity (A) of a crab held in LD (white-gray areas) and then in DD (full gray areas). Phase reference (Φ) for M and E activity bouts in DD are indicated by dotted lines on the right-hand side of the actogram. White circle indicates the 2 h pulse at CT 17, and phase shifting (ΔΦ) is indicated by squares for each activity bout. Periodogram of data in LD (B) and corresponding waveform (C) are shown.

Results

Morning peak

The phase response curve corresponding to the morning peak and its corresponding phase transition curve are shown in Figure 2. A short advance region is observed at the early subjective day (CT3) and delays at CT1 and CT7. No significant differences were observed among pulses given during the subjective night. The corresponding phase transition curve in (b) indicates a type 1 response with short phase shifts. Table I shows the groups with statistical difference (Student's *t*-test, $P < 0.05$).

Evening peak

The evening peak displays a PRC with larger phase shifts compared with the morning peak. Figure 3 presents the PRC (A) and its corresponding PTC (B). Larger phase shifts are also observed during the subjective day (advances at CT 3 and delays at CT 1, 7 and 9). Before the beginning of the subjective night, advances are observed at CT 11, followed by a large region of delays during the early and subjective night. Table II shows statistical difference between CT and phase shifts for the evening peak (Student's *t*-test, $P < 0.05$).

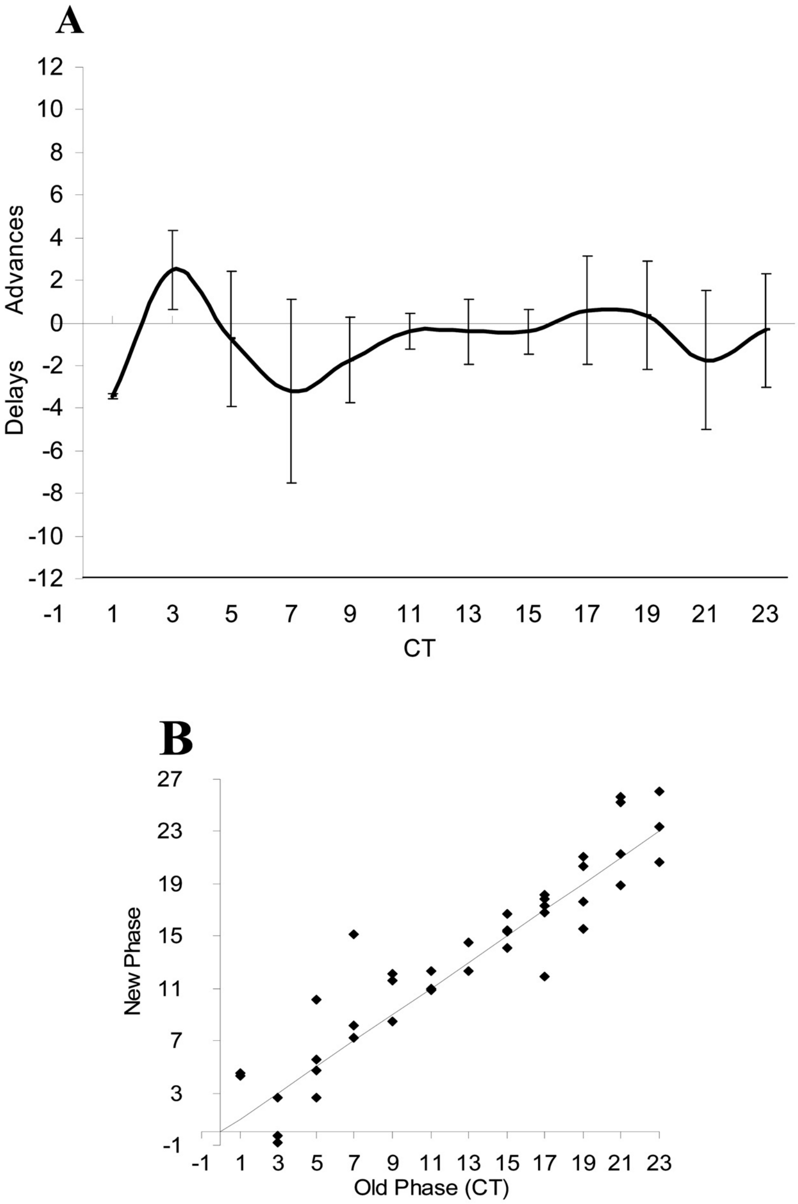

Figure 2. PRC in A, and PTC in B for the morning peak. Phase shifts are larger during the subjective day, while non-significant during the subjective night. For the PRC, values are average ± SE. In B, each value obtained is plotted, indicating a type 1 PRC.

Discussion

The freshwater crab *Pseudothelphusa americana* is not an animal exposed to tides, however, the PRC obtained for the evening peak resembles the phase response of a circatidal oscillator observed in the crustacean *Excorolana* (Enright, 1976). The nature of rhythm regulation,

Table I. Comparison between circadian time hours that show statistically significant phase shifts (Student's *t*-test $P < 0.05$).

	Circadian time			
	1	3	11	15
1		⋆	⋆	⋆
3	⋆			⋆
11	⋆			
13	⋆			
15	⋆	⋆		

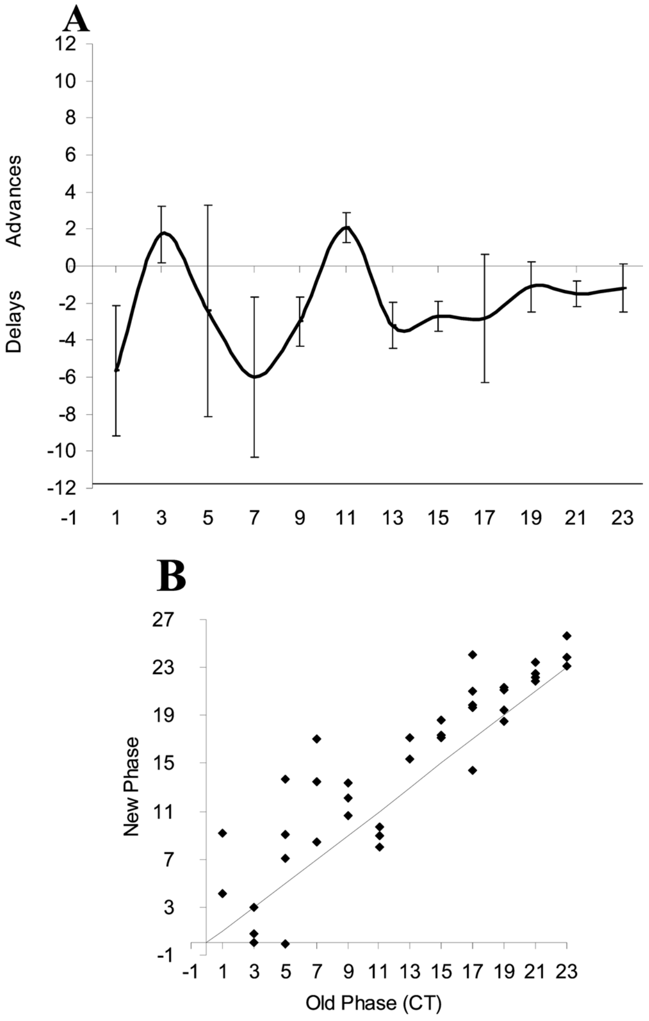

Figure 3. PRC and PTC for the evening peak. Phase shifts are observed in both subjective days and subjective night. PRC values (A) are indicated by the average ± SE. PTC is shown in (B), each value obtained is plotted, indicating a type 1 response.

Table II. The circadian times that show statistically significant phase shifts (Student's t-test $P < 0.05$).

	Circadian time									
	1	3	7	9	11	13	15	19	21	23
1		*			*		*			
3	*		*	*		*	*		*	
7		*			*					
9		*			*					
11	*		*	*		*	*	*	*	*
13	*	*			*					
15		*			*					
19					*					
21		*			*					
23					*					

whether circadian or circatidal, in decapods is still unclear (Naylor, 1996; Palmer, 2000) and the latter seems to depend on the exposure to tides. Previous results indicate that locomotor activity rhythm in *Pseudothelphusa americana* is circadian in nature (Miranda-Anaya et al,. 2003a, 2003b). Bimodal circadian rhythms have also been observed in marine crabs, *Uca subcylindrica,* collected from freshwater environments and unexposed to tidal changes (Thurman, 1998; Thurman & Brohammer, 2001), as do exclusively freshwater decapods (Page & Larimer, 1975; Miranda-Anaya & Fanjul-Moles, 1997, and present study).

A light pulse phase shifts both morning and evening bouts of circadian activity during the subjective day in *Pseudothelphusa americana*. During the subjective night, only the evening peak presents a small area of advances (CT 11) and a large zone of delays (CT 13–23). The difference observed between the PRC for M and E activity bouts indicates that the former is sensitive only during the first half of the subjective day while the latter is also sensitive to light during the subjective night. Differences observed in the evening peak suggest that an oscillator, different from the one driving the morning peak, may control it. Also, the differences in phase shift magnitude indicate that each oscillator has different light sensitivity.

The anatomical location for such oscillators in decapods is still speculative. It has been proposed that least one oscillator is located in each eyestalk and another in the supra-esophageal ganglion of freshwater crayfish (Fuentes-Pardo & Hernández-Falcón, 1993, Page & Larimer, 1975). The freshwater crab *Pseudothelphusa americana* still maintains circadian rhythm after eyestalk ablation, but the rhythm is no longer bimodal as in intact animals (Miranda-Anaya et al., 2003a).

After morning activity, the crab *Pseudothelphusa* hides under sediment or stones and reappears by late evening. During the night, crabs wander on the surface along rivers (Barrera-Mera, unpublished observations); therefore, the animal may not be receiving direct illumination during the day and entrainment may be achieved by non-parametric mechanisms. The sensitivity during the subjective day could be important to maintain a stable phase relationship with the zeitgeber when day length is artificially changed to long days or short days (Miranda-Anaya et al., 2003b). The bimodal locomotor activity may allow the animal to shift from diurnal to nocturnal, according to the daylight in different seasons (unpublished observations), as is observed in other species of crabs (Thurman, 1998). The sensitivity during the subjective night of the evening bout of activity may be related to the variable phase relationship that this bout displays when the day length changes (Miranda-Anaya et al., 2003b). The ecological interpretation of the different PRC in *Pseudothelphusa* *americana* is speculative. Freshwater decapods have evolved adaptations that may be related to reproductive fitness in seasonal environments (Decoursey, 1983), and the difference in light sensitivity of the morning and evening activity bouts may be related to seasonal variation in the circadian rhythm of locomotor activity in this species.

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