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## Circatidal and Circadian Rhythms of Locomotion in *Limulus polyphemus*

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*The nocturnal increases in the sensitivity of the lateral eye of Limulus polyphemus, the species of horseshoe crab found along the Atlantic coast, have been firmly established as being controlled by an endogenous circadian clock (1, 2, 3) located in the brain (4). Virtually nothing is known, however, about the control of the animal's behavioral rhythms of mating and spawning that are observed in the intertidal zone during high tides in late spring (5, 6, 7). Many other marine species, especially intertidal crabs, exhibit similar rhythmic behaviors that have been demonstrated to be under the control of endogenous clocks that are circatidal (8, 9, 10, 11, 12), circadian (10, 12), or both. While there is some evidence that the activity of juvenile horseshoe crabs is primarily nocturnal (13, 14), and possibly controlled by a circadian clock (14), we know of no published work showing that locomotor activity in the adult is endogenously controlled on either a 12.4-h (circatidal) or 24-h (circadian) basis. We report here that locomotor activity in adult individuals of L. polyphemus is endogenously modulated on both a circatidal and a circadian basis and that when the animals are subjected to a light-dark (LD) cycle, most activity occurs at night.*

The locomotor activity of individual adult horseshoe crabs was recorded using activity chambers located in recirculating aquaria. Animals were exposed to three conditions: a 12:12 LD cycle, at 11–14 °C (“fall” conditions, LD<sub>1</sub>), a 14:12 LD cycle, at 17–21 °C (“summer” conditions, LD<sub>2</sub>), and constant darkness (DD). Typical records of the locomotor activity of three horseshoe crabs exposed to these three different photoperiods are presented in Figure 1. Circatidal rhythms were observed in all animals. While significant activity rhythms (15) in the tidal range (12.4 h) were found in only 3 of 6 animals ( $\tau = 12.83 \pm 0.78$  h

[mean  $\pm$  SEM]) during LD<sub>1</sub>, in LD<sub>2</sub>, significant tidal rhythms ( $12.2 \pm 0.1$  h) were observed in all animals. In some cases in LD<sub>2</sub> (4 of 6 animals), clear free-running rhythms were sometimes apparent, (Fig. 1; middle, bottom panels), while in other cases the activity appeared to synchronize to the LD cycles (Fig. 1; top). In DD, circatidal rhythms ( $12.6 \pm 0.2$  h) were found in 5 of 6 animals (Fig. 1; all panels).

Most animals (5 of 6 in LD<sub>1</sub>; 6 of 6 in LD<sub>2</sub>) exhibited significant rhythms in the circadian range ( $\tau = 24.29 \pm 0.14$  h). Periodogram analyses (15) and visual inspection indicated that 5 of the 6 animals tested synchronized their activity to the initial 12:12 LD cycle (LD<sub>1</sub>). The single animal that did not thus synchronize had a very low level of activity. Significantly more activity occurred during the dark phase than the light phase in 4 of 6 animals (Fig. 1; top and bottom [but not middle] panels). The average period ( $\tau$ ) for these animals in the daily (24-h) range in LD<sub>1</sub> was  $24.12 \pm 0.09$  h. Upon subsequent exposure to “summer” conditions (LD<sub>2</sub>), 3 (of 6) animals remained synchronized to the LD cycle (Fig. 1; top panel). In others (2 of 6), this apparent synchronization was not stable (Fig. 1; middle, days 10–18 and days 29–42) and, in still another animal, the synchronization, if any, was unclear (Fig. 1; bottom). Animals that both synchronized and showed a clear onset of activity initiated their activity a significant amount of time ( $1.7 \pm 0.1$  h;  $P < 0.005$ ) before the lights went out during LD<sub>2</sub> but not LD<sub>1</sub> ( $1.1 \pm 0.5$  h;  $P > 0.15$ ). Significantly more activity occurred during D versus L periods in 3 of 6 animals (Fig. 1; top panel only). In constant darkness (DD), all animals also expressed significant circadian rhythms ( $25.27 \pm 0.69$  h; Fig. 1, all panels). In addition, the activity patterns of 3 of 6 animals in DD exhibited evidence of entrainment based on the similarity of phasing with the previous LD cycle (Fig. 1; top, middle). *L. polyphemus* was significantly more active overall during LD<sub>2</sub> than during LD<sub>1</sub> and DD ( $P < 0.03$ ). Neither circatidal ( $P > 0.78$ ) nor

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circadian ( $P > 0.51$ )  $\tau$  values were significantly affected by photoperiod.

Our results provide the first evidence for an endogenous circatidal rhythm in *L. polyphemus*. While field studies (5, 6, 16, 17, 18) and a wealth of anecdotal observations in popular literature have cited the propensity of horseshoe crabs to mate during high tides, this is the first study to demonstrate that an endogenous clock may set the timing for this behavior. Other marine invertebrates, including fiddler crabs and green crabs, have also been shown to possess endogenous circatidal locomotor rhythms that can be entrained by tidally related stimuli (19) such as inundation (20), salinity changes (21), and temperature changes (20). The cues that horseshoe crabs use in their natural habitat to synchronize their mating activities to the natural tidal cycle are currently unknown.

We also provide evidence that the locomotor activity of adult individuals of *L. polyphemus* can be synchronized to an LD cycle, and that these rhythms will persist in constant conditions. This finding is consistent with a previous report showing that juveniles (14) of *L. polyphemus* exhibit circadian rhythms of locomotion. Interestingly, however, whereas 100% of the adult animals in the current study (6 of 6) exhibited significant circadian rhythms in DD, only 40% (2 of 5) of the juvenile animals did (in DD; 14). It is not surprising that *L. polyphemus* exhibits circadian rhythms of locomotion, because circadian modulation of lateral eye visual sensitivity—especially the electroretinogram (ERG)—has been very well documented, and it is clear that horseshoe crabs possess one or more circadian clocks (22, 23, 24, 4, 25, 26, 1, 3). However, we have two reasons to suspect that the timing mechanism that mediates this rhythm of visual sensitivity may be different than the mechanism that controls the timing of locomotion. First, in none of the papers cited above that document circadian control of visual sensitivity is there any mention or indication of a tidal rhythm. Second, although there was clear evidence of rhythmic activity in most of the animals exposed to LD, in several (Fig. 1; middle, bottom panels) the activity was coordinated with the LD cycle only for short periods of time—that is, there was not consistent synchronization or entrainment. This type of modulation of ERG activity also has not been reported in the literature on electroretinograms in *L. polyphemus*.

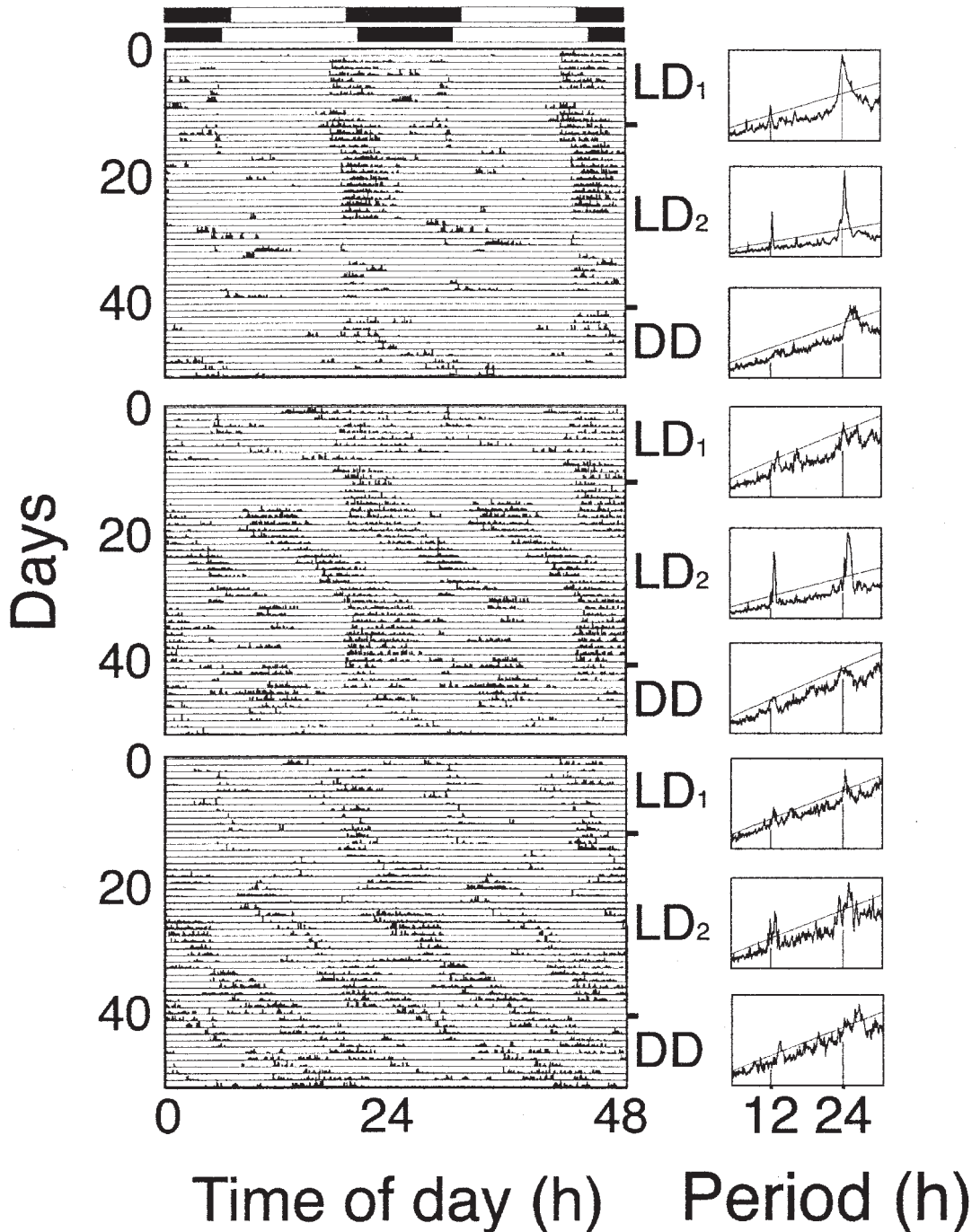
Our results also indicate that under the laboratory conditions to which our animals were exposed, horseshoe crabs are primarily nocturnally active. Of the animals that appeared stably synchronized to the LD cycle (3 of 6), all were significantly more active during the night than during the day. These findings are consistent with previous studies of locomotor activity in larvae (27) and juveniles (13, 14) as well as with the large body of literature demonstrating

greatly increased visual sensitivity at night (3). One field report on a Florida population of *L. polyphemus* also indicates a preference for mating at night (6), but interestingly, other studies in Florida report a preference for diurnal patterns of mating (17). Furthermore, juveniles of *L. polyphemus* in Florida also show a similar preference for diurnal activity in the intertidal zone (5, 16). The issue is further complicated by a study of *L. polyphemus* in Cape Cod, Massachusetts, in which animals were found mating both during the day and at night (7), which is also the pattern in New Hampshire (Watson, unpubl. obs.) where our animals were caught, and in Maine (S. Schaller, pers. comm., Bar Mills Ecological). While the factors associated with the differences in behavior observed between the studies remain to be determined, it is clear from our study that LD cycles do affect both circadian and circatidal rhythms in the laboratory. Several of our animals did not remain completely entrained when exposed to LD (Fig. 1; middle, bottom panels), suggesting that the LD cycle may be a less important stimulus in the temporal organization of locomotor activity than it is for the physiological rhythm of visual sensitivity. While our results do not directly show that the circatidal activity rhythms observed in *L. polyphemus* actually entrain to LD cycles, the expression of the timing of these rhythms appears to at least be modulated by the LD cycle. Similar modulating effects of LD on circatidal locomotor rhythms have been observed in some species of crabs (19) but not in others (28).

Our results also show that *L. polyphemus* can exhibit clear circatidal rhythms in locomotion in the laboratory, even during nonbreeding times of years (November–February). In the field, the appearance of millions of horseshoe crabs along the eastern seaboard, primarily during April through July, clearly indicates a strong seasonal preference for mating. *L. polyphemus* has been reported to be much less active at other times of the year (18) and, in winter, either to burrow in the mud in or around estuaries or to move to the open ocean where it has been found far out on the continental shelf (29). While annual changes in the timing of behavior (that are often strongly influenced by photoperiod) have been well documented in a wide variety of species (30), we are surprised that circatidal rhythms were expressed during this time of year. Whether horseshoe crabs normally express tidal rhythms throughout the year, but these are not observed, or whether the rhythms we observed were initiated by the longer photoperiod or the increased temperature, remains to be elucidated.

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**Figure 1.** Locomotor activity of 3 (of the 6 tested) individuals of *Limulus polyphemus* housed in activity chambers (39 cm wide  $\times$  32 cm long  $\times$  9-cm deep) in recirculating aquaria (left panels). Data are double-plotted to facilitate visual inspection. Periodograms corresponding to the respective photoperiods and animals are presented in the right panels. Animals were exposed to three consecutive photoperiods. (1) LD<sub>1</sub> = 12:12 (light source: 20-W broad-spectrum fluorescent bulbs, Simkar Corp., Pittsburgh, PA; light intensity during L = 150 lux/2.8  $\mu$ mol and during D = 0 lux/0  $\mu$ mol), with water temperature = 11–14 °C. These conditions simulated fall temperature and photoperiod, during which *L. polyphemus* may be expected not to exhibit tidal rhythms); (2) LD<sub>2</sub> = 14:10; temperature = 17–21 °C. These conditions simulated summer conditions during which *L. polyphemus* would be expected to exhibit tidal rhythms. (3) DD = constant darkness; temperature = 17–21 °C. These conditions were employed to determine whether the rhythms observed in LD would persist in DD. Water was collected at the Jackson Estuarine Laboratory (Durham, NH), and the salinity was kept between 25‰ and 27‰. Although temperature varied either between 17 and 21 °C or 11 and 14 °C, neither temperature nor salinity cycled with periods near 12 or 24 h (HOBO data loggers, Onset, Pocasset, MA). In October 2002, horseshoe crabs (2 females, weighing 519 g and 676 g; 4 males, weighing 197–310 g) were caught in lobster traps in Great Bay, New Hampshire, an area that experiences semidiurnal tides. Immediately after the crabs were brought to the laboratory in Plymouth, New Hampshire, magnets were affixed to their dorsal carapace, between the lateral eyes, using cyanoacrylate glue and duct tape. Magnetic reed



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switches (one per activity chamber) that produced a voltage change whenever a crab passed under one were used to monitor activity. The animals were not fed after being caught and were always individually housed in activity chambers. A “ceiling” (9-cm high) was used to prevent the animals from flipping over and becoming immobilized. In addition, three bricks were placed on the ceiling to weigh it down and to create a shielded, darker area over about half of each activity chamber to provide a shelter. Activity was recorded on a CPU-based data collection system and analyzed using the ClockLab suite of programs for analysis of time-series data (Actimetrics, Evanston, IL). Significance of rhythmicity was determined both visually and by chi-square periodogram analysis ( $P < 0.01$ ; 15). The period ( $\tau$ ) in the circadian range for each individual during each experiment was determined by recording the highest significant peak on the periodogram between 22 and 26 h or for circatidal rhythms, between 10 and 14 h. Entrainment to LD cycles was ascertained by visually comparing the onsets of activity during the last several days in LD<sub>2</sub> to the onsets during the first several days in DD. Phase angles were determined by comparing the difference between the onset of the LD cycles and the onset of activity for each animal (as determined by best-drawn eye-fit lines). To determine a preference for activity during L (diurnality) or D (nocturnality), the amount of activity during L and D for each day for each animal was summed. Paired Student’s *t*-test or repeated measures ANOVA ( $P < 0.05$ ; Statview, ver. 4.51, Abacus Concepts, Berkeley, CA) was used to determine statistical significance between means.