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Rhythms of Locomotion Expressed by *Limulus polyphemus*, the American Horseshoe Crab: I. Synchronization by Artificial Tides

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Abstract. *Limulus polyphemus*, the American horseshoe crab, has an endogenous clock that drives circatidal rhythms of locomotor activity. In this study, we examined the ability of artificial tides to entrain the locomotor rhythms of *Limulus* in the laboratory. In experiments one and two, the activity of 16 individuals of *L. polyphemus* was monitored with activity boxes and “running wheels.” When the crabs were exposed to artificial tides created by changes in water depth, circatidal rhythms were observed in animals exposed to 12.4-h “tidal” cycles of either water depth changes (8 of 8 animals) or inundation (7 of 8 animals). In experiment three, an additional 8 animals were exposed to water depth changes under cyclic conditions of light and dark and then monitored for 10 days with no imposed artificial tides. Most animals (5) clearly synchronized their activity to the imposed artificial tidal cycles, and 3 of these animals showed clear evidence of entrainment after the artificial tides were terminated. Overall, these results demonstrate that the endogenous tidal clock that influences locomotion in *Limulus* can be entrained by imposed artificial tides. In the laboratory, these tidal cues override the influence of light/dark cycles. In their natural habitat, where both tidal and photoperiod inputs are typically always present, their activity rhythms are likely to be much more complex.

Introduction

Adult American horseshoe crabs, *Limulus polyphemus*, migrate into the intertidal zone along the eastern coast of North America in the late spring-early summer to attempt to

mate (Rudloe, 1979; Brockmann, 2003). Spawning appears to be triggered by both elevated temperatures and photoperiod (Cohen and Brockmann, 1983; Shuster and Botton, 1985; Barlow *et al.*, 1986; Penn and Brockmann, 1994) as well as by rising water levels (Ehlinger *et al.*, 2003). During this time, breeding activity is synchronized to high tides, with horseshoe crabs moving into mating areas 1–2 h before high tide and returning to deeper waters about 2 h after high tide (Barlow *et al.*, 1986; Penn and Brockmann, 1994). This tidal pattern of activity appears to be further modulated by a general preference for the highest high tide (Barlow *et al.*, 1986), which may explain why in some areas most mating occurs around the high tides associated with the new and full moons (Rudloe, 1980; Smith *et al.*, 2002).

Many other animals also synchronize various behaviors with tidal cycles. In some cases, environmental factors that may serve to synchronize the animal’s activity to the tidal cycle have been identified, and in general, they are species-specific (DeCoursey, 1983; Naylor and Williams, 1984). Inundation cycles (Williams and Naylor, 1969), hydrostatic pressure changes (Naylor and Atkinson, 1972), as well as 12.4-h cycles of increased or decreased temperatures and salinities (Reid and Naylor, 1990) are sufficient to entrain the locomotor rhythms of *Carcinus maenas*, the green crab. Hydrostatic pressure changes also synchronize the behavioral activity rhythms of the portunid crabs *Liocarcinus holsatus* and *Liocarcinus depurator* (Abelló *et al.*, 1991), as well as at least two amphipod species—*Nymphon gracile* (Morgan *et al.*, 1964) and *Excirologa chiltoni* (Enright, 1965). Salinity changes appear to be effective synchronizing agents for the crabs *Rhithropanopeus harrissii* (Forward *et al.*, 1986) and *C. maenas* (Reid and Naylor, 1990). Finally, periodic agitation is sufficient to entrain tidal rhythms in two species of isopods (Klapow, 1972; Hastings,

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1981) and in juvenile horseshoe crabs (Ehlinger and Tankersley, 2006). Although there are many field reports of tidally synchronized beach approaches by male and female *Limulus* during the late spring mating season, we know of no studies that have systematically investigated the cues they may use to synchronize their movements to natural tide cycles.

Periodic environmental signals can synchronize behavioral rhythms in many intertidal species (Palmer, 1995), and many of these species have been shown to have endogenous tidal clocks. Fiddler crabs have clear endogenous circatidal rhythms that free-run (the rhythms persist in constant conditions, with periods close to 12.4 h) in constant conditions (Bennett *et al.*, 1957; Palmer, 1963; Lehman, 1975). *C. maenas* also exhibits a circatidal locomotor activity rhythm, with peaks at the time of high tide (Naylor, 1958). *Uca crenulata*, the California fiddler crab, also has an endogenous rhythm that may be synchronized by tidal cues (Honegger, 1973). Importantly, recent studies found that horseshoe crabs express clear circatidal rhythms under non-tidal laboratory conditions during the summer, fall, and winter (Chabot *et al.*, 2004, 2007). A major goal of this study was to determine whether cycles of water depth changes (artificial tides) entrain these rhythms.

Although *Limulus* mating activity is clearly synchronized to high tides, time of day apparently plays a role as well. The large body of evidence that *Limulus* eyes are more sensitive to light at night (Barlow, 1983) suggests that they might prefer to be most active at night. This view is supported by Rudloe's (1980) finding of nocturnal peaks of mating activity in Apalachee Bay, Florida. However, other field data indicate that nocturnal peaks of activity are not the rule. For example, a study done on Seashore Key, Florida (<100 miles from Apalachee Bay) showed that peaks of activity occurred primarily during the diurnal high tide (Cohen and Brockmann, 1983). Increased activity during the day has also been seen in juvenile *Limulus* in the field (Rudloe, 1978, 1979), and *Limulus* actively breeds on some Massachusetts beaches during day and night high tides (Barlow *et al.*, 1986). Our own observations of *Limulus* mating in the Great Bay estuary, New Hampshire, support the view that mating occurs during both daytime and nighttime high tides (unpubl. data), and our laboratory studies (Chabot *et al.*, 2007) indicate that some animals prefer moving at night (12%), some prefer moving during the day (36%), and the remainder show no preference (52%).

Here we report clear synchronizing effects of simulated tidal cycles on activity patterns. In addition, although most of the animals exposed to light/dark (LD) cycles alone express 24-h patterns of activity, the tidal cues dominated when both LD and simulated tidal cycles were present, and tidal patterns of activity were observed in nearly all animals.

Materials and Methods

Animals and environmental conditions

Adult (200–335 g) male horseshoe crabs, *Limulus polyphemus* (Linnaeus), were collected from the beach during the middle of the mating season (mid-June) at the University of New Hampshire Jackson Estuarine Laboratory at Adams Point, Durham, New Hampshire. The collection site was a rocky shoreline with an abundance of gravel and hard-packed mud. Animals were collected at high tide while they were mating or seeking mates near the shoreline. After being collected, the animals were placed in a cooler and quickly (within 2 h) transported to Plymouth State University, Plymouth, New Hampshire, where they were placed in either “running wheels” (Experiment 1) or activity-monitoring “squares” (Experiments 2 and 3) in a laboratory setting. A previous study using *Limulus* exposed to light/dark (LD) and dark/dark (DD) conditions demonstrated that these two devices yield comparable activity data in the laboratory (Chabot *et al.*, 2007). LD conditions and temperature were monitored using Hobo data loggers (Onset Corporation, Pocasset, MA). Light levels were also measured using an Extech 401036 light meter (Waltham, MA) or a LunaPro light meter (Gossen, Germany). The animals were not fed during the experiments (50–75 days).

Experiment 1: Artificial light/dark and tidal cycles (water depth changes)

To measure activity, 8 animals were placed in running wheels in 110-liter recirculating tanks (three animals/tank; each tank was 50 cm × 95 cm, and 45 cm deep) located in a room controlled for temperature (17 ± 1 °C) and light. Practical salinity was kept at 26 ± 3 by the addition of distilled water and monitored using a DeepSix handheld hydrometer (Coralife). The pH was maintained between 7.8 and 8.2 by weekly monitoring and addition of Sea Buffer (Aquarium Systems, Inc., Mentor, OH) as needed. Nitrate levels were always below 100 mg/l. Lighting was provided by a single 40-W fluorescent bulb (Coralife 10,000K) suspended above the tanks. During the simulated daytime, the light intensity at water level was 100–150 lux (lumens/m²; ~ 0.12 – 0.9 $\mu\text{mol}/\text{m}^2$); at night and during DD, it was 0 lux. The lights were on a 24-h timer that provided a 14:10 LD photoperiod, and the L to D and D to L transitions were instantaneous.

Running wheels were made from the bottom 12 cm of two 20-liter buckets. These were connected to each other by a fixed piece of 2-inch-diameter PVC in such a way as to create a 2-cm slot between the two halves. This slot allowed the animal's tail to extend outside the wheel. The animals were confined to this wheel for the duration of the experiments. Two bar magnets mounted on the outside wall of the running wheels and a magnetic reed switch mounted on the

running wheel frame allowed for wheel rotations to be detected and recorded with a data collection system. For further details, see Chabot *et al.* (2007) and figure 1 in Watson *et al.* (2008).

Tidal cycles were established using water pumps controlled by timers. Water flow rates and timing were adjusted so that every 12.4 h each animal was exposed to a “high tide” that alternated with “low tides”. The depth of water above the carapace of the animal during high tide was 23 cm; during low tide it was about 3 cm. The timing of the pumps was adjusted to produce about 4 h of rising (or falling) water levels followed by 2.2 h of constant water levels. After 18–22 days, the period of the “tides” was adjusted to 12.1 h in order to determine whether the activity would “follow” the new period. Animals were still exposed to LD cycles during this time. When the LD and simulated tidal cycles were terminated, the water levels of the tanks were kept at the average between low and high tide by interrupting the last tidal cycle at the approximate midpoint (mean approximate depth above carapace was 13 cm). Animals were allowed to “free-run” under atidal and DD conditions for 21 days.

Experiment 2: Natural photoperiods and artificial tidal cycles (inundation)

The primary purpose of the second experiment was to compare the effects of artificial LD cycles (Experiment 1) to natural photoperiods. In addition, we also wanted to test tidal cycles of changes in water depth (Experiment 1) and in inundation (during which animals were allowed to become partially exposed at low tide). Eight animals were held in recirculating tanks in a greenhouse located on the roof of a three-story building where they were exposed to natural LD cycles. Ambient lighting intensities during the day ranged from 1,000 to 60,000 lux; during the night the range was from 4 to 90 lux. Each animal was placed in an individual enclosure (“square”) in one of two recirculating tanks (Jewel Industries Inc., Chicago, IL, Model Oceanic-55). The water used was either seawater collected from near the Jackson Estuarine Laboratory or seawater of the same salinity prepared using distilled water and salts (Natural Sea Salts, Oceanic Systems, Dallas, TX).

Four activity-monitoring “squares” (40 cm × 30 cm; Chabot *et al.*, 2007) were created within each aquarium (80 cm × 60 cm) by using plastic lighting grating (1 cm × 1 cm eggshell grating). A “ceiling” (9 cm high) was used to prevent the animals from flipping over and becoming stuck. Three bricks were placed on the ceiling to hold it in place and create a shielded, darker area over roughly half of each activity chamber. Magnets were attached, using duct tape and cyanoacrylate glue, to the dorsal carapace of each animal. This made their movements detectable when they moved near a reed switch that was located on the side of the

square not covered by bricks. For further details, see Chabot *et al.* (2007).

During the first phase of this experiment, the animals were placed in the greenhouse and exposed to the natural photic environment for 48 days. Because the animals were kept in a greenhouse that heated significantly during the day and cooled significantly at night, the water temperature in these tanks also varied significantly, between 10 and 27 °C. Because of these fluctuating temperatures, during the next phase of the study (when the crabs might be exposed to higher temperatures because water could not be chilled when low water levels were to be effected) the tanks were moved to an adjacent greenhouse preparatory room against a large glass window. Under these conditions, temperatures continued to fluctuate with similar, though reduced, amplitudes, and because the southern and western walls of this room were mostly glass, natural photic conditions were still available (light levels were attenuated <10%). Soon after the animals were moved to the preparatory room, they were exposed to 12.4-h inundation cycles by pumping water between the two aquaria as described above. During this time, the water depth in the tanks varied systematically between 2 cm (“low tide”) and 28 cm (“high tide”), and when the water was low, a portion of the dorsal carapace was exposed to air.

Experiment 3: Entrainment of tidal rhythms to water depth changes

The purpose of this experiment was to examine the effect of cycles of water level on locomotor activity and to demonstrate entrainment. Animals were collected on mating beaches and treated in much the same manner as in Experiment 1. They were exposed to “instant-on, instant-off” 14:10 LD cycles throughout this experiment and to simulated high and low tides that varied by 0.5 m for about 30 days. In a change from Experiment 1, 4 cm of sand was placed in the bottom of each activity box to attempt to obtain better “free-runs” when the tides were stopped. In addition, animals were allowed to free-run in LD, under atidal conditions, for about 20 days after 30 days of exposure to artificial tides.

Data analysis

Activity data were collected in 5-min intervals on a computer-based data collection system and analyzed using the ClockLab suite of time-series data analysis programs (Actimetrics, Evanston, IL). Significance of rhythmicity was determined both visually (Chabot and Menaker, 1992; Chabot *et al.*, 2004) and by Lomb-Scargle periodogram analysis ($P < 0.05$). To reduce the occurrence of artificial harmonics created by the major periodicities of the data, we used the Lomb-Scargle method (Ruf, 1999) instead of the more traditional chi-square method (Sokolove and Bushell,

1975); the periods calculated by the two procedures are virtually the same (Chabot *et al.*, 2007). This analysis was used to determine the maximal value of any primary component of rhythmicity in the circadian (between 22 and 26 h) or circatidal (between 10.4 and 14.4 h) range for each animal during each experimental condition. With the exception of DD, at least 10 days of data were used to calculate Lomb-Scargle periodograms. Less than 10 days were used in DD because the visual clarity of rhythmicity often deteriorates within a week in DD in *Limulus* (Chabot *et al.*, 2007) and other intertidal animals (Palmer, 1995). To determine the phase angle of activity to environmental cues (LD or water depth changes), best eye-fit lines were drawn, where possible, through the onsets of activity, using a single-blind protocol. The same method was used to determine alpha, the length of time of the main bout of activity. We also examined all of our data using the “onset” and “offset” plots in ClockLab (Actimetrics) and compared them to our best-fit lines. They were virtually the same as long as we extrapolated between missing onsets and offsets.

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*-tests ($P < 0.05$; *Statview* ver. 4.51, Abacus Concepts, Berkeley, CA) were used to determine statistical significance between means.

Results

Experiment 1: Effects of photoperiod and tidal cues (water depth changes)

Locomotor activity records of two animals first exposed to photoperiodic light/dark (LD) cycles and subsequently to tidal cues are presented in Figure 1. In LD, both the activity records (left panels) and the periodogram analyses (right panels) indicate rhythms in the 24-h range. This pattern was seen in 6 of 8 animals ($\tau = 24.3 \pm 0.2$ h; Mean \pm SEM), while primary periodicities in the 12-h range were observed in the remaining 2 animals (data not shown; $\tau = 11.9 \pm 0.1$ h). Two of 8 animals exhibited significantly more activity during L than D (Fig. 1; top; $P < 0.05$), while in the remaining 6 animals the activity levels exhibited during L and D were not statistically distinguishable (Fig. 1, bottom panel). When the length of the main bout of activity (alpha) was consistent for all days in LD and could be measured ($n = 2$), both animals were active essentially all of the time during L when exposed to LD (alpha = 14 h for both; data not shown). Others exhibited a high degree of variability in alpha ($n = 3$). In these animals, alpha was also generally 14 h when it could be measured. For instance in Figure 1, alpha was 14 h for the first several days and then was very inconsistent until the last 3–4 days. In these 5 animals, when alpha could be measured, activity always began at lights on (phase angle = 0 ± 0.0 h). Alpha could not be

determined in the remaining animals ($n = 3$; Fig. 1, bottom).

When exposed to tidal cues and LD, the patterns of activity became organized around rhythms in the 12.4-h range in all (8/8) animals (Fig. 1). Both visual inspection and periodogram analysis clearly indicate the emergence of a 12.4-h rhythm ($\tau = 12.5 \pm 0.1$ h). The activity patterns of most of the animals (6 of 8; Fig. 1, both panels) became organized around the water depth changes within 2 days (four tidal cycles), while 2 animals took 14 tidal cycles (data not shown). When the period of tidal cues was shortened to 12.1 h, 7/8 animals continued to exhibit significant periodicity, but at significantly shortened tau values (12.1 ± 0.3 h; $t(6) = 4.43$, $P < 0.005$). The phase angle of entrainment was about 2 h before peak high tides whether the animals were exposed to the 12.4-h (1.9 ± 0.4 h) or 12.1-h (2.1 ± 0.5 h) tidal cycles. At these times, water levels were at about 50% of maximum “high tide” levels. The length of the main bout of activity (alpha) was similar when the animals were exposed to either 12.4- (6.2 ± 0.8 h) or 12.1-h (6.8 ± 0.3 h) tidal cycles. Neither phase angles ($t(2) = -0.05$, $P = 0.96$) nor alphas ($t(2) = 2.0$, $P = 0.18$) differed significantly when the animals were exposed to either 12.4- or 12.1-h tidal cues.

Five of the animals exposed first to LD and then LD and artificial tides were subsequently exposed to constant conditions (DD and constant water depth). Although statistically significant circatidal rhythmicity persisted in all of these animals ($\tau = 12.7 \pm 0.2$ h), only 3 were clearly visually tidally rhythmic (Fig. 1, bottom panel). Since the free-running rhythms could not be judged to be clearly in phase with the previous synchronized activity patterns, entrainment to the imposed tidal cycles was not clearly demonstrated in these animals.

There were clear effects of “lights on” and “lights off” on locomotor activity. Most animals (7/8) immediately increased activity in response to lights on (Fig. 1, top). When they were subsequently exposed to tidal cues, only 2 of the 8 animals showed these acute effects of photoperiod change, but even in these animals the effects were much less noticeable during exposure to tidal cues (data not shown).

Experiment 2: Effects of natural photoperiod and tidal cues (inundation)

Locomotor activity records of 2 animals exposed to a natural photoperiod and subsequently to a natural photoperiod and tidal cues are presented in Figure 2. Under these conditions, most animals (5 of 8) showed significantly more activity during L versus D ($P < 0.05$; Fig. 2); for the remaining 3 animals, activity levels in L and D could not be statistically distinguished (data not shown). In a natural photoperiod, both the activity records (left panel) and the periodogram analyses (right panel, top) indicate a rhythm in

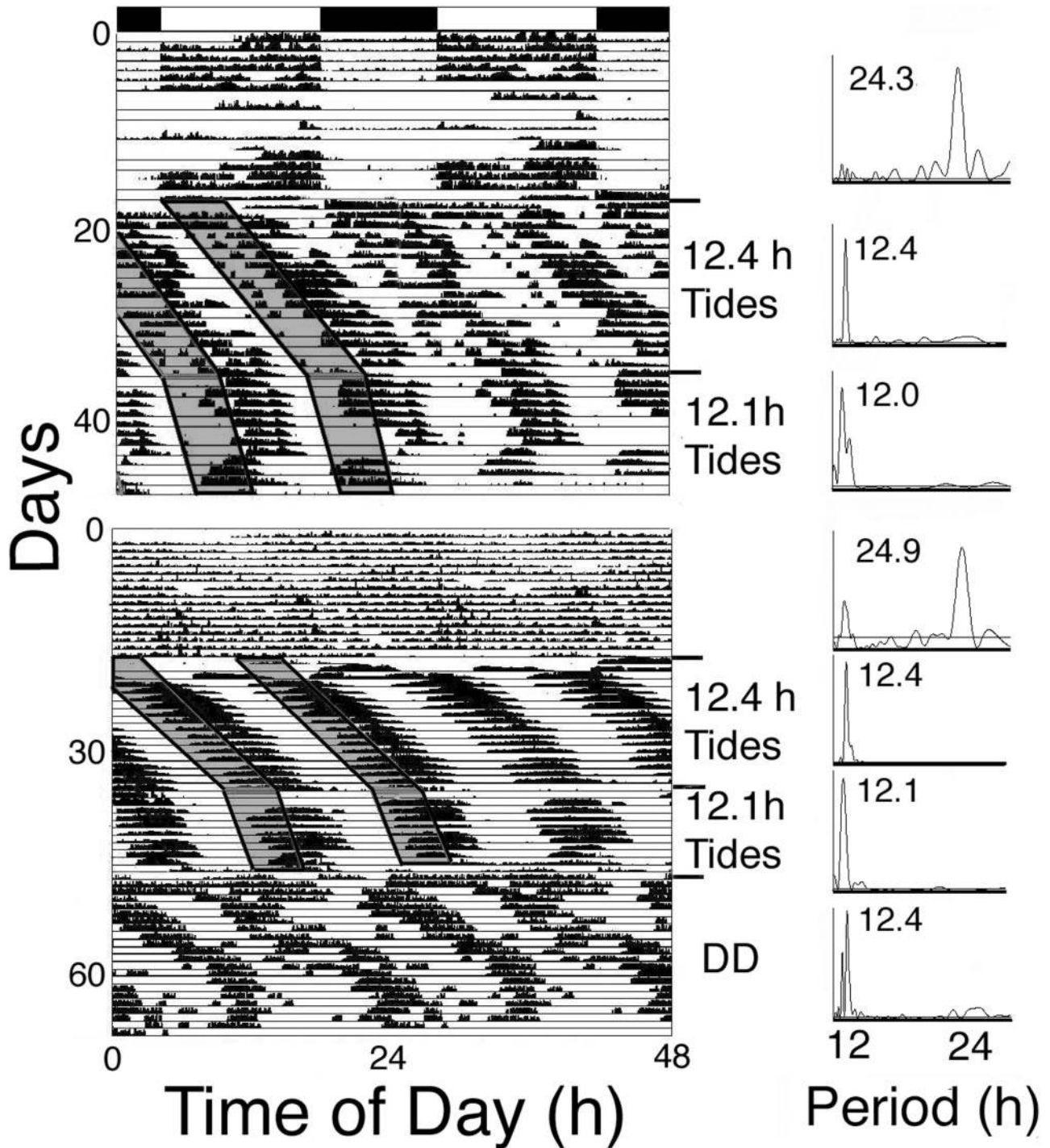


Figure 1. The effects of photoperiod and water depth on locomotor activity of two individuals of *Limulus polyphemus*. Left panel: actograms double-plotted to improve visualization of the patterns. The 14:10 light/dark cycle (LD) is indicated by black/white bars at the top. Artificial tides (changes in water depth) were delivered at periods of 12.4 h and 12.1 h, as indicated by shaded boxes on the actogram. Water depth began to increase at the time indicated by the left side of the boxes, and maximum depths occurred at the time indicated by the right side of the boxes. Animals were first exposed to LD and not tides, then LD and tides of two different periods, and finally (bottom actogram only) no tides (constant water depth) and constant darkness (DD). Right panels: Lomb-Scargle periodogram analyses of respective actogram sections; vertical scale is relative $Q(p)$. Largest peak value above horizontal line of significance ($P < 0.01$) indicated by numerical value.

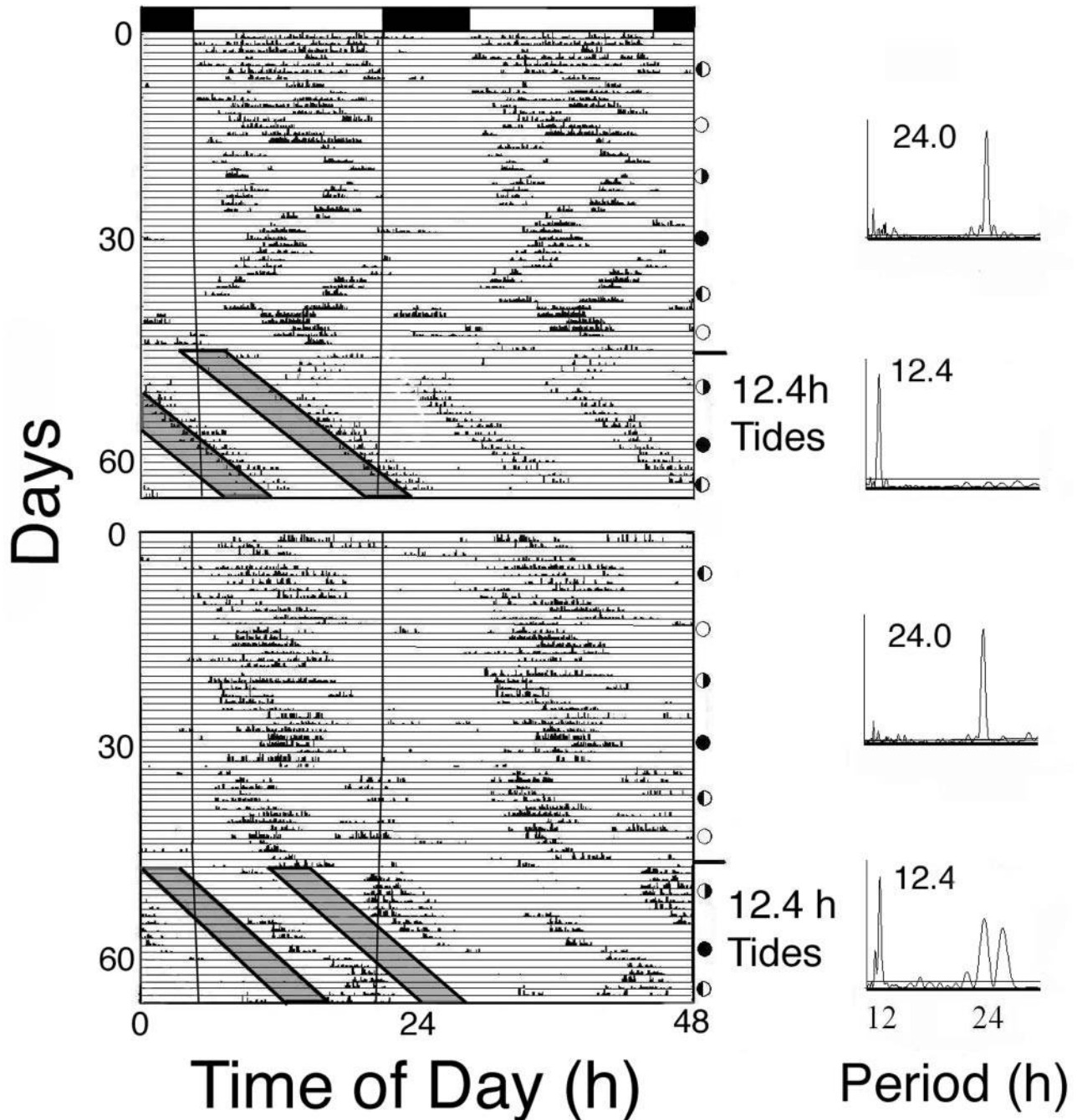


Figure 2. The effects of natural photoperiod cues and inundation cycles on the locomotor activity of two individuals of *Limulus polyphemus*. Approximate photoperiod indicated by black/white bars at top of actograms. Actual sunrises and sunsets are plotted as vertical curvilinear lines. Moon phase is plotted to the right of the actograms. Timing of artificial tides is indicated, as described in legend for Fig. 1, by shaded bars.

the 24-h range. This pattern was seen in 6 of 8 animals ($\tau = 24.1 \pm 0.2$ h; Fig 2). In these animals, there was occasional visual evidence of rhythmic activity on a cycle of about 12.4 h (Fig. 2, last 1–3 weeks of photoperiod exposure only). Significant rhythms in the 12.4-h range were observed in the remaining 2 of 8 animals (data not shown;

$\tau = 12.0 \pm 0.1$ h). When the length of the main bout of activity could be determined ($n = 4$), the animals were active for much of the time during L when exposed to LD ($\alpha = 12.5 \pm 3.5$ h; total day length = 15.5 h). This alpha was statistically indistinguishable ($t(9) = 0.82$, $P > 0.43$) from the mean alpha from Experiment 1 (where ani-

imals were exposed to an artificial instant on/off 14:10 photoperiod). Unlike the animals in the artificial LD cycles, these animals generally began to move about 1 h after sunrise (phase angle = -1.2 ± 0.2 h). There was a significant difference between the phase angles of entrainment to the photoperiod of the animals in the artificial LD cycle versus those in the natural photoperiod ($t(9) = -6.5$, $P < 0.0001$). Although animals were exposed to the night sky during D, there were no obvious effects of lunar phase on either amounts or patterns of activity (Fig. 2).

When exposed to tidal cues and the natural LD cycle, the patterns of activity became organized around rhythms in the 12.4-h range in most animals (Fig. 2). Both visual inspection and periodogram analysis clearly indicate the emergence of a 12.4-h rhythm in 7 of 8 animals (Fig. 2; $\tau = 12.4 \pm 0.1$ h). Although the remaining animal did exhibit a clear statistical peak in the 12.4-h range ($\tau = 12.45$ h), a significant peak in the 24-h range was larger ($\tau = 24.8$). The activity patterns of most of the animals that were clearly synchronized to the water depth changes (6 of 7; Fig. 2) became organized around those changes between two and six tidal cycles after the tidal cycles were initiated (one animal took 16 tidal cycles; data not shown). The phase angle of entrainment for these animals was about 1 h after peak high tides (-1.2 ± 0.5 h), which was significantly different than the phase angles of entrainment to the tidal cycles of the animals in Experiment 1 ($t(14) = -4.8$, $P < 0.0004$). It is not clear whether this was due to cycles of artificial versus natural light or to inundation versus changes in water depth. The duration of the main bout of activity was 5.0 ± 0.6 h and was statistically indistinguishable from the mean alpha from Experiment 1 ($t(9) = 0.82$, $P > 0.43$). Again, during this portion of Experiment 2, lunar phase had no obvious effect on either amounts or patterns of activity (Fig. 2).

Experiment 3: Water depth changes and free-runs

When a third group of animals was exposed to water depth changes in LD and then allowed to free-run in the absence of tides, almost half (3 of 8) showed evidence of entrainment (Fig. 3), while an additional 4 animals exhibited some weaker evidence of entrainment (Fig. 4). The activity of most of the animals (5 of 8) appeared to synchronize to the imposed water level changes delivered on a 12.4-h cycle (Fig. 3), while the remaining animals showed evidence of partial synchronization (at least one bout of activity was synchronized for at least 10 days) to the given cues (Fig. 4).

Discussion

Here we present the first evidence that the circatidal activity rhythms in *Limulus polyphemus* can be synchronized and entrained by tidal cues. Most animals in this study

(20 of 24) exhibited clear tidal rhythms when exposed to artificial "tides" (Figs. 1–3), whether these consisted of cycles of depth or inundation. In most cases, the activity appeared to be synchronized with stable phase angles, and in Experiment 1, when the period of the imposed cycle was shortened, the resultant activity period shortened commensurately and significantly (Fig. 1). Finally and crucially, in Experiment 3, when the artificial tides were stopped, 3 of 8 animals were clearly entrained, and 7 of 8 animals showed at least some evidence of entrainment. These data, taken in combination with results from previous studies, indicate that horseshoe crabs possess an endogenous tidal clock that is capable of driving locomotor rhythms and can be entrained by the rhythmic fluctuations in water depth that characterize natural tide cycles.

The American horseshoe crab displays one of the best-known examples of tide-associated activity. Its mating behavior takes place around high tides, during the spring and summer months, along the Atlantic seaboard of North America (Shuster, 2001; Shuster *et al.*, 2003). During this time of year, males and females approach the high water marks of beaches at around high tides to mate (Barlow *et al.*, 1986), and because it is so easy to observe them at this time, much is known about their breeding behavior (Brockmann, 2003). In animals that are not exposed to robust water level changes due to tides, wind-driven changes in water levels are often more important for stimulating breeding activity than these "micro-tides" (Ehlinger *et al.*, 2003). Our results suggest that water depth changes associated with the changing tides are important cues that American horseshoe crabs use to synchronize breeding activity. Animals in both Florida (Brockmann, 2003) and Massachusetts (Barlow *et al.*, 1986) begin to arrive on the breeding beaches about 1–2 h before high tide, whereas Delaware animals tend to arrive at maximum high tides and stay for 4 h (Penn and Brockmann, 1994). Since our animals began to move about 2 h before high tide, our measured phase angles in the laboratory compare favorably to both the Florida and Massachusetts animals. The average length of alpha (the length of time of the main bout of activity) was about 6 h and was similar in both experiments. This amount of activity corresponds quite well with the amount of time animals spend mating during each tide (2–4 h: Barlow *et al.*, 1986; Penn and Brockmann, 1994; Brockmann, 2003) when one also factors in the activity that would be needed to get the animals to and from the high water marks. Animals that were exposed to the natural photoperiod had a very different phase angle than those exposed to artificial lights that came on and off immediately; they began to move a little more than 1 h after high tide. Since both groups of animals were captured in the same area, the differences are almost certainly not due to using animals from different subpopulations, but may instead be caused by differences in the

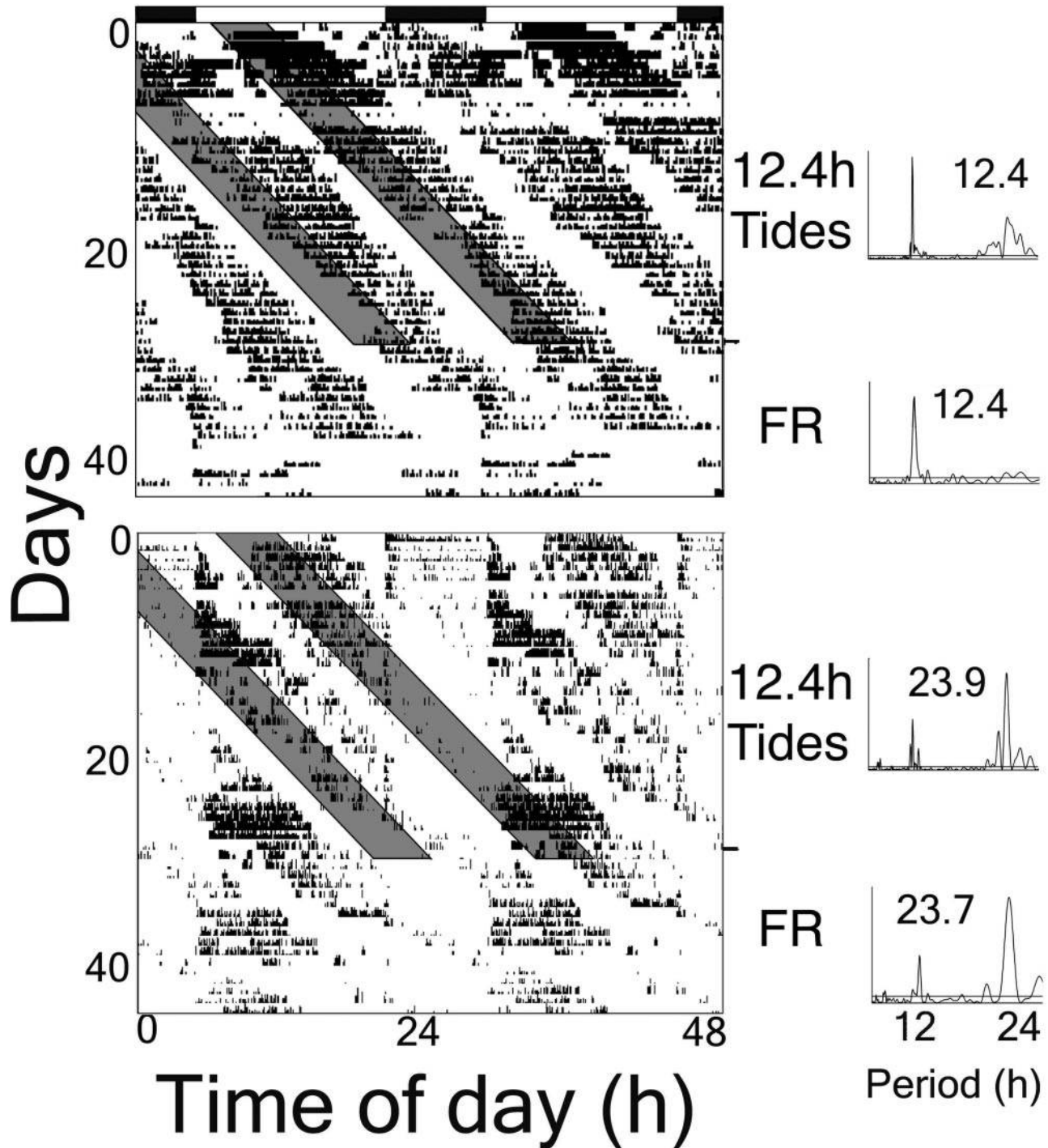


Figure 3. Entrainment of tidal rhythms by artificial tides. These two animals were first exposed to 12.4-h artificial tides, and then the water depth was held constant and the entrained tidal rhythm of locomotion was allowed to free-run (FR). The light/dark cycle remained the same throughout the experiment (14:10).

photoperiods or to the differences in the entraining signals delivered (inundation *versus* water depth changes).

The presence of an endogenous oscillator necessitates entrainment of that oscillator to environmentally appropriate stimuli. Temperature changes associated with the tidal

cycle are sufficient to entrain some crabs (Williams and Naylor, 1969), while other species are sensitive to fluctuations in salinity, or both salinity and temperature (Forward *et al.*, 1986; Reid and Naylor 1990). In some crabs, pressure fluctuations are effective entraining agents (Naylor and

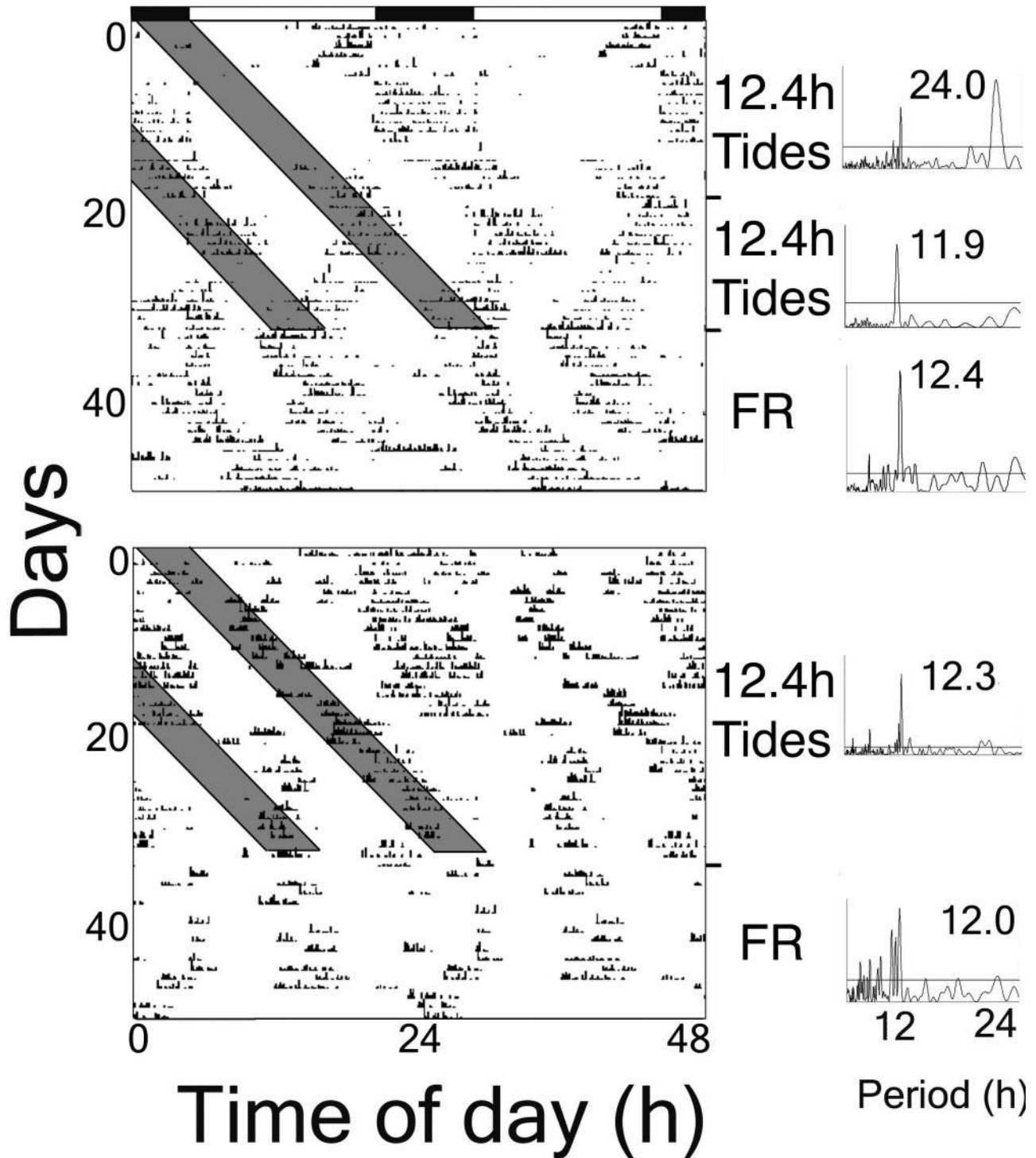


Figure 4. Entrainment of tidal rhythms by artificial tides. Two periodograms are shown in the top panel for the 12.4-h tide period to illustrate a significant change in tau over time. Experimental conditions as described for Fig. 3.

Atkinson, 1972; Abelló *et al.*, 1991), while in two species of isopods and juvenile *Limulus*, periodic agitation is sufficient to entrain tidal rhythms (Klapow, 1972; Hastings, 1981; Ehlinger and Tankersley, 2006). Recent results from our

laboratory indicate that current, temperature, and salinity are all much less effective zeitgebers (Chabot and Watson, unpubl. data), suggesting that cycles of water pressure changes may be the most important environmental cue for

entraining the activity rhythms of *Limulus* in its natural habitat.

After initiation of these cycles, in most animals (and in 3 of 4 animals shown) it took two to six cycles for the activity patterns to become synchronized to the imposed cycles. In one case, there appeared to be 10 cycles of transients before the patterns became synchronized (Fig. 2A). In addition, one of the two daily bouts of activity was missing at times, suggesting that the water depth cycles were not simply either “forcing” the animals to move or inhibiting them from moving. Both of these observations are evidence of involvement of underlying oscillators (Johnson *et al.*, 2004). Interestingly, entrainment was not observed in the first experiment. When the water depth cycles were stopped, the animal’s new phase under conditions of constant water depth could not be clearly traced to the phase during the preceding imposed tidal cycles (Fig. 1, bottom). There are several reasons why clear entrainment may not have been observed in Experiment 1. First, unlike circadian rhythms, which generally show robust persistence in the absence of periodic cues, circatidal rhythms tend to deteriorate in constant tidal conditions after a few cycles (Palmer, 1995). Second, because we stopped the water depth cycles at the midpoint of the tides, the animals were exposed to only “half-tides,” and this may have been a confusing signal for the animals. Third, they might not have entrained to the 12.1-h cycle because it was out of their range of entrainment (Johnson *et al.*, 2004). Finally, some animals may need a burrowing substrate in order to exhibit stable free-running rhythms, although evidence from Watson *et al.* (2008) demonstrates that a burrowing substrate is not necessary for all animals.

We also report here that light/dark (LD) cycles can strongly affect patterns of locomotion in American horseshoe crabs. Many of our animals (11 of 16) expressed daily rhythms of activity when exposed solely to LD cycles. Nearly half (7 of 16) exhibited a preference for diurnal movements, while the others exhibited no preference. Similar modulating effects of LD on locomotion were seen in a previous study (Chabot *et al.*, 2007). In *Carcinus maenas*, it has been hypothesized that both circatidal clocks and circadian clocks drive rhythms of behavioral activity (Naylor, 1996, 1997). However, in horseshoe crabs, these daily patterns are much less apparent when tidal cycles are present (Figs. 1–3). In addition, they tend to fade in constant dark (DD) (Chabot *et al.*, 2007) but not necessarily in constant light (LL) (Watson *et al.*, 2008). Interestingly, our animals exhibited better free-runs in LD (Experiment 3) than in DD (Experiment 1), although it is possible that the addition of sand in the bottom of the tanks during Experiment 3 was a factor.

Limulus is also known to have a circadian clock that drives daily cycles of eye sensitivity (Barlow, 1983), and there is some evidence of circadian rhythms of activity in

juvenile horseshoe crabs (Borst and Barlow, 2002). However, the lack of free-running circadian rhythms of locomotion in DD in the present study (Fig. 1) as well as in previous studies (Chabot *et al.*, 2004) does not support the hypothesis that this circadian clock helps to drive locomotor rhythms in adults. Importantly, recent results from our laboratories (Watson *et al.*, 2008) show clear evidence of electroretinogram rhythms and locomotor rhythms free-running at very different periods (respectively 24.2 h and 12.4 h) in the same animals. The most parsimonious explanation for these results is that the clocks controlling eye sensitivity and locomotion are functionally separate. Thus, in this context, our current and previous results (Chabot *et al.*, 2004, 2007) support the hypothesis that LD cycles occasionally tend to mask endogenous tidal locomotor rhythms. Interestingly, in the laboratory at least, most of this LD-induced masking disappears when tidal cycles are initiated (Figs. 1, 2).

However, it is possible that LD cycles play a more fundamental, but currently unclear, role in the maintenance of tidal rhythms in *Limulus*. Along with their masking effects, LD cycles also apparently help to maintain circatidal rhythms as well as to cause some transient synchronization of these rhythms in *Limulus* (Fig. 4; Chabot *et al.*, 2007) and several crustacean species (Honegger, 1973; Naylor, 1985; Saigusa, 1988, 1992; Palmer, 1990; Saigusa and Kawagoye, 1997). Transient synchronization, otherwise known as relative coordination (Johnson *et al.*, 2004), is indicative of a relatively weak entraining agent influencing an oscillator. Why LD cycles would affect tidal rhythms in this way in *Limulus* and other species remains to be elucidated, though one working hypothesis is that changes in light levels are essentially redundant signals in estuaries such as Great Bay, where the turbidity of the water significantly attenuates light (see Discussion in Watson *et al.*, 2008).

There was no clear effect of moon phase on activity patterns. In at least two Florida populations of *Limulus*, breeding activity increases around the new and full moons at certain times of year (Rudloe, 1978, 1980; Cohen and Brockmann, 1983), while in two northern populations, a lunar influence on activity is less apparent (Cavanaugh, 1975; Barlow *et al.*, 1986). There are at least two ways in which *Limulus* in the field could be sensitive to lunar phase. The first is a change in light intensity at night, but not during new moons. The second is variable tide heights that are synchronized to the phases of the moon. However, although the phase of the moon would lead to differences in tide height in the field, our artificial tides were always the same height, independent of moon phase, so this cue was not available to our laboratory animals. This suggests that relative tidal heights may be more important than changing nighttime light intensities. However, this hypothesis requires further investigation because our experimental pro-

toloc was not specifically designed to test for the effects of moon phase on activity patterns. Barlow *et al.* (1986) proposed that the number of animals mating during any given high tide is proportional to the relative height of that tide. Taken to the extreme, in areas where there is little change in water depth with the tides, such as in the Indian River Estuary in Florida, mating is not very well synchronized to the tides. Furthermore, evidence supporting the view that most mating activity occurs during the new and full moons comes primarily from certain areas in Florida where significant changes in water depth may occur only during this period of the month (Rudloe, 1980). Thus, the results of this study support the hypothesis of Barlow *et al.* (1986) that changes in the numbers of animals mating on a given high tide are due to differences in the relative strength of the entraining cue provided by tides of different heights.

Overall, our results suggest that patterns of behavioral activity in horseshoe crabs can be affected both by cycles of light and dark and by tidal cues. However, tidal cues appear to be more important since they generally override LD cues: when exposed to tidal cycles and LD, all animals entrained to the tidal cycles. Taken together, these results suggest that a hierarchy of cues influence the timing and intensity of locomotion in *Limulus*, with tidal cues being of primary importance.

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