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The effects of water pressure, temperature, and current cycles on circatidal rhythms expressed by the American horseshoe crab, *Limulus polyphemus*

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The American horseshoe crab, *Limulus polyphemus*, expresses tidal rhythms of locomotion that can be entrained to cyclic fluctuations in water depth, but the ability of other tidal cues to entrain locomotor activity has not been assessed. In this study, tidal inundation cycles of ""'12.4 h delivered in the laboratory clearly entrained the locomotor patterns of most animals. However, smaller amplitude water level fluctuations and large amplitude temperature cycles (10°C) were less successful while small-amplitude temperature fluctuations (3°C) and current cycles (0.3 m s^{-1}) were unsuccessful. In the field, animals confined to modified running wheels expressed rhythms of locomotor activity that were clearly synchronized with the tides, but only if they were allowed to experience water level changes. Overall, these results suggest a hierarchy among potential tidal entrainment cues: inundation and water level changes are of primary importance, while other factors such as current and temperature changes appear to play secondary roles.

Keywords: American horseshoe crab; *Limulus polyphemus*; circatidal; biological clock; circadian; entrainment; tides; water pressure

Introduction

Endogenous circatidal rhythms in marine animals were first described in the fiddler crab, *Uca pugnax*, by Bennett et al. (1957) and have since been documented in many other species (Palmer 2000) including the shore crab, *Carcinus maenas* (Naylor 1958), the isopod *Excirolana chiltoni* (Klapow 1972; Enright 1976), and the shrimp *Crangon cragnon* (Aladhub and Naylor 1975). Recently, evidence for such a timing system was also demonstrated in the American horseshoe crab, *Limulus polyphemus* (Chabot et al. 2004). These circatidal clocks are thought to confer adaptive advantages to organisms that live in, or visit, the inter-tidal zone by allowing them to synchronize their activity, and in some cases anticipate the periodic conditions associated with

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changing tides. One of the defining characteristics of these clocks is that they must be synchronized to the tides in order to be adaptive.

Natural tidal fluctuations are associated with rhythmic cycles of many possible water qualities, including temperature, salinity, turbulence, current, turbidity, and depth, and therefore any of these could serve as cues to synchronize and entrain tidal rhythms. The influence of water level changes on circatidal rhythms have been most thoroughly investigated, and this cue can entrain the activity of the amphipod (*Corophium volutator*; Harris and Morgan 1984), the portunid crab (*Liocarcinus holsatus*; Abelló et al. 1991), the fiddler crab (*Uca crenulata*; Honegger 1973), and the American horseshoe crab (*L. polyphemus*; Chabot et al. 2008), as well as many other species. Agitation or turbulence is an effective entraining agent for mole crabs, *Emerita talpoida* (Forward et al. 2007), the amphipod *Synchelidium* spp. (Enright 1963), and the isopod *Eurydice pulchra* (Hastings 1981), while current reversal is effective in glass eels, *Anguilla anguilla* (Bolliet and Labonne 2008). The effects of tidal cues such as these have been studied most thoroughly in *C. maenas*. While this species can be entrained to cycles of temperature, inundation (Williams and Naylor 1969), or hydrostatic pressure (Reid and Naylor 1990), in most species the effects of more than one cue has not been studied. *Carcinus maenas* is also one of the very few species in which the relative importance of tidal cues has also been assessed in situ (Styrishave et al. 1999, 2003).

The goals of this study were twofold. The first goal was to assess the relative importance of several different potential tidal cues, including current, water pressure, inundation, and temperature cycles, in entraining the circatidal rhythm of locomotion in the American horseshoe crab in the laboratory. The second goal was to test the hypothesis that, in situ, the depth changes associated with natural tides are necessary in order to entrain horseshoe crab tidal rhythms of activity. The data obtained suggest that, while tidal cues such as fluctuating temperatures and currents can influence the expression of tidal rhythms, water level changes are the dominant cues used by horseshoe crabs to synchronize their circatidal rhythms of activity in their natural habitat.

Materials and methods

Animals and environmental conditions

This article is the result of work done during portions of three years: May–September 2005, August–December 2006, and June–August 2007. During 2005 and 2007, adult male (190–350 g) and female (250–360 g) *L. polyphemus* were collected from mating beaches in the vicinity of the UNH Jackson Estuarine Laboratory (JEL) at Adams Point, NH. The animals used in 2006 were collected from the Great Bay estuary, NH, by scuba divers. Animals that were studied in situ were immediately placed in running wheels (RWs) and the wheels were deployed in the estuary within 40 m of JEL. Animals that were to be studied in the laboratory were quickly (within 2 h) transported in a cooler to Plymouth State University, Plymouth, NH, and immediately put into individual activity chambers in custom-made recirculating tanks located in a light-tight laboratory. The animals were not fed throughout the duration of the experiments (50–75 days).

Activity monitoring in the laboratory

Activity monitoring chambers (40 x 30 cm^2) were created within recirculating seawater tanks (80 x 60 cm^2; four chambers/tank) using plastic lighting grating (1 x 1 cm^2 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 both hold it in place and to create a shielded, darker area over approximately half of each activity chamber. Magnets were attached to the dorsal carapace of each animal using duct tape, epoxy, and cyanoacrylate glue. This made their movements detectable when they moved near a reed switch that was located on the side of the chamber not covered by bricks. Since the behavior of animals treated in this manner does not differ significantly from the behavior of animals in RWs (without carapace-affixed magnets), their behavior was unlikely to be affected by the affixed magnets (Chabot et al. 2007). For further methodological details, see Chabot et al. (2007). Salinity was kept between 25 and 29 psu by the addition of either dH_2O, seawater collected at the JEL, or artificial seawater (Natural Sea Salts, Oceanic Systems, Dallas, TX), as needed. Nitrate levels remained below 100 mg L^{-1} throughout all experiments. Light was provided by a 40-watt fluorescent bulb (Coralife 10,000 K) suspended above the tanks. Light:dark (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). During the simulated daytime, the light intensity at water level was 100–150 lux (lumens m^{-2}; ""'0.12–0.9 mmol s m^{-2}) and at night it was 0.0 lux. During the times that animals were exposed to a light cycle, a 14:10 LD (14 h of light and 10 h of darkness) photoperiod was administered using an electronic timer. The L to D and D to L transitions were instantaneous. pH was maintained between 7.8 and 8.2 by the addition of Sea Buffer (Aquarium Systems, Inc., Mentor, OH).

In situ monitoring

Male (205–310 g) and female (280–350 g) horseshoe crabs that were captured as described above were immediately placed in ‘‘running wheels’’ (RWs; Chabot et al. 2007; Watson et al. 2008) that were attached, in groups of eight RWs, to weighted wooden pallets. Some pallets were then lowered to the bottom of the estuary in an area that was 2 m deep at low tide and some were attached to the bottom of a floating raft (crabs were always 0.5 m below the surface). Two bar magnets mounted on the outside walls of the RWs and a magnetic reed switch mounted on the RW frame allowed for wheel rotations to be detected and recorded with a data collection system. Nearly 70% of horseshoe crabs housed in these devices exhibit endogenous circatidal rhythms in laboratory conditions, a percentage similar to those housed in activity chambers with or without access to sand for burrowing (Chabot et al. 2007). Long cables connected the RWs to the data collection system that was located in the laboratory. Light levels, temperature, and salinity were constantly monitored in the area using in situ probes. Over the course of the experiment, water temperature ranged from 16^{o}C to 25^{o}C and varied with both the tidal phase and month. Tidal differences in temperature varied considerably, with maximum differences during May–July: up to a 13^{o}C range with typical differences of approximately 8^{o}C. August and September differences were attenuated: up to a 5^{o}C difference between high and low tides with a typical difference of 3°C. Salinity values in the estuary ranged from 12 to 30 psu and was influenced by both the phase of the tidal cycle and rain events. Salinity varied considerably by tidal phase though with less seasonal variation than temperature: for each month (May–September) the maximum salinity differences due to tidal phase averaged 8.5 psu. Rain events caused up to a 10 psu drop over 1–2 day periods with greatly decreased tidal phase differences during these times.

Light levels during the day were extremely variable and depended on cloud cover and water depth and turbidity. Light intensity values at a depth of 1 m from the surface ranged from 1500 lux on a sunny, low turbidity day to 200 lux on a cloudy, high turbidity day. Light was attenuated by approximately 50% for each 1 m increase in depth. During the night, light levels were less variable but depended on both moon phase as well as water depth and ranged from 25 lux at 1 m depth to near 0 lux at 8 m. Based on a previously published study, currents in our study area likely ranged from 0 m s^{-1} (slack tides) to 0.2 m s^{-1} (ebb and flow tides; Short 1992).

Experimental procedures – laboratory studies

The impact of small water level and temperature cycles on activity rhythms

While water level changes of 0.4 m have been shown to synchronize and entrain *Limulus* activity rhythms (Chabot et al. 2007, 2008; Watson et al. 2008), the purpose of this first laboratory experiment was to examine the effects of smaller changes in water level (0.2 m) as well as temperature cycles, on locomotor activity. Animals (*N* ¼ 8 males) were collected on 20 June 2005 and exposed to a 14:10 LD cycle throughout the experiment. To determine the influence of temperature fluctuations on the expression of tidal rhythms, animals were exposed to temperature cycles of ±3°C, delivered with a 12.4 h period by periodically turning 300 W aquarium heaters on and off (two heaters per 50-gallon tank; ViaAqua titanium heaters). In the estuary, colder water from the Gulf of Maine flows into the estuary during the rising tides, so the coldest water temperatures occur at the peak of high tide, while the warmest water temperatures occur at low tide. Temperature maxima in the experimental tanks were designed to coincide with the temperature maxima in the field that the animals were previously exposed to during low tides prior to capture (Bolt et al. 1989). The absolute temperature cycle changes (low tide to high tide) ranged from D1.8°C to D4.1°C with an average temperature change of D3°C.

After 4 weeks, temperature cycles were terminated (temperature was held constant at 16.1 ± 1.3°C) and water level changes of 0.2 m were administered at a 12.4 h cycle for 12 days. These ‘‘tidal’’ cycles were established using water pumps controlled by timers (Chabot et al. 2007). 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 time required to change the water level from high to low was 3.7 ± 0.2 h. Since the rate of flow was very low (approximately 0.4 L min^{-1}), little turbulence or current was created during water level changes.

When clear synchronization to the aforementioned water depth changes did not occur (in 7/8 animals), an inundation cycle was initiated for 2 weeks. All animals (*N* ¼ 8) were alternately exposed to air, and inundated with water, every 12.4 h (water level change ¼ 0.3 m; approximately 50% of their carapace exposed to air; Chabot et al. 2007). After approximately 2 weeks, constant water levels (at maximum 0.25 m depth) were maintained for an additional 2 weeks.

The impact of large temperature cycles on activity rhythms

The purpose of this experiment was to examine the effects of larger temperature cycles (±10°C) on the expression of tidal rhythms of locomotion. Animals (males, n ¼ 7) were collected on 14 June 2006, brought into the laboratory, and immediately exposed to temperature cycles. Because LD cycles appeared to directly induce activity associated with lights on and lights off (and thus might ''mask'' some of the underlying activity patterns), we used constant light (LL; 25–30 lux) in this and subsequent experiments. Temperature cycles with periods of 12.4 h were delivered by alternately turning on and off a large chiller in anti-phase with four of the aquarium heaters described above. This created a temperature cycle that oscillated between 16°C and 26°C, with a period of 12.4 h. After 3 weeks, the period of the cycle was changed to 12.1 h in order to determine if the activity would ''follow'' the new period. After 3 weeks, temperature cycles were terminated and water temperature was maintained at 17 ± 2°C to determine if any rhythms that appeared synchronized to the fluctuating temperatures had, in fact, been entrained.

The influence of current cycles on locomotion

The purpose of this experiment was to examine the effects of oscillating changes in current flow on locomotor activity. Males (n ¼ 6) and females (n ¼ 6) were collected by scuba on 20 September 2006 (n ¼ 10) and 4 October 2006 (n ¼ 2). While these animals were not taken during the breeding season, animals previously collected during this time of year have been shown to produce clear circatidal rhythms like those collected during the breeding season (Chabot et al. 2007). Animals were immediately brought into the laboratory and exposed to cycles of currents at periods of 12.4 h under LL conditions. Current cycles were created using timers connected to water pumps (RIO model 600 or 800; TAAM Inc., Camarillo CA) that were adjusted to create a flow of 0.3 m s^{-1}. Currents were on for approximately 4.2 h and then off for 2 h in order to approximate incoming, outgoing, and slack tides. Thus, currents alternated across the tank every 6.2 h. After 2 weeks, the period of the cycle was changed to 12.1 h in order to determine if the activity would ''follow'' the new period. Under these conditions, alternating pumps were on for 4.05 h and then off for 2 h. Two weeks later the current cycles were terminated and activity was collected for an additional 2 weeks.

Experimental procedures – in situ studies

The purpose of this experiment was to test the hypothesis that water level changes were necessary for entrainment of horseshoe crab tidal rhythms of activity in their natural habitat. Three sets of animals were placed in the Great Bay estuary adjacent to JEL from 19 June 2006 to 15 September 2006. One set of nine *Limulus* (four females, five males) was placed in RWs attached to a wooden pallet that was anchored on the bottom in shallow water approximately 5 m from the shoreline at low tide. The water in this location was 4 m deep at high tide and approximately 1 m deep at low tide, yielding a 300% change in water level between tides. A second set of five males was placed in RWs attached to a wooden pallet that was anchored to the bottom of the estuary in deeper water (5 m of water at low tide), approximately 20 m from shore. Since the mean depth at high tide in this location was approximately 8 m, these animals experienced smaller water level changes of approximately 60%. The third set of animals (12 males) was placed in identical RWs and attached underneath a floating dock so that they would experience all the cues associated with changing tides, except the typical fluctuations in water depth. This group of animals was located at a consistent depth of 1 m from the surface and within 25 m of the animals secured to the bottom. At no time during the tide cycle were any of the RWs or animals out of the water. Each of the in situ animals experienced natural photoperiods and the full complement of tidal cues (salinity, current, temperature, and other physicochemical changes) with the exception of the animals in the floating RWs, which did not experience tidal water level changes because the float to which they were attached went up and down with the tides.

Data analysis

Activity data were collected at 5-min intervals using a computer based data collection system and analyzed via the ClockLab suite of time series data analysis programs (Actimetrics, Evanston, IL). Since previous studies have shown a lack of significant differences between male and female behavioral activity (Chabot et al. 2004) and since the τ's exhibited by males and females in this study did not differ significantly ($t(6) = 0.68$, $p > 0.53$), male and female data were combined in this study. Significance of rhythmicity was determined both visually (Chabot and Menaker 1992; Chabot et al. 2007) and by Lomb–Scargle periodogram analysis ($p < 0.01$). The Lomb–Scargle method (Ruf 1999) was used because it reduces the occurrence of artificial harmonics created by the major periodicities of the data, compared to the chi-squared method, while calculating periods that are virtually the same (Chabot et al. 2007) as those calculated using the more traditional Chi-square method (Sokolove and Bushell 1975). 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 range (between 10.4 and 14.4 h) for each animal during each experimental condition. At least 10 days of data were generally used to calculate Lomb–Scargle periodograms. To determine the phase angle of activity to environmental cues (water depth, temperature, or current changes), the best eye-fit lines were drawn through the onsets of activity, when possible, using a single-blind protocol.

Results

The impact of various tidal cues on rhythms expressed in the laboratory

Small amplitude temperature and large and small water level changes

The effects of small amplitude temperature cycles (±3°C), small amplitude water level changes (0.2 m), and inundation cycles (0.3 m) on the activity patterns expressed by *L. polyphemus* are shown in Figure 1. During the temperature cycles, most animals (7/8) exhibited circatidal rhythms (12.4 ± 0.1 h; Figure 1). However, almost all these rhythms were apparent only for the first 10–14 days of the experiment

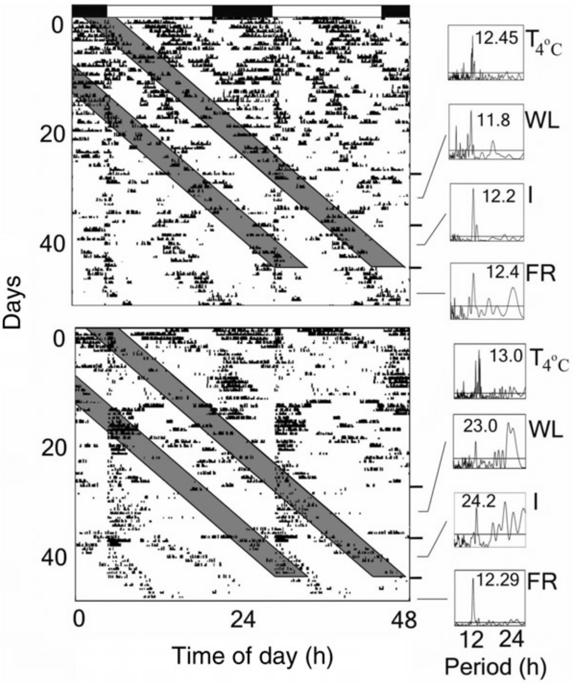

Figure 1. The effects of water level and temperature changes on the locomotor activity of two horseshoe crabs exposed to LD 14 : 10. Gray boxes indicate the times when animals were experiencing rising temperature or water levels. Labels on the right of the figure indicate the treatment that the animals were exposed to during different times in the experiment (T_4, 4°C temperature change (18–22°C); WL, water level changes of 0.2 m; I, inundation cycles of 0.3 m; FR, free run). Right panels: periodogram analyses of the sections of actograms separated by horizontal bars. Highest significant peaks in the circatidal (10.4–14.4 h) or circadian (22–26 h) range are noted. Inundation cycles were effective as entraining cues while small temperature changes and small WL changes were not.

(Figure 1, top and bottom panels) and only two of these animals showed some evidence of synchronization to the imposed cycles (Figure 1, top panel), while the remaining animals showed no evidence of synchronization (Figure 1, bottom). The remaining animal lacked significant circatidal rhythmicity. Thus, it appears as if the horseshoe crabs were merely expressing an endogenous tidal rhythm of activity and small changes in water temperature were not sufficient to entrain the rhythm.

When small amplitude cycles (0.2 m) of water level changes were later imposed on this same group of animals, only one of the seven animals showed a clear circatidal rhythm and clear evidence of synchronization (r ¼ 12.3 h, data not shown). In this animal, increased locomotor activity occurred 3 h before high water level. However, the remaining animals did not express rhythms that were circatidal (Figure 1). Some of these animals (3/6) did express rhythms of approximately 12 h (r ¼ 12.1 ± 0.2 h), but these appeared to be induced by the LD cycle and not by the tidal cycles (Figure 3, top). In addition, three of these animals exhibited primary rhythmic components in the daily range (Figure 1, bottom; r ¼ 23.1 ± 0.1 h). These data indicate that small fluctuations in water depth were not capable of influencing the expression of horseshoe crabs rhythms in the laboratory.

When larger water level cycles that exposed horseshoe crabs to air during periods of low tide (inundation) were introduced, most animals (6/7) exhibited circatidal rhythms (r ¼ 12.2 ± 0.1 h) and the activity of five of these animals appeared to be synchronized to the cycles (Figure 1, top and bottom). The remaining animals appeared to be synchronized to the inundation cycles, but did not exhibit statistically significant rhythms in the circatidal range (data not shown). Phase angles to these imposed cycles showed that increased locomotor activity occurred 3.5 h (±0.1) before peak high water (approximately the time when the animals would again be fully covered by water). When the inundation cycles were terminated, four of these animals showed evidence of entrainment to the inundation cycles (Figure 1), confirming previous data indicating that moderate to large changes in water depth are sufficient to entrain horseshoe crab tidal rhythms of activity.

Larger amplitude temperature changes (10°C)

The effects of large amplitude temperature cycles (±10°C) on the locomotor activity of two representative horseshoe crabs are shown in Figure 2. When cycles of temperature changes were delivered at periods of 12.4 h, most animals (6/7; r ¼ 12.7 ± 0.2 h) exhibited circatidal rhythms (Figure 2, top). However, only one of the six animals that exhibited circatidal rhythms showed clear synchronization to the imposed temperature changes (data not shown) and two of the remaining five

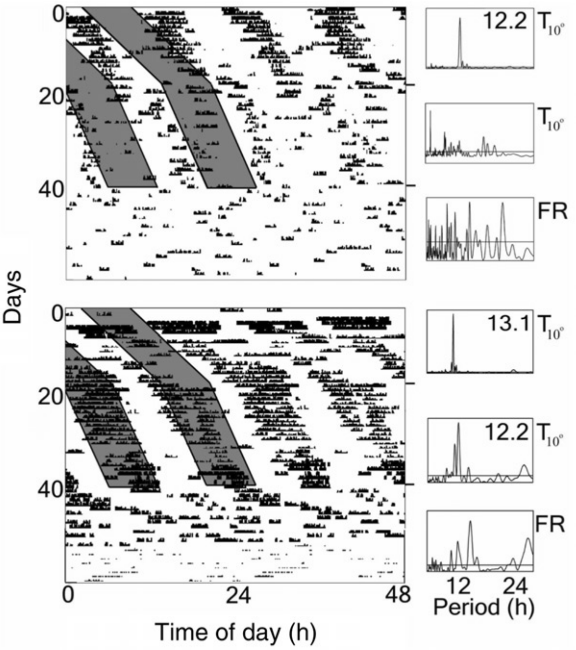

Figure 2. The effects of large temperature fluctuations (10°C; from 14°C to 24°C) on locomotor activity. Temperature cues were delivered at two different periods (12.4 h, first 18 days; 12.1 h, next 19 days). Gray boxes indicate rising water temperatures. T_{10}, 10°C temperature change; FR, temperature held at 17°C. Large temperature fluctuations were more effective as entraining cues than small temperature fluctuations but much less effective than inundation.

animals showed partial evidence of synchronization (Figure 2, bottom). The remainder (3/6), while expressing clear circatidal rhythms, lacked clear evidence of synchronization (Figure 2, top). When the temperature cycles were delivered at a 12.1 h period, most animals (5/7) again exhibited circatidal rhythms (Figure 2, bottom; r¼12.2 ± 0.1), while only two of them showed moderately clear evidence of synchronization (Figure 2, bottom). In addition, another two of the seven showed partial evidence of synchronization (data not shown), while the remaining animals (3/7) did not exhibit clear synchronization (Figure 2, top). When the tidal temperature cycles were later terminated and the animals were exposed to constant temperature conditions, some animals (2/7) showed evidence of entrainment to the previous cycles (Figure 2, bottom; although for both animals, the resultant periods of the free runs (r¼15.5 and 25 h) were outside of the circatidal range), while the remainder did not show any evidence that they entrained to the imposed temperature cycles. Thus, while large percentage of animals expressed tidal rhythms of locomotion during large amplitude temperature cycles, the extent to which they synchronized their activity to the cycles was both variable and limited.

The influence of current cycles

Two examples of the effects of current cycles (0.3 m s^{-1}) on the locomotor activity of *L. polyphemus* are shown in Figure 3. When the cycles of current were delivered at

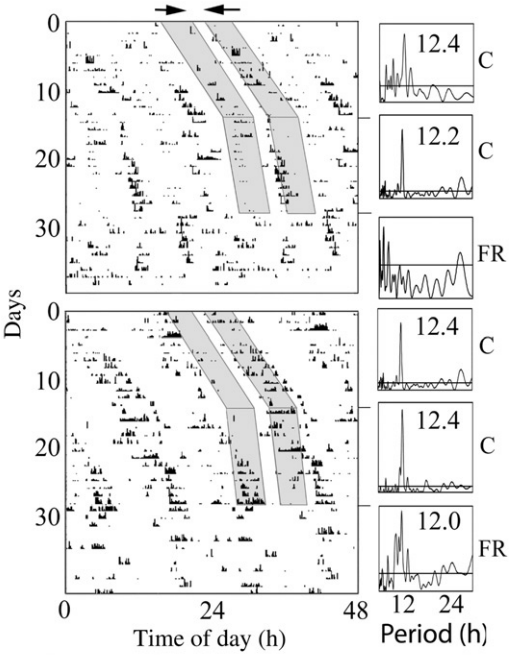

Figure 3. The effects of current cycles (0.3 m s^{-1}) on locomotor activity. Currents were imposed at two different periods (12.4 h, first 15 days; 12.1 h, subsequent 20 days). C, current cycles effected. Arrows indicate the direction of the simulated incoming and outgoing tidal currents, which alternated across the tank every time the pumps came on (every 6.2 h during the 12.4 h phase and every 6.05 h during the 12.1 h phase). Current cycles were ineffective as entraining cues.

periods of 12.4 h, 6 out of 12 animals exhibited circatidal rhythms (r¼ 12.62 ± 0.13 h) that appeared to be somewhat synchronized to the current cycles (Figure 3), while the remaining animals did not show any significant rhythms (data not shown). When current cycles were delivered at a 12.1 h period, over half (7/12) showed circatidal rhythms that appeared to be somewhat synchronized (Figure 3; r¼ 12.3 ± 0.2 h), while the remaining did not have any significant rhythms (data not shown). While 8 of the 12 animals showed some synchronization to the cues delivered at either 12.4 or 12.1 h periods, there was no clear evidence of entrainment by any of the animals when these current cycles were terminated (Figure 3).

Activity of Limulus *in situ*

Representative actograms illustrating the patterns of locomotor activity expressed by two male horseshoe crabs in RWs anchored on the bottom of the Great Bay estuary and experiencing natural tidal water level changes (300% change from low to high) are presented in Figure 4. Seven of the nine animals exhibited clear circatidal rhythms (r¼ 12.4 ± 0.1 h) with generally positive phase angles of entrainment to peak high tide, but with high variability (0.2 ± 1.3 h). Of these seven animals, five also exhibited significant 24 h secondary peaks (Figure 4). The remaining two animals exhibited primarily circa 24 h rhythms (r¼ 24.0 ± 0.2) with clear secondary circatidal peaks (data not shown). When rhythms were assessed 1 month later (mid-late August) in eight of these animals, three of them exhibited primary peaks in the circatidal range (r¼ 12.6 ± 0.2 h), while five exhibited peaks in the circadian range (r¼ 24.4 ± 0.4 h).

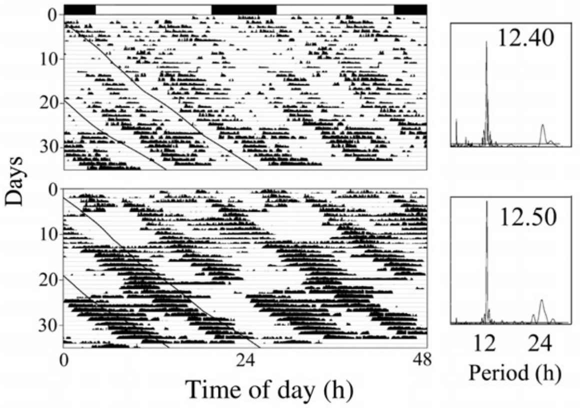

Figure 4. Locomotor activity recorded from two American horseshoe crabs housed in RWs and anchored on the bottom of the Great Bay estuary, at a depth of 1 m (mean low tide, total tide excursion is 3 m in this location. Data shown on the left panels are double-plotted to facilitate visual inspection. The approximate LD cycle is indicated by black/white bars at the top. Dark sloping lines indicate the time of peak high tide. Right panels: Lomb–Scargle periodogram analyses of the activity shown in left panels. Vertical scale – relative $Q(p)$ (strength of rhythmicity). Largest peak value above horizontal line of significance (p5 0.01) is indicated by numerical value. Both animals expressed a tidal rhythm of activity that was synchronized to the natural tides. Interestingly, each animal preferred to be active during a different phase of the tidal cycle.

Two of the five animals that were placed deeper in the estuary in September (60% water level change from high to low) exhibited significant tidal rhythms (data not shown; r¼ 12.9 ± 0.5 h; 3.4 ± 0.1 h phase angle), while the remaining three exhibited significant daily rhythms (r¼ 25.0 ± 0.2 h). In each of the two groups, one animal also exhibited significant secondary peaks in the circatidal or circadian range.

All 12 of the animals in RWs that were attached to a floating raft exhibited strong daily rhythms (Figure 5; r¼ 23.9 ± 0.1 h). All of these animals were primarily diurnally active and exhibited a negative phase angle to time of sunrise (-2.7 h ± 0.4 h). Five of these twelve animals also exhibited significant peaks of activity in the circatidal range (Figure 5, top). When the activity patterns of nine of these animals were assessed 1 month later (mid-late August, data not shown), most still exhibited daily rhythms (7/9), one exhibited circatidal rhythmicity and the remaining animal exhibited a lack of rhythmicity in either the circadian or circatidal range.

Discussion

This study is the first to examine the relative importance of several cues, associated with changing tides, on the expression of tidal and daily rhythms exhibited by horseshoe crabs both in the field and the laboratory. Overall, our data support the hypothesis that water level changes are the dominant timing cue used by horseshoe crabs to synchronize their activity to the tides (Watson et al. 2009a). Most animals (7/9; Figure 4) that were anchored to the bottom of the estuary and experienced the

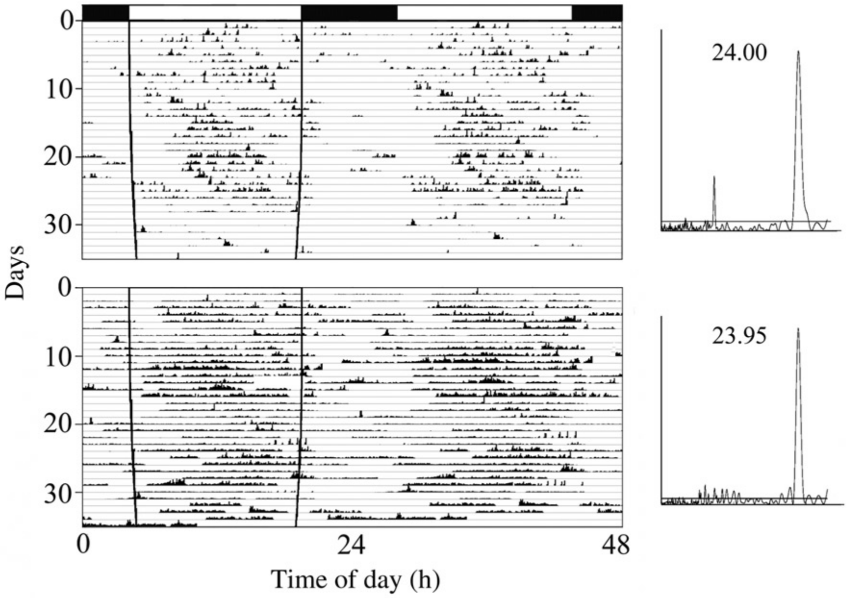

Figure 5. Locomotor activity recorded from two horseshoe crabs housed in RWs and attached to a floating dock in situ (left panels). Actual sunrise and sunsets are plotted as vertical curvilinear lines. These animals experienced all the cues associated with changing tides, except for changes in water depth. As opposed to the animals shown in Figure 4, both these animals primarily expressed a diurnal pattern of activity.

complete suite of tidal cues in the estuary exhibited tidal rhythms. However, those that were attached to a floating raft and were thus exposed to all the natural tidal cues, except water pressure changes, did not express clear tidal rhythms. Instead, they exhibited clear daily rhythms. This suggests that while factors such as current changes (0–0.2 m s^{-1}), temperature changes (D10°C), salinity changes (D10 psu), and other physicochemical parameters may influence the behavior of horseshoe crabs, they are generally not sufficient to synchronize tidal rhythms. In the only other comparable field study that we are aware of, water pressure was also the primary tidal cue used by amphipods to synchronize their activity (Enright 1965): animals exposed to water pressure rhythms in situ entrained to the tidal cycles while those tethered to a buoy, like our animals tethered to a floating dock, did not.

The primary importance of water level changes for *Limulus* tidal entrainment was also supported by our laboratory results. In the laboratory, most (5/7) animals were clearly entrained to cycles of inundation (Figure 1), while other potential tidal cues such as temperature cycles (Figures 1 and 2) or current cycles (Figure 3) were much less effective. While the effectiveness of inundation cycles had been previously demonstrated in *Limulus* (Chabot et al. 2007, 2008), the importance relative to other tidal cues had not been established. Water level and inundation cycles also appear to be the most important cues for *C. maenus* (Naylor et al. 1971). The amphipod *C. volutator* (Holmstrom and Morgan 1983) the rocky shore fish *Blennius pholis*, (Northcutt et al. 1991), and the cumecean crustacean *Dimorphostylis asiatica* (Akiyami 2004) also entrain to water level changes. In fiddler crabs, the ability to synchronize to water pressure changes appears to be mediated by a statocyst (Fraser 2006), but a similar organ has not been located in horseshoe crabs (Botton and Loveland 1987).

The ability to entrain to cycles of inundation is usual for animals that either frequently visit or live in the inter-tidal zone (Naylor and Atkinson 1972) and this tidal cue may help to synchronize the breeding behavior of *L. polyphemus*. This species exhibits well-known seasonal visitations to the inter-tidal zone to breed in the early summer along the eastern seaboard of the USA, with peaks of breeding activity beginning at or 1–2 h before high tide and ending 2–4 h after peak high tide as water levels are receding (Rudloe 1985; Penn and Brockmann 1994; Shuster 2001; Shuster et al. 2003). During the breeding season, individuals breeding at the high-tide line often experience an ‘‘inundation’’ event (a brief exposure to air followed by re-immersion) as water levels fall (Brockmann 2003; Watson et al. unpublished observations) although the frequency of inundation for individuals has not been reported. It is possible that, in the field, these inundation events are strong breeding synchronization cues.

However, while inundation may help to synchronize breeding behavior in *Limulus*, water depth changes are also sufficient. Many inter-tidal residents or visitors, including horseshoe crabs (Chabot et al. 2008; Watson et al. 2008), synchronize to tidal water depth changes (Naylor and Atkinson 1972). While horseshoe crabs have long been known to exhibit behavioral tidal rhythms during the breeding season, this tidal behavior has recently been extended to non-breeding seasons when they would not be expected to experience inundation cycles. In the laboratory, horseshoe crabs exhibit endogenous circatidal rhythms (Chabot et al. 2004) that can be entrained to water level changes (Chabot et al. 2008) during both breeding and non-breeding times of year. In the field, *L. polyphemus* has recently been shown to be a regular inter-tidal zone visitor at times other than the 1-month breeding season (Watson et al. 2009b). These excursions into the inter-tidal zone at high tide are often associated with digging pits in the mud and it is likely that they are foraging for food in these areas at high tide (Watson et al. unpublished). Thus, based on previous (Chabot et al. 2008) and present results (Figures 4 and 5), it seems likely that water depth change is a primary cue that enables this species to determine the best times for foraging on submerged tidal flats.

We also provide further evidence for a potential ‘‘threshold’’ for the water pressure cue necessary to entrain tidal rhythms. While inundation accompanied by larger water level changes was a very effective entraining agent, small water pressure changes were much less effective (1/7 entrained; data not shown). While this might suggest that inundation itself, as opposed to water pressure changes only, may be a critical component, previous work has shown that 7/8 animals showed evidence of entrainment to water level changes of 0.5 m while depth changes of 0.2 m allowed for synchronization but not clear entrainment (Chabot et al. 2008). Our in situ results also support the finding that stronger depth change signals increase the percentage of animals that entrain: 2/5 animals exhibited tidal rhythms in deeper water that experienced 60% change in water levels, while 7/9 of those anchored in shallower water and experiencing 300% water pressure changes showed tidal rhythms. While the ‘‘threshold’’ of between 0.2 and 0.5 m is somewhat greater than those reported for *C. maenas* (Naylor et al. 1972) and some planktonic species (0.1 m; Knight-Jones and Morgan 1966), this study appeared to use a more stringent definition of entrainment than the previous studies. The importance of water levels/depth changes are also seen in breeding rhythms in the field: populations that are exposed to tides of at least 0.5 m or so synchronize to the high water changes (Rudloe 1979, 1980, 1985; Cohen and Brockmann 1983; Barlow et al. 1986) while populations that experience only micro-tides (of a few centimeters) do not synchronize their breeding with these tidal cycles (Ehlinger et al. 2003). Further, wind action leading to increased or decreased tidal heights significantly affects breeding in this species (Brockmann 2003). The importance is also indicated by the matching of breeding frequency with tidal periodicity in the environment: populations that experience twice-daily tides exhibit breeding activity corresponding to those twice daily high tides (Rudloe 1985; Barlow et al. 1986), while populations along the Gulf Coast of Florida synchronize to the diurnal (once daily) tides they experience (Rudloe 1979, 1980, 1985; Cohen and Brockmann 1983). Populations that experience only ‘‘micro’’ tides of a few centimeters (essentially no tidal water level changes) do not synchronize their breeding with these water level cycles (Ehlinger et al. 2003).

Our results concur with previous reports indicating that *L. polyphemus* generally begins its activity before ‘‘high tide’’. Chabot et al. (2007, 2008) reported that activity began approximately 2 h before high tides in laboratory situations compared to 0.2–3.4 h (field) and 3.5 h (lab) in this study (Figures 1 and 4). These positive phase angles are similar to those seen by fish in situ where this species invades the shallows and increases its activity on the flood tide (Ralston and Horn 1986). Other species, however, seem to increase their activity with the falling tides, a behavior thought to decrease the risk of stranding (Gibson 2003). Interestingly, in this study, submerged in situ animals began moving only 0.2 h before peak high tides although there was a large degree of variability (± 1.3 h) among animals (Figure 4). Similar differences in phasing have also been observed between in situ and laboratory results in some *Uca* sp. (Honeggar 1973) and the author attributed those differences to a more complex suite of tidal factors that are present in the field. This is certainly a possibility with *Limulus* as well.

As previously reported in *L. polyphemus* and other species, LD cycles can have a significant effect on the behavioral activity patterns in animals exhibiting tidal rhythms. The most dramatic effects in this study were seen in the animals that were attached to the floating raft. All these animals exhibited significant daily rhythms that began over 2 h after sunrise. Interestingly, many of these animals also expressed a weak circatidal rhythm, which is virtually identical to the results of a previous study in which we recorded the activity of *Limulus* that were housed in aquaria located in a greenhouse and exposed to natural sunlight (Chabot et al. 2008). In a further parallel between these two studies, when tidal rhythms are apparent in the activity records of either in situ (Figure 5) or greenhouse animals (Chabot et al. 2008), they always occurred during the day. This suggests a masking effect of LD cycles, such that tidal activity is inhibited during darkness and ‘‘permitted’’ during the day. Similar modulatory effects of the solar day are seen in some breeding populations of *L. polyphemus* in the field (Barlow et al. 1986) although the modulation was not as clear. In *Limulus* (Chabot et al. 2007) and at least three crab species (*U. crenulata* (Honegger 1973); *Sesarma pictum* (Saigusa 1992); *Hemigrapsus sanguineus* (Saigusa and Kawagoye 1997)), LD cycles also appear to be able to transiently synchronize, or phase shift, circatidal rhythms. Thus, these species exhibit ‘‘relative coordination’’ of their circatidal rhythms to LD cycles. LD also affects behavioral rhythms in *Uca* such that tidal cycles influence the activity of some individuals while LD cycles influenced others (Honneger 1973). Interestingly, beach isopod behavior is also affected by LD cycles but primarily only if well fed, when they avoid swimming during the day (Reid 1988). It is worth noting that our animals were not fed during the experiments so hunger could have influenced their propensity to express tidal rhythms. Finally, while hydrostatic pressure changes entrain larval release in the crab *Rhithropanopeus harrisii* in tidal areas, in non-tidal areas daily rhythms of larval release are seen (Forward and Bourla 2008).

While our data indicate that temperature cycles can, in some cases, entrain *Limulus* activity rhythms (Figure 2), temperature cycles do not appear to be as effective as water level changes. During large temperature cycles (10^{o}C), 3/7 of the animals appeared to synchronize their activity to the imposed rhythm, and when the animals were allowed to free run, two of the seven showed evidence of entrainment. While this percentage is markedly lower than for water level changes (Chabot et al. 2008), or inundation (Figure 1), these data indicate that temperature changes have some, albeit weaker, input into the circatidal entrainment pathway. It should be noted that these high-temperature changes might be considered out of the normal range of temperature excursions that *Limulus* would be exposed to in their natural habitat, unless they became stranded out of water between tides. In contrast, small to moderate temperature cycles ($\pm 3^{o}$C) are very similar to what they might experience as the tides change in the Great Bay estuary. Depending on the season, the incoming Gulf of Maine water at high tide could be 0–5^{o}C colder (summer) or warmer (winter) than the estuarine water temperature at low tide. In this study, exposure to similar small ($\pm 3^{o}$C) temperature cycles, whether in the field or in the laboratory, were even less effective as synchronizing cues: only two of the eight animals appeared to synchronize to the imposed cycles (entrainment was not assessed). Thus, not surprisingly, the magnitude of the cue seems to matter, suggesting a threshold effect for temperature as well as for water depth changes. Similar results were found in

Carcinus: when subjected to 4°C temperature cycles they did not show evidence of entrainment, but when they were subjected to cycles of 11°C there was entrainment (Williams and Naylor 1969). However, these larger temperature differences may be more commonly experienced by this species, and hence more relevant, since *C. maenas* is often out of water during low tides.

We have shown that artificial currents are not able to entrain the locomotor behavior of *L. polyphemus* in the laboratory (Figure 3). Likewise, animals attached to a floating raft in the estuary also did not entrain to tides even though exposed to relatively strong currents of $0.2\,\mathrm{m\,s^{-1}}$ (Figure 5). We did see some evidence of synchronization to the turbulence cues we administered but we postulate that, since these rhythms did not persist when the currents were terminated, the currents were simply initiating short-term activity (masking) and not synchronizing the underlying circatidal clock. Further, several animals exhibited four bouts of activity per day (every time a cue was administered; Figure 3, top) instead of synchronizing their activity to a particular ‘‘high’’ or ‘‘low’’ tide. Interestingly, there is good evidence that larval *Limulus* entrain to cycles of mechanical agitation simulating the turbulence created by the incoming tide (Ehlinger and Tankersley 2006). The same type of current reversal technique used in our study has been recently shown to be effective in synchronizing eel behavior (although entrainment was not assessed; Bolliet and Labonne 2008). Moreover, in animals such as mole crabs (Forward et al. 2007), amphipods (Enright 1963), and isopods (Hastings 1981) that inhabit the swash zone, this type of mechanical stimulation appears to be the most important stimulus for entraining rhythms. However, other than during the mating season, adult horseshoe crabs rarely inhabit areas where they would encounter significant wave or wind induced turbulence or agitation on a regular basis and this is probably why they are much less sensitive to the synchronizing influences of agitation than juvenile horseshoe crabs.

While it appears that water level changes are the strongest entraining agent for horseshoe crab activity, turbulence and temperature may also contribute to entrainment. In a number of marine animals one cue is not sufficient to entrain their behavior, but when presented with a key zeitgeber the entrainment is further enhanced. One species, the amphipod *Corophium volunator*, entrains to cycles of 10°C but shows greater entrainment when subjected to temperature cycles in conjunction with cycles of immersion (Holmstrom and Morgan 1983). Water pressure and immersion cycles also appear to be important synchronizers in the *C. maenas,* while temperature may also serve to enhance entrainment to these cues (Naylor et al. 1971). Similar findings have been observed in other crustacean species as well (Williams and Naylor 1969; Naylor and Williams 1984). Nevertheless, when horseshoe crabs were subjected to the combined stimuli associated with all the natural tidal cues, except the pressure changes associated with changing water depths, they did not express tidal rhythms of activity. Therefore, our findings suggest that water level changes are the strongest entraining cue for *L. polyphemus* with temperature, currents, salinity, and other physicochemical changes playing a minor role.

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