

# Endogenous rhythms of locomotion in the American horseshoe crab, *Limulus polyphemus*

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## Abstract

American horseshoe crabs (*Limulus polyphemus*) exhibit clear circadian rhythms of visual sensitivity in the laboratory and in the field they exhibit seasonal patterns of mating behavior that are closely associated with the tides. Recent reports suggest that *Limulus* locomotor activity may be controlled by endogenous circadian and/or circatidal clocks and that light:dark (LD) cycles may affect the rhythmic output of both of these clocks. In this study, we examined locomotor behavior in the laboratory to determine the extent of this endogenous activity and to examine the influence of LD cycles on these rhythms. Thirty-three *L. polyphemus* were captured during the breeding season and their activity was monitored with activity boxes and “running wheels” in seawater kept at constant temperature and salinity. Activity patterns were analyzed using visual inspection of actograms and Chi-square and Lomb–Scargle periodograms. Overall, 36% of the animals was significantly more active during L, while only 12% was more active during D (52% showed no preference). Circatidal rhythms were observed in LD in 67% of the horseshoe crabs. Surprisingly, LD cycles appeared to synchronize these rhythms at times. In DD, the majority of animals tested (63%) exhibited circatidal rhythms that persisted for at least seven days. Overall, the results demonstrate that an endogenously controlled tidal rhythm of locomotion operates during, and significantly after, the breeding season in this species. In addition, the present results are consistent with the presence of circalunidian oscillators controlling these rhythms.

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## 1. Introduction

*Limulus polyphemus*, the American horseshoe crab, is a well known model animal for investigating visual physiology. While early studies concentrated on funda-

mental processes of the visual system, such as lateral inhibition (Hartline et al., 1956), recent studies have focused on the modulation of the visual system by an endogenous circadian clock (Barlow, 1983; Gaus et al., 1997; Pieprzyk et al., 2004). As a result of efferent signals from the brain, the sensitivity of the lateral eyes increases nearly 100,000-fold at night compared to the day (Barlow, 1983). This increase in sensitivity appears to be caused by several different factors, including

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changes in lateral eye structure (Barlow et al., 1980), lateral inhibition (Renninger and Barlow, 1979), and metabolism (Chamberlain and Barlow, 1979, 1984). However, while visual sensitivity is clearly organized on a 24-hour or circadian basis, during the breeding season when horseshoe crabs are most easily observed (Brockmann, 2003), locomotion is clearly influenced by the 12.4 hour tidal cycle.

Tidally associated breeding behavior has long been observed in *L. polyphemus*. Adult American horseshoe crabs migrate into the intertidal zone along the Eastern coast of North America in the late spring–early summer in order to attempt to mate (Rudloe, 1979; Brockmann, 2003). Spawning appears to be triggered by both elevated temperatures and photoperiod (Cohen and Brockmann, 1983; Barlow et al., 1986; Penn and Brockmann, 1994; Ehlinger et al., 2003). During this time, breeding activity is synchronized to high tides: horseshoe crabs migrate to mating areas approximately 1 h before high tide and return to deeper waters about 2 h after high tide (Barlow et al., 1986). This tidal pattern of activity is further modulated by a general preference for the highest high tide (Barlow et al., 1986). For example, Rudloe (1980) and Smith et al. (2002) found that most mating occurs around the high tides associated with the new and full moons.

While periodic environmental signals can synchronize behavioral rhythms in many intertidal species (Palmer, 1995a,b), these behaviors have also been shown to be endogenous in several species. 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; Lehmann, 1975). The shore crab (*Carcinus maenas* L.) also exhibits a circatidal locomotor activity rhythm, with peaks at the time of high tide (Naylor, 1958). The rhythm persists in constant dim light at constant temperatures, whether the crabs are kept moist in air or constantly immersed in seawater. The California fiddler crab, *Uca crenulata*, also has an endogenous rhythm that may be synchronized by tidal cues (Honeggar, 1973). Importantly, a recent pilot study involving 6 horseshoe crabs, found that they express clear circatidal rhythms in controlled laboratory conditions during the winter (Chabot et al., 2004). Interestingly, this is a time of year when this species is thought to be relatively quiescent and would not be expected to express circatidal activity. One of the goals of this study was to examine the patterns of locomotion expressed during the spring and summer when, because of their breeding activity (Brockmann, 2003), circatidal patterns of activity would be expected to be most evident. A

further goal was to determine the extent to which these rhythms are under the control of endogenous clock(s) during mating season.

While *Limulus* mating activity is clearly synchronized to high tides, time of day appears to play a large role as well. The large body of evidence demonstrating that *Limulus* eyes are more sensitive to light at night strongly suggests that they might prefer to be most active at night. This view is supported by Rudloe (1980) the finding that in Apalachee Bay, FL nocturnal peaks of mating activity are quite apparent. However, other field data indicate that nocturnal peaks of activity are not the rule. For example, a study done on Seashore Key, FL (<100 miles from Apalachee Bay) showed that peaks of activity primarily occurred 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) while on some Massachusetts beaches, *Limulus* actively breeds during day and night high tides (Barlow et al., 1986). Our own observations of *Limulus* mating in the Great Bay estuary, NH, support the view that mating occurs during both daytime and nighttime high tides (unpublished data). Given the conflicting reports concerning the breeding activity of *Limulus* in the field, it would be interesting to determine if they have an endogenous tendency to be most active at night or during the day. Two abstracts (Casterlin and Reynolds, 1979; Borst and Barlow, 2002) of studies performed with juvenile *Limulus*, indicate that they have a tendency to be nocturnal. Another major objective of this study was to extend their work and assess whether adult *Limulus* are most active during the day or night when exposed to summer photoperiods.

While one would certainly predict that circadian rhythms would be greatly influenced by LD cycles, this is not a prediction one would make about circatidal rhythms. However, circatidal rhythms appear to be modulated by light in at least three species of crabs: *Sesarma* sp. (Palmer, 1990; Saigusa, 1992), *C. maenas* (Naylor, 1985) and the California fiddler crab, *Uca crenulata*, (Honeggar, 1973; although the response for individual fiddler crabs is highly variable). The final goal of this study was to assess the effects of light on circatidal locomotor activity. Our results demonstrate that adult horseshoe crabs express robust circatidal and daily rhythms of locomotion during the breeding season, as well as for 2 months thereafter and that both rhythms were affected by LD cycles. Finally, more than a third of the *Limulus* examined exhibited significantly more activity during the day than versus night, at this time of year.

## 2. Materials and methods

### 2.1. Animals and environmental conditions

This paper is the result of work done during portions of two years: May–September, 2002 and May–August, 2003. Each year, adult male *Limulus polyphemus* were collected from the beach during late May–early June (the early part of the breeding season) at the UNH Jackson Estuarine Laboratory at Adams Point, New Hampshire. The collection site was a rocky shoreline with an abundance of gravel, cobble and hard-packed mud. Animals were collected at high tide while mating or seeking mates near the shoreline.

Following collection, the *L. polyphemus* were quickly (within 2 h) transported in a cooler to Plymouth State University, Plymouth, NH, and immediately put into individual enclosures in one of two recirculating tanks (Jewel Industries Inc., Chicago IL, Model Oceanic-55) located in a light-tight laboratory. The water temperature in these tanks was maintained between 18–22 °C and salinity was kept between 25 and 29 ppt using either seawater collected at the Jackson Estuarine Laboratory or artificial seawater (Natural Sea Salts, Oceanic Systems, Dallas, TX). Nitrate levels were kept below 100 mg/L. Lighting was provided by a single 40-watt fluorescent bulb (Coralife 10,000 K) suspended above the tanks. During the simulated daytime, the light intensity at water level was 100–150 lx (Luna Pro light meter, Gossen, Germany); at night, and during DD, it was 0 lx. The lights were on a 24-h timer and photoperiod varied with the experiments. The animals were not fed throughout the duration of the experiments, which typically lasted 80 days.

### 2.2. Activity monitoring

*L. polyphemus* were placed in either “running wheels” or activity monitoring squares. “Running wheels” (Fig. 1) were made from the bottom 12 cm of two 5-gallon buckets. These were connected to each other by a fixed piece of 2" 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. An axle was inserted through the center of the PVC wheel so that it was suspended at both ends on a triangular PVC support structure. The animals were confined to this wheel for the duration of the experiments. Two features were added to prevent them from becoming stuck in the running wheels: 1) A “tail limiter” (a 10 cm disc of stiff polypropylene plastic) was threaded 1/3 of the way up on the tail and secured with cable ties. This prevented the tail

from being withdrawn into the running wheel and becoming stuck. 2) The front of the carapace was tethered to the running wheel frame using cable ties that passed through the slit in the wheel. 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.

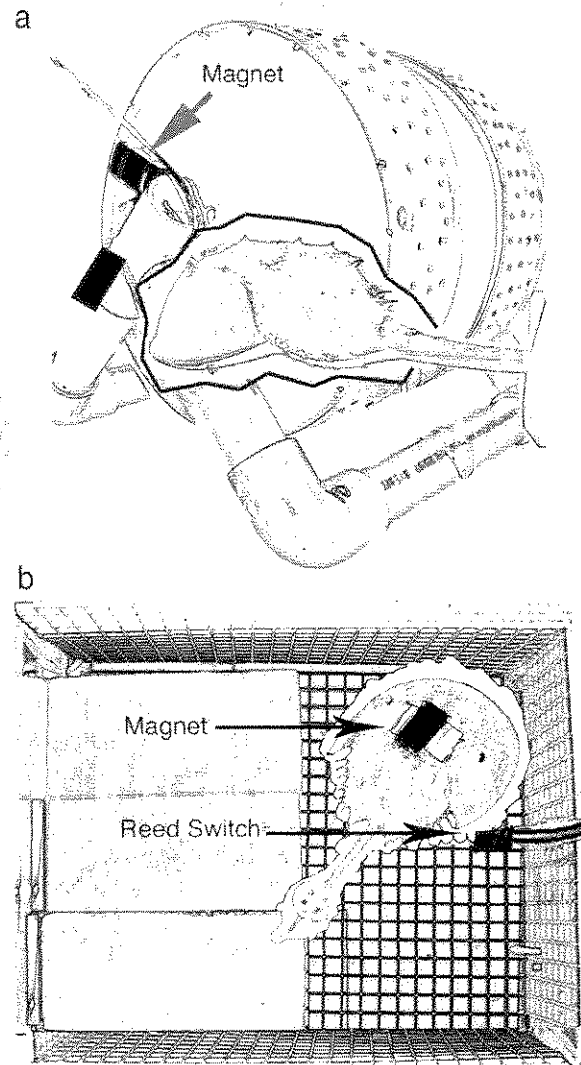


Fig. 1. Computer rendered photo of a *Limulus* (a) “running wheel” and (b) activity “square”. a. Part of the running wheel has been “cut away” to reveal the position of the animal. Note the circular “tail limiter”, magnetic reed switch (black rectangle attached to RW leg) and magnet (black rectangle). The anterior tether cannot be seen in this diagram. b. The ceiling of the square as well as some of the brick “roof” (left) has been cut away to reveal the animal. Note the magnet affixed to the dorsal carapace and the magnetic reed switch (black rectangle at right of diagram).

Eight, 40 cm × 30 cm activity monitoring “squares” (Chabot et al., 2004) were created within two recirculating aquaria (80 cm × 60 cm each) using plastic lighting grating (1 cm × 1 cm eggshell grating; Fig. 1). 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 approximately half of each activity chamber. A plastic practice golf ball was threaded approximately 2 cm on to the tail of each animal and secured with cable ties in order to prevent their telsons from becoming stuck in the plastic grating. Magnets were attached to the dorsal carapace of each animal using duct tape and cyanoacrylate glue. 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. (2004).

### 2.3. Experiment 1: rhythms in LD

The primary purpose of this experiment was to examine the effects of LD cycles on locomotor activity. A secondary purpose was to assess any possible effects of 1) time of day of capture, 2) “paired” (amplexed on a female) or solitary status of the animal, and 3) apparatus type (activity chambers or running wheels) on the expression of activity rhythms. Experiment 1 took place from 4 June, 2003 to 22 August, 2003. Sixteen male *L. polyphemus* (257–368 g) were collected and placed into one of the previously described activity chambers within 4 h of capture. They were kept in a 15:9 LD cycle throughout the duration of the experiment (80 days). The lights came on at 5:15 and went off at 20:25 (virtually the same as the natural sunrise and sunset times in the area from which they were taken).

Eight (of the aforementioned sixteen) male horseshoe crabs were collected during the high tide at 14:30 on 4 June and the other eight were collected during the following high tide at 4:45 am on 5 June. Half the animals captured on each high tide were paired with females and half were satellite or solitary males (i.e. — they were not amplexed to a female). Whether paired during capture or not, all males were tested as individuals. In Experiment 1, there were no significant effects of time of day of collection or “paired status” on tidal Tau, ( $P > 0.13$ ) or circadian Tau ( $P > 0.05$ ). Therefore, these data were combined for subsequent analysis. All sixteen of these animals were placed in either running wheels ( $n=8$ ) or activity monitoring squares ( $n=8$ ). Apparatus type had no significant effect on any rhythmic measure ( $P > 0.05$ ), therefore these data were also combined for subsequent analysis.

### 2.4. Experiment 2: rhythms in DD

The primary purpose of this experiment was to examine the effects of constant darkness (DD) conditions on locomotor activity. This experiment took place from 29 May–5 September, 2002. Eight male *L. polyphemus* (172–351 g) were collected on 29 May during the afternoon high tide and were placed in running wheels and immediately exposed to constant conditions (DD). This occurred at 21:30; 1/2 h after sundown. DD was maintained for 16 days until the evening of 13 June. Because the clarity of the rhythms in DD was diminished, we wanted to determine if the decline was due to declining poor health or to the exposure to DD (circatidal rhythms often lose their clarity after exposure to constant conditions; Palmer, 1998). Thus, they were placed back into LD on 14 June, (14:10 cycle; lights on at 11:25 am; off at 1:25 am).

An additional nine male *L. polyphemus* (199–298 g) were collected during the morning high tide on July 3 and exposed to LD (14:10); five of these were placed in running wheels and four in activity monitoring squares. Like Experiment 1, there was not a significant effect of time of capture on tidal Tau, circadian Tau, or the ratio between movement in the daytime and movement at night ( $P > 0.05$ ). Further, there were no significant effects of activity recording method on any rhythmic measure ( $P > 0.05$ ). Thus the data collected in this experiment were combined for subsequent analysis, irrespective of time of capture or apparatus used.

On 22 July, the remaining 13 *L. polyphemus* (of the 17 total used in Experiment 2) were put into DD for 13 days.

### 2.5. Data analysis

Activity data were collected in 5-minute intervals on a computer-based data collection system and analyzed via 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 Chi-square periodogram analysis (Sokolove and Bushell, 1978;  $P < 0.01$ ). With the exception of DD, at least 10 days of data were used to calculate Chi-squared periodograms. Less than 10 days were used in DD because the visual clarity of rhythmicity deteriorated within a week, on average (this is similar to what has been seen in other marine species; Palmer, 1998). The period (Tau) in the circadian range for each individual during each experiment was determined by recording the highest significant peak (if any) on the Chi-square periodogram

between 22 and 26 h. For circatidal rhythms the highest peaks between 10 and 14 h were used. In order to attempt to statistically distinguish between primary components of rhythmicity and harmonics, we also analyzed the data using the Lomb–Scargle periodogram, a test that has been suggested to be superior to the Chi-square periodogram (Van Dongen et al., 1999). Since the average Taus generated by either of these periodograms were statistically indistinguishable ( $P > 0.05$ ) only Chi-square periodograms are presented here.

To simplify presentation of the results, when an animal expressed a bimodal rhythm we considered it to have an activity cycle that was primarily organized in a circatidal manner. These “bimodal” (circatidal) animals inevitably exhibit both circa-12 h rhythms AND circa-24 h rhythms, most likely because the periodogram analyses report harmonics of the basic (circatidal) rhythm (e.g. Fig. 2 — top panel). However, when we observed a unimodal rhythm with a period of between 22–26 h we considered this animal’s activity cycle to be primarily organized on a daily (in LD) or a circadian (in DD) basis. In two cases, the animals exhibited alternate unimodal and bimodal activity. These animals were grouped with the circatidal animals for purposes of analysis.

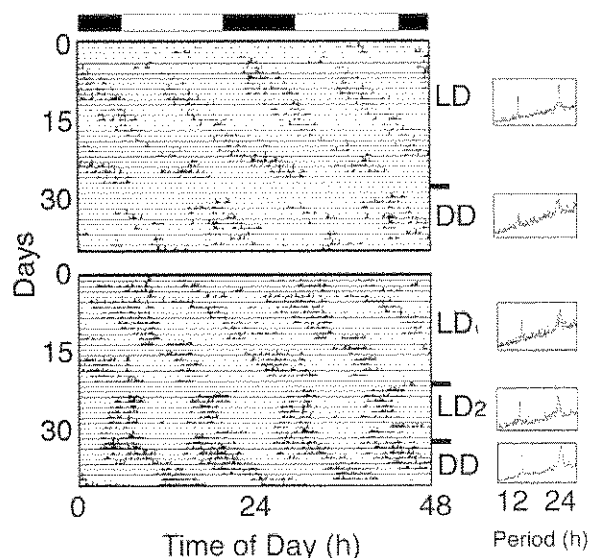


Fig. 2. Free-running locomotor activity rhythms of two individual *L. polyphemus* exposed to LD 14:10 that indicate clear tidal patterns of activity (left panels). Data are double-plotted to facilitate visual inspection. Periodogram analyses of sections of the actograms (separated by horizontal bar) are presented in the right panels. The two different LD sections of the bottom actogram were analyzed separately to illustrate either free-running (LD<sub>1</sub>) or entrained (LD<sub>2</sub>) periods.

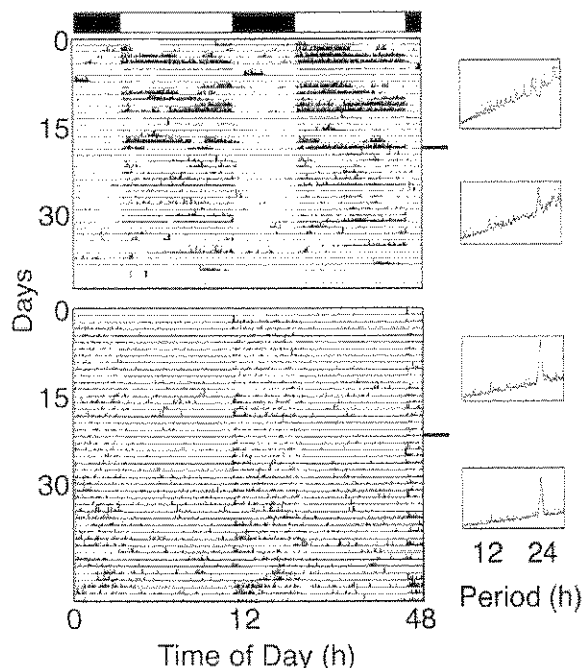


Fig. 3. Running-wheel activity of two horseshoe crabs exposed to 14:10 LD and exhibiting a preference ( $P < 0.05$ ) for daytime activity (top) or nighttime activity (bottom).

To determine an animal’s possible preference for activity during L (suggesting “diurnality”) or D (suggesting “nocturnality”), the amount of activity recorded each day for each animal during the L portion of the day was summed and compared to the amount of activity expressed during the D portion of the day (paired Student’s *t* tests for each animal,  $P < 0.05$ . Paired Student’s *t*-test or repeated measures ANOVA ( $P < 0.05$ ; Statview, Abacus Concepts, Berkeley CA) were used in other cases to determine statistical significance between means.

### 3. Results

#### 3.1. Light dark conditions

##### 3.1.1. Tidal rhythms

Locomotor activity records of two animals exposed to a “summer” LD cycle and subsequently to constant darkness (DD) are presented in Fig. 2. Both the activity records (left panels) and the periodogram analyses (right panels) clearly show robust rhythms in the 12.4-hour range, with one animal (top of Fig. 2) expressing a circatidal rhythm that drifts relative to the LD cycle, and the other (bottom of Fig. 2) displaying patterns that both transiently synchronize to the LD cycle and also drift

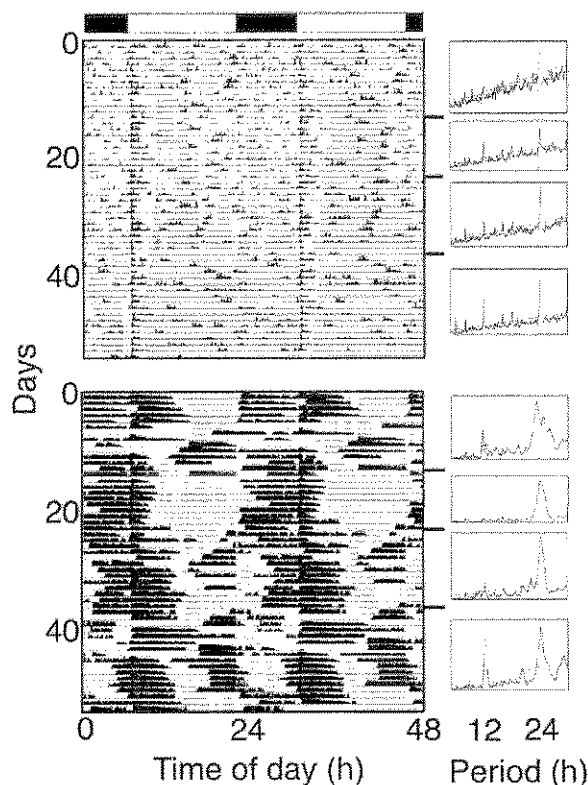


Fig. 4. Running-wheel activity of two horseshoe crabs exposed to 14:10 LD and exhibiting “masking” (direct effects of lights on or lights off) as well as spontaneous changes in Tau (bottom panel). Top Panel — lights on increased in activity; Bottom panel — lights off increased activity.

with respect to LD. Over the course of two summers (2002 and 2003), 67% of the *L. polyphemus* tested displayed significant ( $P < 0.01$ ) circatidal rhythmicity when exposed to summer LD conditions (22/33  $\text{Tau} = 12.25 \pm 0.40$ , (SEM)). Of the animals that displayed circatidal activity, 50% expressed circatidal rhythms that were often synchronized to the LD cycle (11/22; Fig. 2 — bottom panel) while 45% exhibited occasional free-running circatidal patterns in LD, alternating with periods of synchronization to LD (10/22; Fig. 2 — bottom panel). One *L. polyphemus* showed a circatidal pattern with no apparent synchronization to LD (Fig. 2, top panel). Overall, circatidal patterns of activity were similar both during (Fig. 2; days 1–30) and after (Fig. 2; days 31–end) times when animals were observed breeding in the Great Bay estuary.

### 3.1.2. Daily rhythms and LD activity patterns

While most horseshoe crabs exhibited circatidal rhythms as their primary component, many of the remaining animals (8/11) exhibited significant rhythms

( $P < 0.01$ ) in the 24 hour range (Fig. 3;  $\text{Tau} = 23.89 \pm 0.80$ ). Interestingly, in two cases, circatidal rhythms disappeared and then reappeared over the course of the 8 weeks of the experiment (Fig. 4 — bottom panel). We were somewhat surprised to find that 36% of the 33 animals tested moved significantly more during L ( $P < 0.05$ ; 12/33; Fig. 3 top panel), while only 12% (4/33) moved significantly more in the dark ( $P < 0.05$ ; Fig. 3 bottom panel). The majority (52%; 17/33) did not exhibit significantly more activity in either the light or the dark (Fig. 2 — top and bottom panels; Fig. 4 — top panel). Overall, daily patterns of activity were similar both during (Fig. 3 — days 1–30) and after (Fig. 3 — days 31–end) the seasonal breeding activity observed in the field.

### 3.1.3. Lights on/off effect

There were clear effects of “lights on” and “lights off” on locomotor activity. Most animals (88%; 29/33) exhibited immediate changes in activity in response to either lights on or lights off. All of the animals (12/12) that moved significantly more in L became immediately active when the lights came on (Fig. 3, top panel). Furthermore, when the lights went off, most of these animals (58%; 7/12) immediately stopped activity (Fig. 3 — top panel). In addition, of those animals that were most active during D, most (75%; 3/4) became immediately active when the lights went off (Fig. 3, bottom panel) and 50% (2/4) of them decreased their activity immediately when the lights went on (Fig. 3, bottom panel). Even among those that did not exhibit significant tendency for activity in L or D; 65% (11/17) showed immediate increases in activity due to lights on (Fig. 4, both panels) while only 29% (5/17) showed immediate increase due to lights off (data not shown).

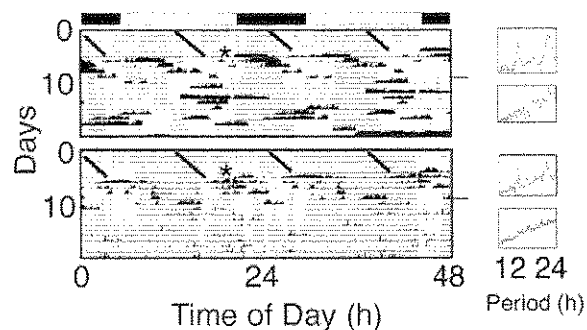


Fig. 5. Running-wheel activity of two horseshoe crabs captured (\*) in Durham (Great Bay), NH and immediately placed into running wheels in a lab with DD conditions in 2002. Dark/light bars: LD conditions that animals were previously exposed to. Sloping lines at top of records indicates times when natural high tides took place at the site of capture.

### 3.2. Constant conditions (DD)

#### 3.2.1. Circatidal and circadian rhythms

Two representative examples of the effects of exposure to DD on the day of capture are presented in Fig. 5. Both of these animals exhibited significant circatidal rhythms during the initial 6 days of recording. Of the eight *L. polyphemus* that were immediately placed in constant darkness following capture, seven (88%) showed significant circatidal rhythmicity ( $\text{Tau} = 12.03 \pm 0.03$ ; Fig. 5). The phase of these rhythms could not be clearly correlated with the phase of the previous high tides that the animals had been exposed to in Great Bay. The remaining animal showed a primary component in the circadian range ( $\text{Tau} = 24.08$ ; data not shown). The rhythms expressed by all of these animals generally disappeared within a week (Fig. 5). Rhythmic locomotion subsequently re-emerged in most animals (6/8; 3/8 — circatidal range; 3/8 — circadian range) when later exposed to LD. Of eleven animals that were initially exposed to LD in the lab, and later exposed to DD, 45% (5/11) exhibited significant circatidal rhythmicity in DD ( $\text{Tau} = 13.00 \pm 0.96$ , Fig. 2). Importantly, the phase of activity at the beginning of DD was clearly correlated with the activity phase at the end of LD (Fig. 2). One of these eleven animals later exhibited significant circadian behavior in DD ( $\text{Tau} = 24.56$ , data not shown). In general, although horseshoe crabs consistently expressed both circatidal and circadian rhythms of activity in DD, tidal rhythms were most common. In addition, they were generally less robust and persistent than the tidal rhythms recorded in LD.

## 4. Discussion

### 4.1. Tidal rhythms

We report here that an endogenously controlled tidal rhythm of locomotion is present during, and significantly after, the breeding season in *L. polyphemus*. Most individual *L. polyphemus* in this study showed clear and robust circatidal rhythms of locomotor behavior. Since all of our investigations on *Limulus* were conducted in constant conditions, with respect to tidal cues, the evidence is clear that there are endogenous oscillators that control circatidal rhythms of locomotion in this species. These results are similar to those found in a previous laboratory study on this species conducted during winter months (Chabot et al., 2004). Endogenous circatidal rhythmicity characterizes many truly intertidal species such as the shore crab *Carcinus maenas* (Naylor, 1958), the isopod *Excirologa chiltoni* (Klapow, 1972;

Enright, 1976), and the shrimp *Crangon crangon* (Al-Adhub and Naylor, 1975). The presence of such an oscillator(s) would allow *Limulus* in the field to potentially anticipate and synchronize to the natural tidal cycles when it is an intertidal visitor.

### 4.2. Circatidal rhythms — out of breeding season

Our results also show that *Limulus* can exhibit tidal rhythms in the fall — nearly 3 months after the end of the mating season. Field studies have only demonstrated tidally-oriented behavior during the late spring–early summer when it is easy to observe horseshoe crabs that approach the beach to mate (Rudloe, 1980; Cohen and Brockmann, 1983). A previous study reported that they are able to express tidal rhythms in mid-winter, if provided with “summer time” photoperiods (14:10) and water temperatures (17–20 °C; Chabot et al., 2004). It is interesting that an animal that is thought to be intertidal only during a few weeks during early summer can exhibit circatidal rhythms during other times of year. Either this is indicative of their normal behavior, and such tidal rhythms in subtidal horseshoe crabs serve an adaptive purpose that has yet to be discovered, or the environmental conditions we exposed them to in the laboratory (essentially summertime temperatures and photoperiod) triggered behavioral rhythms that are normally only expressed during the spring/summer mating season. Studies are currently underway to address both these possibilities.

### 4.3. Light effects on circatidal rhythms

We were surprised to find clear effects of LD cycles on circatidal rhythms. While many physical factors are likely to entrain or influence tidal cycles (e.g. — salinity or inundation cycles) an effect of LD cycles on circatidal rhythms is not intuitive. As one would predict, the tidal rhythms of most tidal species that have been studied are not affected by the LD cycle (DeCoursey, 1983; Palmer, 1995a,b). However, in the current study, in all cases but one (1/22; Fig. 2 — top panel), patterns of tidal activity in our animals appeared to be influenced by LD cycles to a certain extent (Figs. 2, 3 and 4). In several cases the circatidal rhythms were entrained by LD cycles and when released into DD the rhythms appeared to freerun from the point of entrainment. Interestingly, similar results have been seen in at least two other marine species. Tidal locomotor activity in *Sesarma pictum* (Saigusa, 1992) and *U. crenulata* (Honeggar, 1973) can be phase shifted by LD cycles. In the present case, the influence that the LD cycle has on tidal rhythms is

intriguing and two-fold: 1) Overall, for *L. polyphemus*, we saw clearer tidal rhythms in LD than DD (e.g. Fig. 2 top vs. bottom panels; Fig. 2 vs. Fig. 5). 2) Tidal rhythms can synchronize to LD cycles for several days (Figs. 2 and 4). In two previous studies, LD cycles seem to clarify circatidal rhythms in the semi-terrestrial crab (Saigusa 1992) while Stillman and Barnwell (2004) have shown a similar LD modulation of locomotor activity in the Pacific fiddler crab *Uca princeps*.

From a functional point of view, one would not expect photoperiod to have any effect on circatidal rhythms as photoperiod is obviously not a strong predictor of tidal phase. However, since moon phase has also been reported to affect breeding activity in *Limulus* (Barlow et al., 1986), it would be interesting to determine whether animals that approach beaches preferentially around full or new moons are the individuals whose circatidal activity is most affected by photic input. Alternatively, it is possible that the effects of light act on the underlying oscillators during locomotor activity in a more indirect way. Among its other effects reported here, the lights on and lights off transitions cause a large percentage of animals to either start or stop movement (Figs. 3 and 4). In particular, these transitions appear to cause a large percentage of animals to begin to move. In mammals, acute increases in activity can induce phase shifts in some species (Wickland and Turek, 1991; Gannon and Rea, 1995; Buxton et al., 2003). This suggests that activity may feedback on the oscillatory system that controls activity in mammals and affect the phasing of their activity. If the induced activity seen in *Limulus* likewise feeds back onto the oscillatory system that mediates circatidal rhythms then this would help to explain how the light signals of photoperiod may (counter-intuitively) affect circatidal rhythms. Studies currently underway, using more natural, gradual, light cycle transitions, indicate that, while these cycles can produce less or even no masking, strong tidal rhythms are still present.

Alternatively, one might argue that, since light affects the two daily bouts of activity, this is instead evidence of a circadian clock system controlling crepuscular (dawn/dusk) activity. However, there are at least three lines of evidence that both argue against this “crepuscular” hypothesis and support the hypothesis that activity in this species is instead tidally organized: 1) robust breeding activity in the field is only observed during high tides and not at dawn and dusk (except during the time of month when the high tides take place at this time; Barlow et al., 1986); 2) there is a general lack of strong, persistent entrainment to LD cycles in many animals in the present study (Fig. 2), in a previous study

(Chabot et al., 2004) and during ongoing investigations with more natural, gradual, light:dark transitions and; 3) We also have independent evidence that tidal cues (inundation cycles) can synchronize these rhythms (Chabot et al., in preparation). If these were solely (crepuscular) circadian rhythms, one would not predict tidal cues to affect the rhythms in any way. Whether the effects of light on activity rhythms in *Limulus* are due to the influence of photoperiods on circadian (Naylor, 1996), circalunidian (Palmer, 1995b) or circatidal (Naylor, 1996) clocks remains to be elucidated.

#### 4.3.1. Daily activity patterns

One of the most surprising results from our study was that fully a third of the animals tested moved significantly more during L than during D. This was a consistent finding during both years of the study. Over half of the animals had no preference for increased activity during L or D while only 12% moved more during D. A large body of evidence from laboratory studies has demonstrated nocturnal increases in retinal sensitivity (Barlow, 1988; Barlow et al., 2001) driven by an endogenous circadian pacemaker in the brain (Kass and Barlow, 1992). In addition, some field studies (Rüdloe, 1979, 1980), suggest that *Limulus* is primarily a nocturnally active species. Thus, we were surprised that such a small percentage of the animals tested preferred to be most active at night. These data are, however, consistent with the results from several other field studies that have indicated a preference for, or ambivalence towards, increased activity during L and D portions of the LD cycle. For example, during breeding season in Cape Cod, MA, *Limulus* approach beaches to mate during both daytime and nighttime high tides (Barlow et al., 1986) and, in one study in Florida, most of the horseshoe crabs were diurnally active (Cohen and Brockmann, 1983). In the population of *L. polyphemus* that we are studying in Great Bay, NH approximately equal numbers of animals approach beaches during the day and night high tides during the breeding season (Schaller, Chabot and Watson, unpublished data). At the very least, the evidence presented in this study, and in some previous field studies, suggests that not all *L. polyphemus* are primarily nocturnally active. Moreover, these findings highlight potential differences between the timing mechanisms underlying the physiological changes in the retina (Barlow et al., 2001) and those governing overall locomotor activity (Chabot et al., 2004).

#### 4.3.2. Daily and circadian rhythms of locomotion

Some animals also expressed daily and circadian rhythms of locomotion. In LD, 8/33 of the animals



exhibited a primary component of 24 h (a “daily” rhythm). Most of these daily rhythms appeared to occur (at least in part) due to direct effects of the onset of L (or the onset of D). Recent results from our lab show that this activity around lights on or lights off may be induced by the suddenness of the transitions: the activity around lights on disappears when we use a system that produces gradual increases and decreases in light instead of the sudden transitions used in the current experiments (Chabot et al., unpublished obs.; Watson et al., unpublished obs.). The suggestion that the daily rhythms that we observed in the current studies are directly induced by the LD cycles is supported by the finding that only 1/8 animals caught and put directly into DD expressed an endogenous circadian pattern as their primary rhythm of locomotory activity (data not shown). Similarly, only 1/4 of animals kept in the lab for >40 days later showed primarily circadian rhythms in DD. Circadian locomotor rhythmicity in *L. polyphemus* has been previously reported during the winter months in adults (Chabot et al. 2004). Circadian rhythms of locomotion have also been reported in juvenile *Limulus* (Borst and Barlow, 2002). These results are not surprising since numerous researchers have demonstrated robust circadian rhythmicity of retinal sensitivity in this species (Kaplan and Barlow, 1980; Barlow, 1983; Barlow et al., 2001; Pieprzyk et al., 2003). This visual sensitivity in *Limulus* has been well documented over the past two decades and the clock regulating this rhythm has been localized to the protocerebrum (Kass and Barlow, 1992). While this study suggests that locomotion is influenced in part by an endogenous circadian oscillator, the small percentage of animals exhibiting primarily circadian rhythms in DD, combined with the relatively weak effects of LD cycles on their activity, suggests a weak influence of a circadian oscillator on locomotor activity. Rather, the most dominant oscillator driving locomotion in *Limulus* appears to operate on a 12.4 h cycle. Further studies are underway to test the hypothesis that different endogenous oscillators modulate visual sensitivity and locomotion.

#### 4.3.3. Clock types

While circatidal activity patterns have been documented in many intertidal species, the nature of the clock system that controls these rhythms is somewhat controversial (Palmer, 1995b; Naylor, 1996; Palmer, 1997; Naylor, 1997). An outstanding question in the field of biological rhythms is whether the locomotor activity of intertidal species is driven by two circalunidian clocks or by one circatidal clock (with circadian influence). Naylor and colleagues (Reid and Naylor,

1989) hypothesize that intertidal animals have one circatidal clock that drives behavioral rhythms and one circadian clock that modifies those rhythms (Naylor, 1958). Alternatively, the circalunidian hypothesis, first proposed by Palmer and Williams (1986), proposes that circatidal rhythms can be best explained by the presence of two circalunidian clocks; each with a period of circa 24.8 h (approx. the periodicity of the moon hence “lunidian”) and 180° out of phase with one another. Behaviorally, this produces major activity bouts every 12.4 h (just like a circatidal clock would) and can be alternatively invoked to explain the type of rhythm seen in virtually all of our data records. While any conclusion is tentative, our data seem to fit the three criteria put forth by Palmer (1997) as part of the circalunidian hypothesis: 1) Evidence of two components of rhythms that scan the day at different periodicities; 2) Evidence of “skipping” — sudden alternations of unimodal and bimodal patterns; and 3) “Splitting” — the separation of one component into two components. All three of these criteria were most clearly met by two of the animals in this study (for example, see Fig. 4 — bottom panel). It is difficult to imagine how a single oscillator (or a circatidal oscillator modulated by a circadian oscillator) could give rise to the data shown in Fig. 4b. Similar arguments to those made by Palmer (1997) have been put forth by circadian biologists to argue the presence of two functionally separate circadian clocks in many species, including such widely diverse classes of animals as fish and rodents (Pittendrigh and Daan, 1976). The data seen in Fig. 4 (bottom panel) is similar to the “splitting” phenomenon observed in rodents (Pittendrigh and Daan, 1976). Similar splitting of two “circalunidian components” has also been seen in the locomotor activity of the crab *Helice crassa* (Palmer and Williams, 1986). However, the relative rarity of these phenomena in our data (2/33 animals) suggests that, if there are two oscillators governing the timing of locomotion in *Limulus*, they are strongly coupled. Thus, although we have some evidence to support the circalunidian hypothesis, convincing evidence supporting a coupled, two-oscillator system (such as is found in trout; Erikson, 1973, hamsters; Pittendrigh and Daan, 1976; and *Drosophila*; Stoleru et al., 2004) has yet to be obtained.

As noted above, it would appear that some of our data support the presence of a circadian oscillator that modulates locomotion because in DD some animals exhibit periodicities in the 24 h range. However, it should be noted that our analyses, whether visual or periodogram, cannot differentiate between circadian (period of circa 24 h) versus circalunidian (period of circa 24.8 h)

periodicities. Indeed, many species express circadian rhythms with periods that are 24.8 h or longer (Pittendrigh and Daan, 1976). In LD, it is also likely that, in some instances, direct responses to the LD cycle are causing periodicities in the 24 h range (Figs. 3 and 4). Another possibility is that the circa-24 h rhythm is, in many cases, an artifact; periodogram analyses often produce harmonics of underlying rhythms, so that, for example, animals expressing a 12.2 hour tidal rhythm will also appear to have a 24.4 hour circadian pattern (Van Dongen et al., 1999). However, it is important to remember that there is exceptionally clear evidence that a circadian clock controlling many physiological and anatomical attributes of the *Limulus* lateral eye (cf. Barlow et al., 2001) and to summarily dismiss the circa 24 hour activity rhythms seen in this study as an artifact seems premature at this juncture. Further, there is evidence that several other *Limulus* behaviors are modulated in a circadian fashion (Powers and Barlow, 1985). Interestingly, while dozens of papers have been published documenting robust circadian rhythms of lateral eye changes, as far as we can determine, there has been no published indication of circatidal modulation of visual sensitivity. This suggests to us that there are at least two functionally separate endogenous timing systems in this species: one oscillatory system controlling locomotion and another controlling visual system changes. Whether these timing systems interact at all with each other remains to be elucidated.

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### References

- Al-Adhub, A.H., Naylor, E., 1975. Emergence rhythms and tidal migrations in the brown shrimp, *Crangon crangon* (L.). J. Mar. Biol. Assoc. U.K. 55, 801–810.
- Barlow, R.B., 1983. Circadian rhythms in the *Limulus* visual system. J. Neurosci. 3, 856–870.
- Barlow, R.B., 1988. Circadian rhythm in sensitivity of the *Limulus* retina nearly compensates for day–night changes in ambient illumination. Invest. Ophthalmol. Visual Sci. 29, 350.
- Barlow, R.B., Chamberlain, S.C., Levinson, J.C., 1980. The *Limulus* brain modulates the structure and function of the lateral eye. Science 210, 1037–1039.
- Barlow Jr., R.B., Powers, M.K., Howard, H., Kass, L., 1986. Migration of *Limulus* for mating: relation to lunar phase, tide height, and sunlight. Biol. Bull. 171, 310–329.
- Barlow, R.B., Hitt, J.M., Dodge, F.A., 2001. *Limulus* vision in the marine environment. Biol. Bull. 200, 169–176.
- Bennett, M.F., Shriner, J., Brown, R.A., 1957. Persistent tidal cycles of spontaneous motor activity in the fiddler crab, *Uca pugnax*. Biol. Bull. 112, 267–275.
- Borst, D., Barlow, R.B., 2002. Circadian rhythms in locomotor activity of juvenile horseshoe crabs. Biol. Bull. 203, 227–228.
- Brockmann, H.J., 2003. Nesting behavior: a shoreline phenomenon. In: Shuster, C.N., Barlow, R.B., Brockmann, H.J. (Eds.), The American Horseshoe Crab. Harvard Univ. Press, Cambridge, MA, p. 33–49.
- Buxton, O.M.C., Lee, W., L'Hermite-Balériaux, M., Turek, F.W., Van-Cauter, E., 2003. Exercise elicits phase shifts and acute alterations of melatonin that vary with circadian phase. Am. J. Physiol. 284, R714–R724.
- Casterlin, M., Reynolds, W., 1979. Diel locomotor activity pattern of juvenile *Limulus polyphemus* Linnaeus. Rev. Can. Biol. 38, 43–44.
- Chabot, C.C., Menaker, M., 1992. Effects of physiological cycles of infused melatonin on circadian rhythmicity in pigeons. J. Comp. Physiol. 170, 615–622.
- Chabot, C.C., Kent, J., Watson, W.H., 2004. Daily, circadian and tidal rhythms of locomotor activity in the horseshoe crab *Limulus polyphemus*. Biol. Bull. 207, 72–75.
- Chamberlain, S.C., Barlow, R.B., 1979. Light and efferent activity control rhabdom turnover in *Limulus* photoreceptors. Science 206, 361–363.
- Chamberlain, S.C., Barlow, R.B., 1984. Transient membrane shedding in *Limulus* photoreceptors: control mechanisms under natural lighting. J. Neurosci. 4, 2792–2810.
- Cohen, J.A., Brockmann, H.J., 1983. Breeding activity and mate selection in the horseshoe crab, *Limulus polyphemus*. Bull. Mar. Sci. 33, 274–281.
- DeCoursey, P.J., 1983. Biological timing. In: Vernberg, F.J., Vernberg, W.B. (Eds.), The Biology of Crustacea, vol 7. Academic Press, San Diego, pp. 107–162.
- Ehlinger, G.S., Tankersley, R.A., Bush, M.B., 2003. Spatial and temporal patterns of spawning and larval hatching by the horseshoe crab, *Limulus polyphemus*, in a microtidal coastal lagoon. Estuaries 26, 631–640.
- Enright, J.T., 1976. Plasticity in an isopod's clockworks: shaking shapes form and affects phase and frequency. J. Comp. Physiol. 107, 13–37.
- Erikson, L.O., 1973. Spring inversion of the diel rhythm of locomotor activity in young sea going trout and Atlantic salmon. Aquilo, Ser. Zool. 14, 69–79 as cited in Pittendrigh, C.S. 1981 Circadian systems: Entrainment. Pp. 95–124 in Aschoff, J., Handbook of Behavioral Neurobiology; Biological Rhythms. Plenum Press, New York.
- Gannon, R.L., Rea, M.A., 1995. Twelve-hour phase shifts of hamster circadian rhythms elicited by voluntary wheel-running. J. Biol. Rhythms 10, 196–210.
- Gaus, G., Casaretto, M., Kass, L., 1997. The effect of neuropeptides from *Limulus* on its circadian rhythm in retinal sensitivity. J. Comp. Physiol. 180, 137–142.
- Hartline, H.K., Wagner, H.G., Ratliff, F., 1956. Inhibition in the eye of *Limulus*. J. Gen. Physiol. 39, 651–673.
- Honegger, H.W., 1973. Rhythmic activity responses of the fiddler crab *Uca crenulata* to artificial tides and artificial light. Mar. Biol. 21, 169–202.
- Kaplan, E., Barlow, R.B., 1980. Circadian clock in *Limulus* brain increases response and decreases noise of retinal photoreceptors. Nature 286, 393–395.

- Kass, L., Barlow, R.B., 1992. A circadian clock in the *Limulus* brain transmits synchronous efferent signals to all eyes. *Vis. Neurosci.* 9, 493–504.
- Klapow, L.A., 1972. Natural and artificial rephasing of a tidal rhythm. *J. Comp. Physiol.* 79, 233–258.
- Lehmann, U., 1975. Interpretation of entrained and free-running locomotor activity patterns of *Uca*. In: DeCoursey, P.J. (Ed.), *Biological Rhythms in the Marine Environment*. University of South Carolina Press, Columbia, South Carolina, pp. 77–92.
- Naylor, E., 1958. Tidal and diurnal rhythms of locomotory activity in *Carcinus maenas* (L.). *J. Exp. Biol.* 35, 602–610.
- Naylor, E., 1985. Tidally rhythmic behavior of marine animals. *Symp. Soc. Exp. Biol.* 39, 63–93.
- Naylor, E., 1996. Crab clockwork: the case for interactive circatidal and circadian oscillators controlling rhythmic locomotor activity of *Carcinus maenas*. *Chronobiol. Int.* 13, 153–161.
- Naylor, E., 1997. Crab clocks rewound. *Chronobiol International* 14, 427–430.
- Palmer, J.D., 1963. "Circa-tidal" activity rhythms in fiddler crabs. *Biol. Bull.* 125, 387 (abstract).
- Palmer, J.D., 1990. Comparative studies of tidal rhythms. X. A dissection of the persistent activity rhythms of *Sesarma*. *Mar. Behav. Physiol.* 17, 177–187.
- Palmer, J.D., 1995a. *The Biological Rhythms and Clocks of Intertidal Animals*. Oxford University Press, New York, NY.
- Palmer, J.D., 1995b. Review of the dual-clock control of tidal rhythms and the hypothesis that the same clock governs both circatidal and circadian rhythms. *Chronobiol. Int.* 12, 299–310.
- Palmer, J.D., 1997. Dueling hypotheses: circatidal versus circalunidian battle basics. *Chronobiol. Int.* 14, 337–346.
- Palmer, J.D., Williams, B.G., 1986. Comparative studies of tidal rhythms II. The dual clock control of the locomotor rhythms of two decapod crustaceans. *Mar. Behav. Physiol.* 12, 269–278.
- Penn, D., Brockmann, H.J., 1994. Nest-site selection in the horseshoe crab, *Limulus polyphemus*. *Biol. Bull.* 187, 373.
- Pieprzyk, A.R., Weiner, W.W., Chamberlin, S.C., 2003. Mechanisms controlling the sensitivity of the *Limulus* lateral eye in natural lighting. *Behav. Physiol.* 189, 643–653.
- Pittendrigh, C.S., Daan, S., 1976. A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 106, 291–331.
- Powers, M.K., Barlow, R.B., 1985. Behavioral correlates of circadian rhythms in the *Limulus* visual system. *Biol. Bull.* 169, 578–591.
- Reid, D.G., Naylor, E., 1989. Are there separate circatidal and circadian clocks in the shore crab *Carcinus maenas*? *Mar. Ecol. Prog. Ser.* 52, 1–6.
- Renninger, G.H., Barlow, R.B., 1979. Lateral inhibition, excitation, and the circadian rhythm of the *Limulus* compound eye. *Soc. Neurosci. Abstr.* 5, 804 (abstract).
- Rudloe, A., 1978. Some ecologically significant aspects of the behavior of the horseshoe crab, *Limulus polyphemus*. Ph.D. Dissertation, Florida State University, Tallahassee, FL. 246 pp.
- Rudloe, A., 1979. Locomotor and light responses of larvae of the horseshoe crab, *Limulus polyphemus* (L.). *Biol. Bull.* 157, 494–505.
- Rudloe, A., 1980. The breeding behavior and patterns of movement of horseshoe crabs, *Limulus polyphemus*, in the vicinity of breeding beaches in Apalachee Bay, Florida. *Estuaries* 3, 177–183.
- Saigusa, M., 1992. Phase shift of a tidal rhythm by light–dark cycles in the semi-terrestrial crab, *Sesarma pictum*. *Biol. Bull.* 182, 257–264.
- Smith, D.R., Pooler, P.S., Swan, B.L., Michels, S.F., Hall, W.R., Himchak, P.J., Millard, M.J., 2002. Spatial and temporal distribution of Horseshoe crab (*Limulus polyphemus*) spawning in Delaware bay: implications of monitoring. *Estuaries* 25, 115–125.
- Sokolove, P.G., Bushell, W.N., 1978. The chi-square periodogram: its utility for analysis of circadian rhythms. *J. Theor. Biol.* 74, 131–160.
- Stellman, J.H., Barnwell, F.H., 2004. Relationship of daily and circatidal activity rhythms of the fiddler crab, *Uca princeps*, the harmonic structure of semidiurnal and mixed tides. *Mar. Biol.* 144, 473–482.
- Stoleru, D., Peng, Y., Agosto, J., Roshbash, M., 2004. Coupled oscillators control morning and evening locomotor behavior of *Drosophila*. *Nature* 431, 862–868.
- Van Dongen, H.P.A., Olafson, E., VanHartevelt, J.H., Kruyt, E.W., 1999. Searching for biological rhythms: peak detection in the periodogram of unequally spaced data. *J. Biol. Rhythms* 14, 617–620.
- Wickland, C.R., Turek, F.W., 1991. Phase-shifting effects of acute increases in activity on circadian locomotor rhythms in hamsters. *Am. J. Physiol.* 261, R1109–R1117.