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Circatidal rhythms of locomotion in the American horseshoe crab *Limulus polyphemus*: Underlying mechanisms and cues that influence them

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Abstract While eye sensitivity in the American horseshoe crab *Limulus polyphemus* has long been known to be under the control of an endogenous circadian clock, only recently has horseshoe crab locomotion been shown to be controlled by a separate clock system. In the laboratory, this system drives clear activity rhythms throughout much of the year, not just during the mating season when horseshoe crabs express clear tidal rhythms in the field. Water temperature is a key factor influencing the expression of these rhythms: at 17°C tidal rhythms are expressed by most animals, while at 11°C expression of circatidal rhythms is rarely seen, and at 4°C rhythms are suppressed. Neither long (16:8 Light:Dark) nor short (8:16) photoperiods modify this behavior at any of these temperatures. Synchronization of these circatidal rhythms can be most readily effected by water pressure cycles both *in situ* and in the lab, while temperature and current cycles play lesser, but possibly contributory, roles. Interestingly, Light:Dark cycles appear to have synchronizing as well as "masking" effects in some individuals. Evidence that each of two daily bouts of activity are independent suggests that the *Limulus* circatidal rhythm of locomotion is driven by two (circalunidian) clocks, each with a period of 24.8h. While the anatomical locations of either the circadian clock, that drives fluctuations in visual sensitivity, or the circatidal clock, that controls tidal rhythms of locomotion, are currently unknown, preliminary molecular analyses have shown that a 71 kD protein that reacts with antibodies directed against the *Drosophila* PERIOD (PER) protein is found in both the protocerebrum and the subesophageal ganglion [*Current Zoology* 56 (5): 499–517, 2010].

Key words Circatidal, Circadian, Circalunidian, Rhythms, Behavior, PER, Tides, Locomotion

1 Rhythms in *Limulus*: An Overview

The goal of this review is to demonstrate that time is a crucial factor to consider when examining behavior in American horseshoe crabs *Limulus polyphemus*. After brief reviews of the importance of time to all organisms, we provide evidence that time of tide, time of day, time of year, and, possibly, time of moon phase all impact the expression of locomotor activity in this species. Further, we summarize the strong evidence for an endogenous clock system that controls behavioral circatidal rhythms in this species. Next, we discuss the different tidal, daily and seasonal factors that may serve to synchronize these behavioral rhythms to the changing environment. Then, we review the evidence that the timing system that controls circatidal behavioral activity in *Limulus* is composed of two clocks, termed circalunidian clocks because their periods are the same as that of the "daily" lunar cycle, 24.8 h. Lastly, we discuss the importance of the PERIOD protein (PER) as a piece of the molecular apparatus of the circadian clock of other model animals and suggest that a homologue of this protein may be part of the timing systems in *Limulus*.

1.1 Circadian rhythms

Most organisms reside in a world influenced by ubiquitous rhythmic physical processes dictated primarily by the revolutions and orbits of the sun, the earth, and the moon. The motions of these heavenly bodies create rhythms of tides, lunar phase, seasons and most universally important to all species, changes in the light/dark cycle associated with time of day. The synchronization of an organism's behavior and physiology to these external signals appears to be adaptive, especially when it comes to time of day: every organism that has been studied sufficiently, with the exception of Archaebacteria, has been shown to have an endogenous timing system (or endogenous clocks) that helps the organism to synchronize to, and anticipate, these daily

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changes. These “circadian” clocks (circa - about; dian - day) have been extensively studied since their discovery several decades ago (Gardner et al., 2006) and today much is known about this timing system from the adaptive advantages (DeCoursey, 2004) to the underlying molecular machinery (Dunlap, 2004).

Synchronization of behaviors to the solar day is ubiquitous among eukaryotic organisms (Johnson et al., 2004), including numerous species of marine invertebrates. When these animals are brought into the lab and exposed to constant conditions, these rhythms persist and are thus termed "free-running" circadian rhythms. For example, free-running locomotor activity rhythms have been reported in intertidal molluscs (*Aplysia*, Block and Lickey, 1973), crustaceans (fiddler crabs, Honegger, 1976), amphipods (Bregazzi, 1972), and isopods (Enright, 1976), to name a few. Circadian rhythms also affect physiological rhythms, such as blood sugar changes (Williams, 1985), oxygen consumption (Arudpragasam and Naylor, 1964), and neuronal activity (Block and McMahon, 1984).

In horseshoe crabs visual sensitivity is increased at night through the actions of a circadian clock (Barlow, 1983). This change in responsiveness, resulting from alterations in a wide variety of anatomical and physiological parameters of the eyes, leads to a 10^6 increase in visual sensitivity at night (Powers and Barlow, 1985; Herzog et al., 1996). As a result, it has been suggested that they can see as well by the light of the full moon as they can in the day (Herzog et al., 1996). While a great deal is known about the changes this clock elicits in the lateral eyes and other photoreceptors, the precise location of this clock in the brain is not known (Saito et al., 2004). The importance of the circadian clock and these physiological changes are detailed elsewhere in this volume (Dalal and Battelle, 2010).

Interestingly, the role of this clock in the temporal partitioning of its behavior is not clear. In the lab, adult horseshoe crabs exposed to Light: Dark (LD) cycles express diurnal (50%) or nocturnal (15%) patterns of locomotion (the remainder either express tidal rhythms or are arrhythmic), while some populations of juveniles have been shown to express nocturnal (Borst and Barlow, 2002) or diurnal (Rudloe, 1981) patterns. However, since these daily patterns have not been shown to persist under conditions of constant dark or light (Chabot et al., 2007), the involvement of the circadian clock in driving activity rhythms has not been demonstrated. In addition, it has been shown that the circadian clock that drives oscillations in visual sensitivity does not drive changes in locomotor activity (Watson et al., 2008). Therefore, at this time, the most parsimonious conclusion is that the daily patterns of locomotion expressed by some horseshoe crabs are the result of masking, or the tendency of some animals to avoid being active during either the light or dark hours of the day.

While the presence of an endogenous circadian clock that controls locomotor behavior has not been convincingly demonstrated, breeding behavior does appear to be affected by time of day, depending on the population studied. Some studies report increased activity primarily during nocturnal high tides (Rudloe, 1980) while others report increased activity during diurnal high tides (Cohen and Brockmann, 1983) in *Limulus* only 100 miles away. Still other studies have documented increased activity during both nocturnal and diurnal high tides depending on which tide is higher (Barlow et al., 2001). Interestingly, in areas where there are no significant tides, breeding activity is asynchronous, and thus not correlated with day or night (Ehlinger et al., 2003).

1.2 Circannual rhythms

Rhythms in the other three time domains (tidal, lunar, and annual) appear to be less common and/or more difficult to measure and, thus, much less is known about their importance or their molecular mechanisms. Annual rhythms are likely limited to more temperate or circumpolar species although the prevalence of these rhythms among animals is unclear likely due to the inordinate amount of time it takes to record multiple cycles. While thousands of species of animals are known to exhibit annual/seasonal changes in behavior and/or physiology, endogenous control has been demonstrated in only a very few avian species (Gwinner, 2003).

Annual changes in behavior are particularly well known in *Limulus polyphemus.* During the spring and summer months (Shuster, 2001; Shuster et al., 2003), males and females approach the high water marks of beaches around high tides (Barlow et al., 1986) to mate and, because it is so easy to observe them at this time, much is known about their breeding behavior (Brockmann, 2003). However, while Horseshoe crabs exhibit very clear annual rhythms of breeding (Brockmann, 2003) and activity (Watson et al., 2009) in the field and in the lab (Chabot et al., 2010), whether these rhythms are endogenous (circannual) or exogenously driven by changing environmental factors is unknown.

1.3 Circalunar rhythms

While lunar rhythms have been described in a number of species, they appear to be much less common than daily rhythms. The endogenous nature of these rhythms has been demonstrated in only a few species of fish (Takemura et al., 2010; Kavaliers, 1982) but these rhythms may also be effected by the interaction of endogenous circadian and circatidal clocks as thought to occur in lizards that forage in the intertidal zone (Wikelski and Hau, 1995).

Horseshoe crabs have also been reported to be influenced by the phase of the moon, although the strength of these rhythms appears to be population dependent. In the field, *Limulus* breeding activity increases around the new and full moons at certain times of year in at least two Florida populations (Rudloe, 1978, 1980; Cohen and Brockmann, 1983), while in three northern populations, a lunar influence on activity is less apparent (Cavanaugh, 1975; Barlow et al., 1986; Watson et al., 2008; 2009). In one laboratory experiment *Limulus* housed in running wheels in a greenhouse exhibited no clear effect of moon phase on activity patterns (Chabot et al., 2007).

1.4 Circatidal Rhythms

Tidal rhythms are generally limited to those species that inhabit or visit the intertidal zone (Palmer, 1990) and are thus much less common than circadian rhythms. Nonetheless, much more is known about these rhythms than either lunar or annual rhythms. Many marine organisms that visit, or live in, the intertidal zone, such as green crabs (*Carcinus maenas*, Naylor, 1958), mud fiddler crabs (*Uca pugnax*, Bennet et al., 1957) and fiddler crabs (*Uca crenulata,* Honegger, 1973a,b) exhibit tidally organized behavioral rhythms that are driven by an endogenous clock system (DeCoursey, 1983; Palmer, 1995a). For example, both green crabs (*Carcinus maenas*; Naylor, 1958) and mud fiddler crabs (*Uca pugnax*, Bennet et al., 1957) have tidal rhythms and the California fiddler crab *Uca crenulata* has an endogenous tidal rhythm that appears to be entrained by tidal cues (Honegger, 1973a, b).

Adult horseshoe crabs express a tidal rhythm of activity during mating season (Rudloe, 1979; Brockmann, 2003), with animals moving close to shore during the high tide portion of the tidal cycle, and then retreating to deeper water during low tide (Barlow et al., 1986; Penn and Brockmann, 1994). This tidal pattern of activity appears to be further modulated by a preference for the highest tide each day (Barlow et al., 1986), which may explain why in some areas most mating occurs around the high tides associated with the new and full moons (Rudloe, 1980; Smith et al., 2002).

While the factors that entrain endogenous <u>circadian</u> rhythms (primarily light cues) are well understood, even at the molecular level (Koh et al., 2006), the cues that synchronize tidal rhythms to environmental cycles are more varied and are less well studied. While inundation cycles (Williams and Naylor, 1969), hydrostatic pressure changes (Naylor and Atkinson, 1972), as well as cycles of increased or decreased temperatures and salinities (Reid and Naylor 1990), are sufficient to entrain the locomotor rhythms in several species (See Palmer, 1995a for review), the results are not as clear as they are for circadian rhythm entrainment.

While there are many field reports of tidally synchronized beach approaches in male and female *Limulus* during late spring, until recently it was not known if horseshoe crabs possessed an endogenous tidal rhythm, and, if such a tidal clock existed, how it was synchronized to cues from the environment. In the last several years we have developed a method for measuring *Limulus* locomotor rhythms in the laboratory and have clearly demonstrated that they have an endogenous tidal rhythm of activity (Chabot et al., 2004; 2007; Watson et al., 2008). Our recent investigations in horseshoe crabs show that water pressure changes are the primary cue for the synchronization of circatidal rhythms (Chabot et al., 2008, Chabot et al., 2010; Watson et al., 2009).

In this paper, we pull together our recent studies of the circatidal rhythms of locomotor behavior in horseshoe crabs and the factors that help to synchronize the clocks driving this behavior. The study of circatidal rhythms is particularly amenable in this species because the clarity of the rhythm is so robust. Finally, we review evidence suggesting that the timing system that controls these rhythms is composed of two coupled circalunidian oscillators.

2 Outputs: Clock Controlled Behavior in *Limulus*

2.1 Breeding season: field observations

Limulus exhibits robust seasonal breeding behaviors that take place primarily during high tides. This season lasts 3–4 weeks on average, with more southern populations beginning to breed in late February, several weeks earlier than northern populations. During this time, horseshoe crabs approach the beaches during high tides, usually in male/female pairs (Brockmann, 2003; Saunders et al., 2010). They do not appear to approach beaches during every high tide, however, often skipping one or more successive tides (Rudloe, 1980; Watson and Chabot, 2010). The females dig pits into the substrate and deposit several dozen (in Northern populations) to several hundred (Delaware populations) eggs that are subsequently fertilized by either attached or satellite males (Brockmann, 2003). There is some indication that single males find nesting females via both visual cues and chemical attraction (Hassler and Brockmann, 2001; Schwab and Brockmann, 2007; Saunders et al., 2010).

2.2 Breeding season: laboratory behavior

In the laboratory, when animals are placed in activity chambers during the breeding season, circatidal rhythms are seen in approximately two-thirds of the animals tested (Fig. 1, top panel; Chabot et al., 2004; 2007; 2008; Chabot and Watson, 2010; Watson et al., 2008). Since all of these investigations were conducted in constant conditions with respect to tidal cues, the evidence is clear that there are endogenous oscillators in this inter-tidal visitor that control circatidal rhythms of locomotion. Similar endogenous circatidal rhythmicity characterizes many other intertidal species such as the shore crab *Carcinus maenas* (Naylor, 1958), the fiddler crab (Bennet et al., 1957), the isopod *Excirolana chiltoni* (Klapow, 1972; Enright, 1976), and the shrimp *Crangon crangon* (Al-Adhub and Naylor, 1975). The presence of such an oscillator(s) would allow these species and *Limulus* to potentially anticipate and synchronize their activity to the natural tidal cycles. Moreover, during the breeding season, synchronization of males and females to the local tidal cycles would likely enhance the probability of both finding a mate and depositing eggs in an optimal location at the high water line.

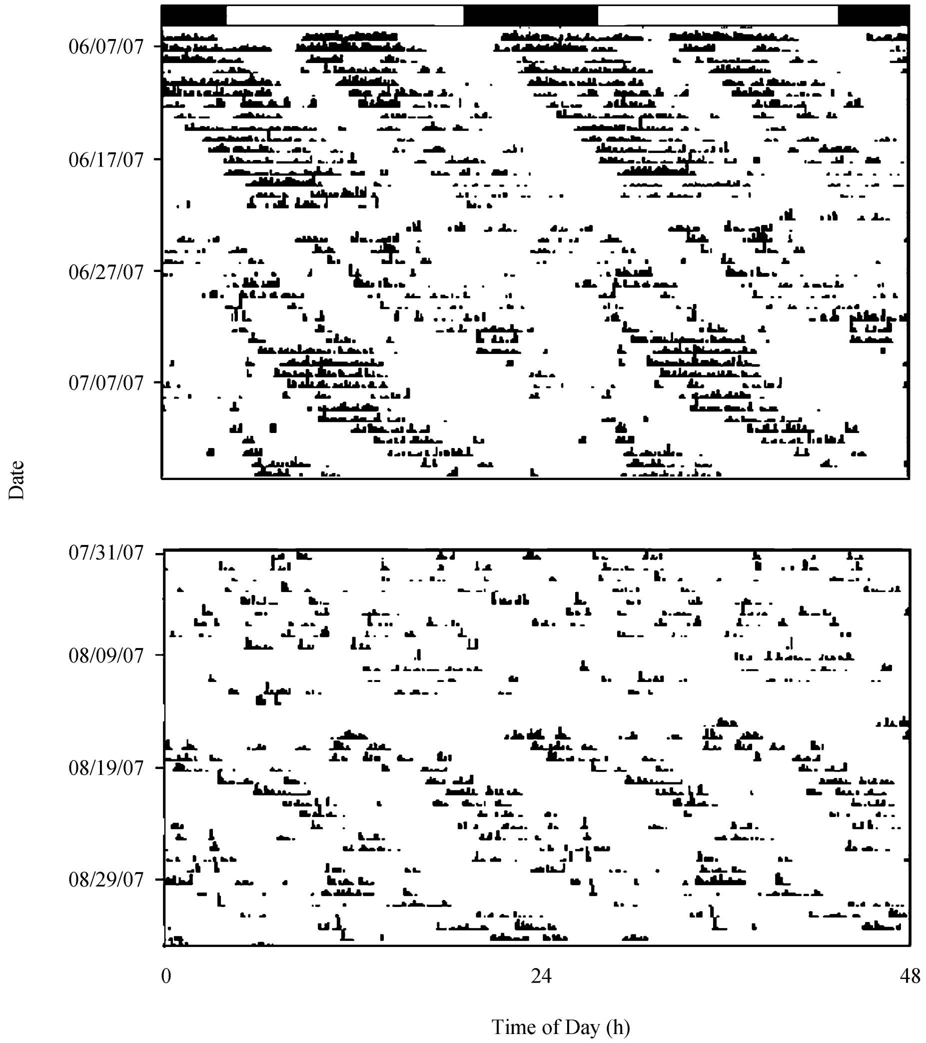

Fig. 1 Locomotor actograms showing endogenous expression of circatidal rhythms in two animals in atidal conditions both during (top panel first two weeks) and after (top panel last three weeks and bottom panel) breeding season

The timing and intensity of activity is indicated by the intensity of the black marks on the actogram. Data are double-plotted to facilitate visual inspection. The 14:10 light/dark cycle (LD) is indicated by black/white bars at the top. Lomb-Scargle periodogram analyses were performed on all of the actograms presented in the manuscript but are not presented here. Note that the clarity of the rhythms changes somewhat over time (less clear in top panel more clear in bottom panel) but is not correlated with breeding (June) versus non-breeding season (July/ August).

2.3 Non-breeding season

We have also shown that *Limulus* can exhibit tidal rhythms at other times of year as well, such as the late summer (Fig. 1, top and bottom panel; Chabot et al., 2007) and winter (Chabot et al., 2004; Watson et al., 2009), as long as the water temperature is above 11°C. While most 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), recent field telemetry studies have also shown a tendency for animals to move onto the tidal flats during high tides and off during low tides during the remainder of the summer months and into the fall (Watson et al., 2008; Watson and Chabot, 2010). It appears as if during these tidal excursions horseshoe crabs are actively foraging on tidal flats during these non-breeding times of year (Lee, 2010).

2.4 Seasonal changes in activity: the effects of temperature and photoperiod

In the field, horseshoe crabs exhibit clear seasonal changes in behavior. As described above, they exhibit very obvious breeding behaviors for about a one month period in the late spring in New Hampshire (NH) (Watson et al., 2008), and a few months earlier in the year in more southern regions (Barlow et al., 1986; Rudloe, 1980; Brockmann and Smith, 2009). They are typically very active in the months sandwiching the breeding season, while in the winter months they are much less active (Brockmann and Smith, 2009; Watson et al., 2009; Schaller et al., 2010; Watson and Chabot, 2010). While these seasonal rhythms may be endogenously controlled or modulated, we have recently shown that warmer water temperatures appear to be permissive: when horseshoe crabs in running wheels were exposed to warm water in the lab (17°C) the majority of them expressed tidal rhythms of activity, even if the L:D cycle was 9:15, typical of winter (Fig. 2, 4/8 animals; Watson et al., 2009,7/8 animals on initial exposure, 5/8 after 4 weeks of intervening colder temperatures). In contrast, when the water temperature was reduced to 11°C or below, they did not express a tidal rhythm of locomotion, even if they were exposed to a L:D cycle of 14:10, typical of the spring and summer. At 4°C, activity rhythms were

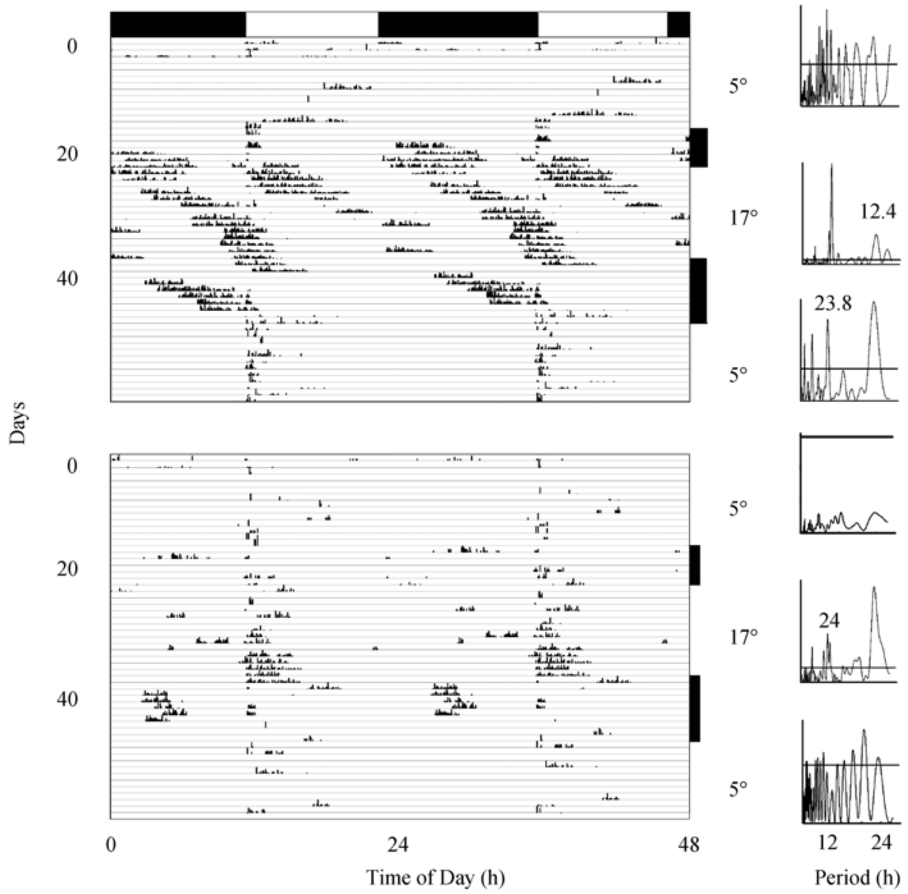

Fig. 2 The effects of water temperature on the expression of endogenous circatidal rhythms in two *Limulus* exposed to LD
Periods of increasing or decreasing temperatures are indicated by black boxes to the immediate right of the actograms. Right Panels: Lomb-Scargle periodograms of portions of the activity records as delineated by temperature. Horizontal line: level of significance ($P<0.01$). Values above line: highest peak period within the circatidal (10.4–14.4 h) or circadian range (22–26 h).

completely suppressed and activity levels were greatly attenuated. Thus, temperatures above 11°C are permissive in that they allow circatidal rhythms to be expressed. In contrast, photoperiodic changes do not appear affect the expression of circatidal rhythms.

This supports the hypothesis first proposed by Watson et al. (2009) that in NH, when water temperature rises rapidly in the spring and exceeds 10°C–12°C, horseshoe crabs become quite active and start moving towards areas where they typically breed. Interestingly, photoperiod (L:D 15:9 or 9:15) had little or no effect on animals in the lab. Because circannual rhythms are thought to be entrained primarily by photoperiod (Gwinner, 2003), while temperature appears to have a greater impact than photoperiod on the expression of tidal rhythms, it is likely that these annual rhythms are not endogenous but rather are driven by seasonal fluctuations in water temperature.

3 Inputs: Entrainment of the Clocks

3.1 Overview: the importance of tidal cues

The presence of an endogenous oscillator necessitates entrainment of that oscillator to environmentally appropriate stimuli. Possible entraining cues that are associated with natural tidal fluctuations include temperature, salinity, turbulence, current, turbidity and depth and therefore any of these could serve as cues to synchronize and entrain tidal rhythms. Water level changes have been most thoroughly investigated and this cue can entrain circatidal rhythms in amphipods *Corophium volutator* (Harris and Morgan, 1984), Portunid crabs*Liocarcinus holsatus* (Abello et al., 1991) and the Fiddler crab *Uca crenulata* (Honegger, 1973a,b), as well as many other species. Temperature changes associated with the tidal cycle are sufficient to entrain several crab species (Williams and Naylor, 1969), as are pressure fluctuations for both crabs and fish (Naylor and Atkinson, 1972; Abello et al., 1991; Northcutt et al., 1991). Periodic agitation or turbulence is sufficient to entrain tidal rhythms in two species of isopods (Klapow, 1972; Hastings, 1981) as well as mole crabs, *Emerita talpoida* (Forward et al., 2007), while current reversal is effective in glass eels, *Anguilla anguilla* (Bolliet and Labonne, 2008).

3.2 Synchronization of *Limulus* breeding in the field

The ability to entrain to cycles of inundation is not unusual for animals that either frequently visit or live in the intertidal zone (Naylor and Atkinson, 1972) and tidal cues may help to synchronize the breeding behavior of *Limulus polyphemus*. As summarized above, this species exhibits well-known seasonal visitations to the intertidal zone to breed, with peaks of breeding activity beginning at or 1–2 h before high tide and ending 2–4 hrs 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, Unpubl. Obs), although the frequency of inundation for individuals has not been reported.

While it is possible that, in the field, inundation events are strong cues for the synchronization of breeding to high tides, the changes in water depth associated with the tides may also be important. In animals that are not exposed to robust water level changes due to tides, wind driven changes in water levels are often more important for stimulating breeding activity than these “micro-tides” (Ehlinger et al., 2003). These water level changes are also likely important synchronizing cues for *Limulus* during non-breeding times of the year when individuals of at least one population (Great Bay, NH; Lee, 2010) invade the tidal flats during high tides to forage, and retreat to channels during low tides. In the field, *Limulus polyphemus* has recently been shown to be a regular intertidal zone visitor at times other than the 1 month breeding season (Watson et al., 2009; Watson and Chabot, 2010). During these tidal excursions, animals would rarely, if ever, be exposed to inundation. Thus, other cues must be used to synchronize their activity and our results demonstrate that in the lab water pressure changes are strong entraining agents (Fig. 3; Chabot et al., 2010).

3.3 The importance of depth changes to entrainment of tidal rhythms

3.3.1 Laboratory studies We have recently completed a series of laboratory and field experiments designed to assess the relative importance of a number of tidal cues in modulating horseshoe crab tidal rhythms. Our main finding is that circatidal activity rhythms in *Limulus polyphemus* can be synchronized and entrained by tidal cues and that water pressure changes appear to be most important cue associated with changing tides in this species. In the lab, most (> 80%) animals exhibit clear tidal rhythms when exposed to artificial "tides" (Fig. 3), consisting of ~12.4 hr cycles of water depth changes or inundation. In most cases, the activity appears to be synchronized with stable phase angles i.e.

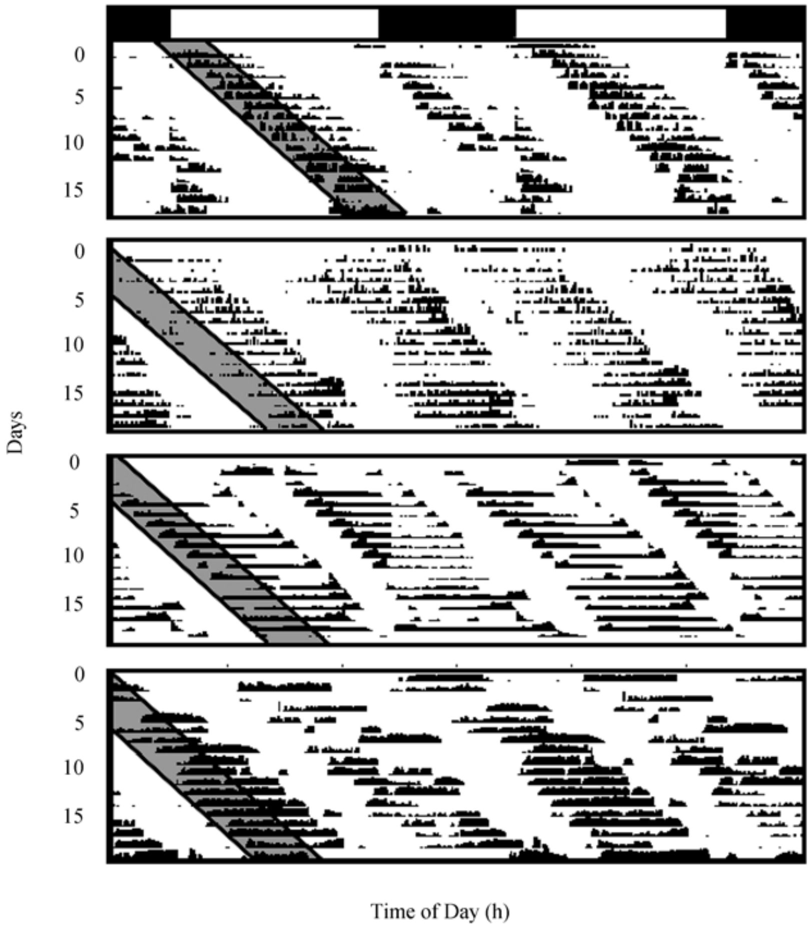

Fig. 3 The effects of 12.4 h water level changes on the locomotor activity of four horseshoe crabs exposed to LD
Gray boxes indicate one of the times (two/day) when animals experienced rising water levels.

the temporal relationship between the activity and the tidal signal is stable over time; results one would expect when entraining an endogenous oscillator. In a previous study, when the period of the imposed cycle was shortened, the resultant activity period shortened commensurately and significantly (Chabot et al., 2008). Importantly, when the artificial tides were stopped, a significant proportion of animals were clearly entrained (Fig. 3; Chabot et al., 2008; 3/8; an additional 4/8 showed some evidence of entrainment), continuing to maintain a rhythm that was synchronized to the previous entrainment signal (see also Fig. 4; Watson et al., 2008). The effects of other factors such as temperature and current cycles are presented below (3.4 –6).

In several other marine species, water pressure cycles also appear to be the most important cues (*C. maenus,* Naylor et al., 1971; the amphipod *Corophium volutator*, Holmstrom and Morgan 1983; the rocky shore fish *Blennius pholis*, Northcutt et al., 1991; and the cumecean crustacean *Dimorphostylis asiatica*, Akiyami, 2004). Interestingly a water pressure sensing organ has not been located in horseshoe crabs (Botton and Loveland, 1987). In fiddler crabs, the ability to synchronize to water pressure changes appears to be mediated by a statocyst (Fraser, 2006).

3.3.2 *In situ* studies The primary importance of water pressure changes, relative to other cues associated with the tides, is supported by *in situ* results. All animals (*n*=11) that were in running wheels that were attached to the underside of a floating raft (and were thus exposed to all the natural tidal cues, except water pressure changes) did not express clear tidal rhythms (Fig. 4, top). Instead, they exhibited clear daily rhythms. In contrast, most animals (7/9; Fig. 4, bottom; Chabot et al., 2010) that were housed in “running wheels” and anchored to the bottom of the estuary (and thus experienced the complete suite of tidal cues in the estuary) exhibited tidal rhythms. Thus, while current changes (0–0.2m/sec), temperature changes (Δ0–5^{o}C), salinity changes (Δ0–10 psu) and other physicochemical parameters associated with

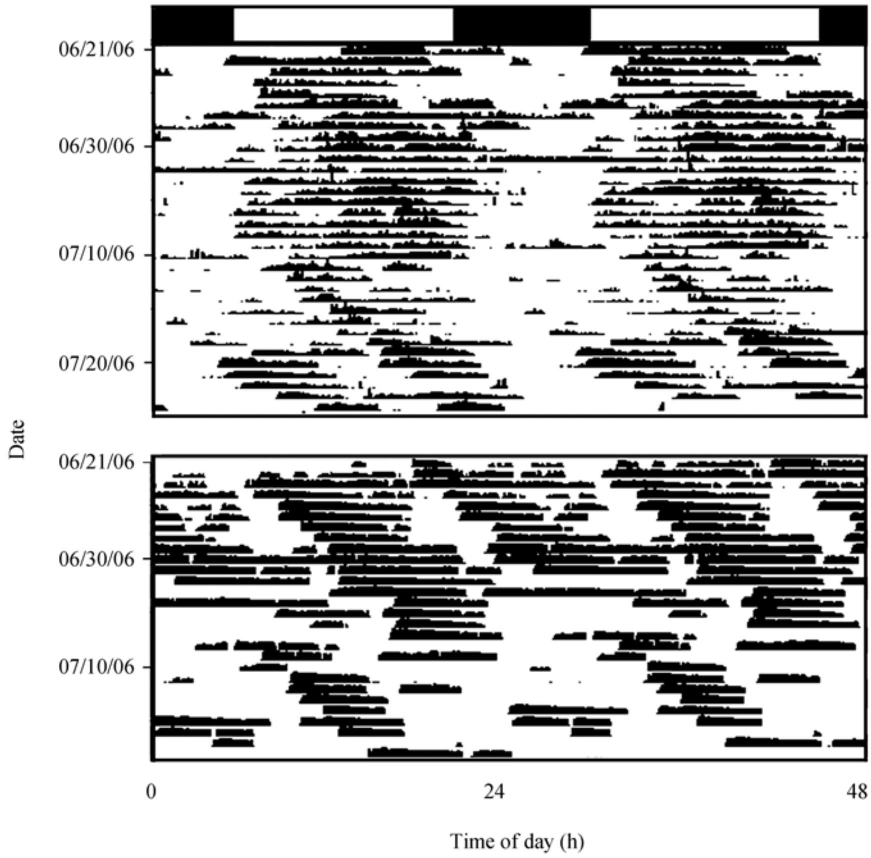

Fig. 4 Behavioral activity patterns in two *Limulus* in running wheels that were either attached to a floating raft (top) or fixed to the bottom of the estuary (bottom)

Approximate photoperiod is indicated by LD bars at top. Note that the animal attached to the floating raft was primarily diurnal (though with some evidence of tidal rhythmicity during the last two weeks) whereas the animal located at the bottom of the estuary was primarily tidal.

the tides may influence the behavior of horseshoe crabs, they are generally ineffective on their own. Additional direct evidence supporting this statement is presented below in Sections 3.3.3 –6. Our results in *Limulus* are similar to another *in situ* study involving amphipods in which water pressure was also the primary tidal cue used to synchronize their activity (Enright, 1965); animals exposed to water level changes synchronized to the tidal cycles while those tethered to a float, like our animals, did not.

3.3.3 Water pressure threshold The amplitude of the signal matters when it comes to the entraining effectiveness of water pressure changes. While inundation or larger water level changes (0.4 m) were effective entraining agents (7/8 showed evidence of entrainment; Chabot et al., 2008), small water pressure changes only (0.2 m) were much less effective (1/7 entrained; Chabot et al., 2010). Our previous *in situ* results also support this hypothesis: 40% of animals exhibited tidal rhythms in deeper water, where they experienced a 60% change in water pressure, while nearly 80% of those anchored in shallower water and experiencing 300% water pressure changes showed tidal rhythms (Fig. 4; Chabot et al., 2010). Previous results from our lab have indicated that horseshoe crabs require at least a depth fluctuation of between 0.2 m (100% change in depth in laboratory tanks) and 0.5m (250% change) in order to entrain to water level changes. While this value is somewhat greater than those reported for *C. maenas* (Naylor and Atkinson, 1972) and some planktonic species (0.1 m; Knight-Jones and Morgan, 1966), we may be using a more stringent definition of entrainment than the previous studies. It is also possible that the rate of change matters when it comes to entraining effectiveness. However, in our lab, increasing the rate of change does not increase the effectiveness of entrainment (Chabot and Watson, Unpubl. Obs.). The importance of relative water levels/depth changes are also seen in *Limulus* breeding rhythms in the field: populations that experience only micro-tides (of a few cm) do not synchronize their breeding with these tidal cycles (Ehlinger et al., 2003) while populations that experience tides of at least 0.5 m or so effectively synchronize to the water level changes (Rudloe, 1979, 1980, 1985; Cohen and Brockman, 1983; Barlow et al., 1986). In addition, increased or decreased tidal heights due to wind action, or phases of the lunar cycle, significantly affects the timing of breeding in this species (Barlow et al., 1986; Brockmann, 2003). Our results concur with previous field studies indicating that *Limulus polyphemus* generally begins its' activity before "high tide" (Brockmann, 2003) but differs slightly from a Delaware study where animals are generally not active until maximum high tide (Smith et al., 2002). Additionally, Chabot et al. (2007, 2008) reported that activity began approximately 2 h before high tides in lab situations compared to 0.2–3.4 h in the field (Brockmann 2003; Watson and Chabot, 2010).

3.4 The role of temperature as an entrainment cue for tidal rhythms

The *in situ* (floating dock, Fig.4-top) results indicating that the temperature fluctuations associated with changing tides are less effective than water level changes are supported by laboratory results. In the lab, artificial tidal cycles consisting of large temperature fluctuations (10^{o}C) caused 50% of the animals tested to synchronize their locomotor activity to the imposed rhythm (Fig. 5). However, less than 33% of them showed evidence of entrainment when the animals were allowed to free run when the temperature was held constant (Chabot et al., 2010). Although this percentage is markedly lower than for water level changes or inundation (Chabot et al., 2008), these data suggest that temperature changes may have some input into the circatidal entrainment pathway. Importantly, these large temperature changes are likely out of the normal range

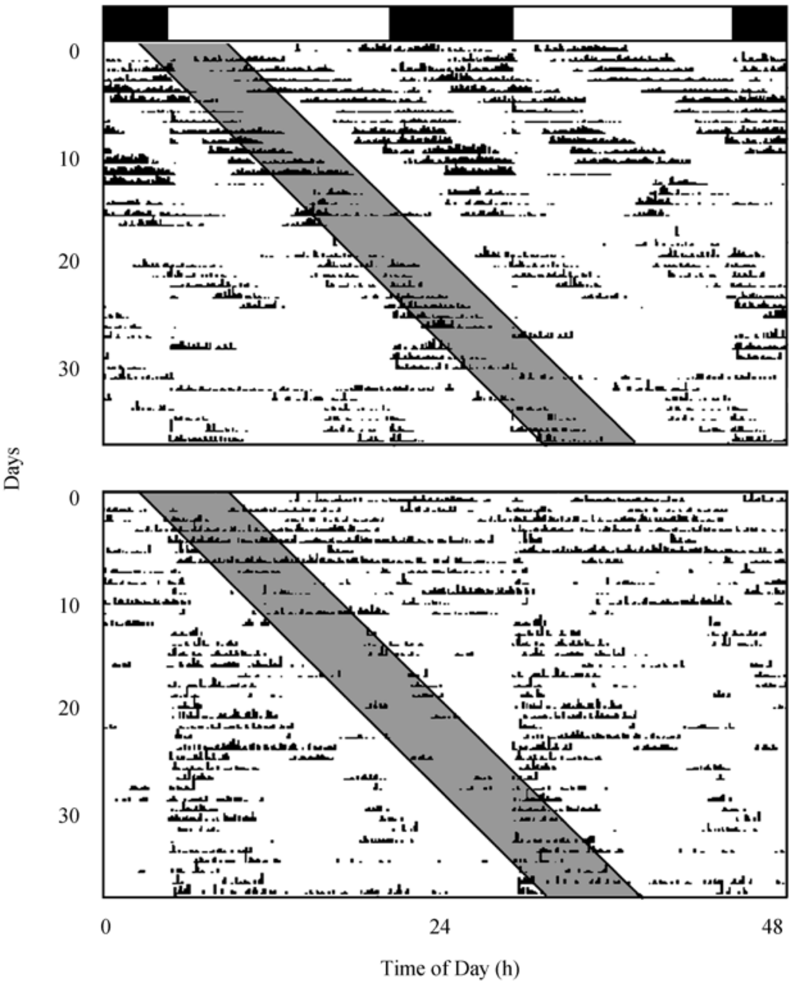

Fig. 5 The effects of circatidal temperature cues on behavioral activity in two horseshoe crabs exposed to LD

Shaded boxes indicate times of warming temperatures (from 16℃ to 26℃). Note that while the top animal exhibited clear circatidal rhythms, the bottom animal did not. In neither case were the rhythms were clearly synchronized to the temperature cycles.

of temperature cycles 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 (± 3°C), like those they might experience in the field during a typical tidal cycle in an estuary, are even less effective (only 25% synchronized -entrainment was not assessed; Chabot et al., 2010). Similar results were found in *Carcinus*: 11°C temperature cycles effectively entrain the tidal rhythms of this species while 4°C temperature cycles are ineffective (Williams and Naylor, 1969). Importantly, 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.

3.5 Current Cycle Effects

Artificial currents appear to have even less of an effect than depth changes or temperature cycles on locomotor rhythms of *L. polyphemus* in the laboratory (Fig. 6; Chabot et al., 2010). Interestingly, there was some evidence of synchronization to the turbulence cues that were administered but it appeared that the currents were simply initiating short-term responses (masking) and not synchronizing the underlying circatidal clock because: 1) these rhythms did not persist when the currents were terminated and; 2) several animals exhibited four bouts of activity/day (every time a cue was administered, Fig. 6, bottom panel; Chabot et al., 2010) instead of synchronizing their activity to a particular "high" or "low" tide. Turbulence does appear to be an important synchronizing cue at an earlier life stage of *Limulus* because larval *Limulus* entrain to cycles of turbulence that simulate the incoming tide (Ehlinger and Tankersley, 2006). In addition, there is evidence that currents could be used as cues to allow horseshoe crabs to time activity to the tides, especially the highest tides when the current is the strongest (Brockmann, 2003), and orient when moving into, and off, mating beaches (Rudloe and Hernnkind, 1976). Furthermore, currents appear to be capable of synchronizing the behavior of other species, such as the European eel (although entrainment was not assessed; Bolliet and LaBonne, 2008). Moreover, in some species mechanical stimulation that simulates incoming and outgoing tides appears to be the most important stimulus for entraining rhythms in animals that

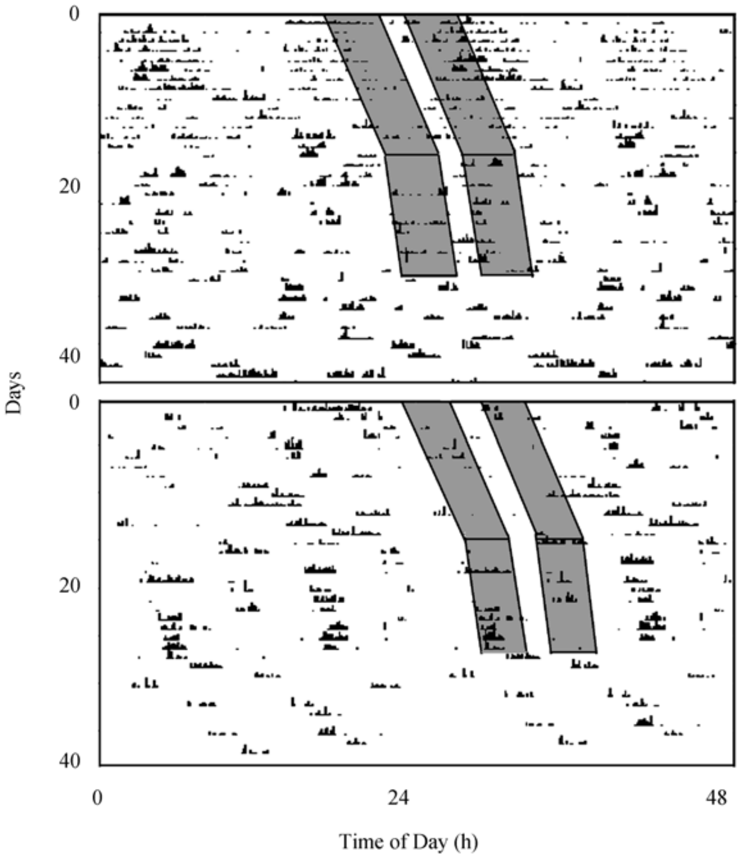

Fig. 6 The effects of circatidal current cues on behavioral activity in two horseshoe crabs exposed to constant light

Shaded boxes indicate times of currents (four total/day) which alternated direction every 6.2 h. Note that the bottom animal appears to synchronize to the current cycles and thus the animal exhibited 4 bouts of activity/day.

inhabit the swash zone such as mole crabs (Forward et al., 2007), amphipods (Enright, 1965), and isopods (Hastings, 1981). In horseshoe crabs, waved caused turbulence affects beach choice with animals preferring beaches with less waves (Smith et al., 2002). The relative lack of importance of turbulence for synchronizing the tidal rhythms of *Limulus* adult horseshoe crabs may make a lot of sense since, 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. However, those animals that inhabit channels in the Great Bay estuary may be exposed to currents as high as 0.3 m/sec (Short, 1992).

3.6 The relative importance of tidal cues

Thus, while it appears that water level changes are the strongest entraining agent for horseshoe crab activity, turbulence and temperature may also contribute. In a number of marine animals the presentation of more than one tidal cue appears to enhance entrainment. For instance, the amphipod *Corophium volutator* entrains to temperature cycles of 10°C but exhibits greater entrainment when temperature cycles are delivered in conjunction with cycles of inundation (Holmstrom and Morgan, 1983). Like in the horseshoe crab, water pressure and inundation cycles appear to be the most important entraining signal in *C. maenas* (Naylor et al., 1971) and in other crustacean species (Williams and Naylor 1969; Naylor and Williams 1984).

3.7 Photoperiod Effects

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). However, LD cycles can strongly affect patterns of locomotion in American horseshoe crabs and a few other species (Saigusa, 1992; Saigusa and Kawagoye, 1997). In horseshoe crabs we have observed two major effects of LD cycles on Limulus activity patterns: 1) Direct effects or “masking” and 2) transient synchronizing effects.

3.7.1 Masking and induction of daily rhythms While circatidal rhythms are commonly expressed by *Limulus*, a high percentage of animals also express significant daily rhythms of activity when exposed solely to LD cycles (Chabot et al., 2007; Fig. 7). Nearly half of these exhibit preferences for diurnal movements, about a third exhibit no preference, and the remainder prefer to move at night (Chabot et al., 2007; 2008). However, in most cases, the diurnality or nocturnality appears to be due to “masking” (direct effects of light [or dark] on locomotor activity) rather than the effects of an entraining agent on an underlying circadian clock. While a circadian clock does drive robust rhythms of increased nocturnal eye sensitivity in *Limulus* (Barlow, 1988; Barlow et al., 2001), we have not observed clear free-running circadian rhythms in constant lighting conditions (Chabot et al., 2004; 2007). If these daily rhythms were endogenously controlled one would expect persistence in constant conditions. While circadian activity rhythms in juvenile horseshoe crabs have been reported (although the sample size was very small; Borst and Barlow, 2002), preliminary results in our lab suggest juvenile *Limulus* express endogenous circatidal rhythms, but not circadian rhythms (Dubofsky et al., Unpubl. Obs.) Importantly, juveniles in our lab do exhibit a clear preference for nocturnal activity when exposed to a light/dark cycle, but that rhythmic component disappears under constant conditions. Interestingly, juvenile crabs from the Gulf of Mexico have been reported to be diurnal in the lab (Rudloe, 1981). Whether these differences are due to population differences or laboratory conditions remains to be elucidated.

The strongest effects of LD cycles have been seen in animals housed in running wheels and either attached to a floating raft or in a greenhouse and thus exposed to high intensity natural lighting. All of these animals exhibited significant daily rhythms that began over two hours after sunrise and also expressed weak circatidal rhythms (Chabot et al., 2008). Interestingly, when tidal rhythms are apparent in the activity records of either *in situ* (Fig. 4 top panel, last two weeks of activity; Chabot et al., 2010) or greenhouse animals (Chabot et al., 2008), they always occurred during the day. Similar results are observed in breeding fish populations (Takemura et al., 2010). This suggests that tidal activity is inhibited during darkness and “permitted” during the day. This is exactly the pattern that has emerged in some animals that have been tracked using ultrasonic telemetry in their natural habitat (Watson and Chabot, 2010). However, a preference for daytime high tides is not always the rule, especially during the breeding season. While a preference for the diurnal high tides is observed in some breeding populations of *L. polyphemus* (Cohen and Brockman, 1983) others appear to prefer the night tides (Florida, Rudloe, 1979, 1980; Cape Cod, Massachusetts, Barlow et al., 1986) or whichever tide is highest (Barlow et al., 1986). Most likely, these differences are not due to light/dark preferences of horseshoe crabs, but to their preference for the highest tides, which occur during the new and full moons, and at night in the north and during the day in the south (Brockmann, 2003). In

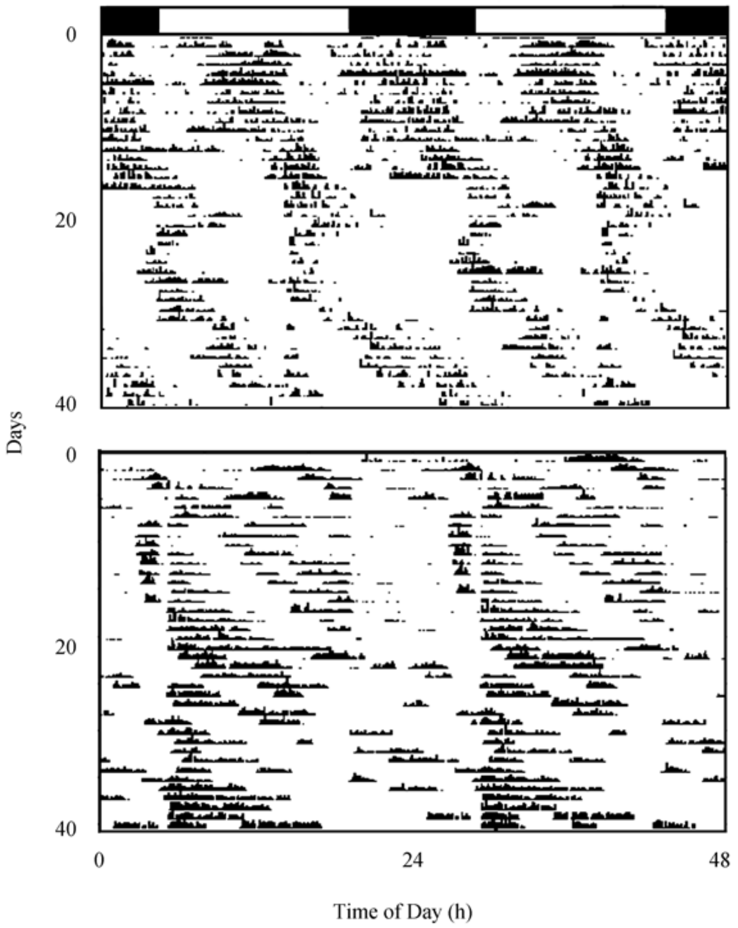

Fig. 7 The effects of LD cycles on locomotor activity in two *Limulus*

Top: Note the apparent transient synchronization to the LD cycles for the first 20–30 days and the subsequent free-run at approximately Day 26. Bottom: Note the bouts of tidal activity that free-run through the first few weeks during L but not D. Note also the instant increase in activity upon lights on and the instant cessation of activity at lights off.

NH approximately equal numbers of animals approach beaches during the day and night high tides during the breeding season and the differences in tide height between night and day high tides is very minor (Watson et al., 2009).

3.7.2 Transient synchronization of circatidal rhythms to LD cycles While LD cycles appear to cause significant masking of endogenous *Limulus* activity patterns in the lab, these cycles also appear to have two additional effects: 1) to help to "maintain" circatidal rhythms, since we often observe clearer circatidal free-runs in LD than in DD (Chabot et al., 2008); 2) to cause some transient synchronization of these rhythms in horseshoe crabs (Fig. 7, Top panel; Chabot et al., 2007) and several other crustacean species (Honegger, 1973a,b; Naylor, 1997; Saigusa, 1988, 1992; Palmer, 1990; Saigusa and Kawagoye, 1997). This type of transient synchronization, otherwise known as relative coordination (Johnson et al., 2004), is indicative of a relatively weak entraining agent influencing an oscillator. Why should photoperiod have any synchronization effect on circatidal activity patterns? Photoperiod is obviously not a strong predictor of tidal phase but there are at least four testable hypotheses that may explain these effects: 1) light levels are a proxy for moon phase; 2) light levels are a proxy for water depth; 3) light level changes directly induce movements which then induces a phase shift of the underlying clock system; 4) In the absence of tides (i.e. some areas in Florida), LD cycles may help to synchronize breeding. A brief discussion of each of these hypotheses follows.

Moon phase has been reported to affect breeding activity in *Limulus* (Barlow et al., 1986), but it is certainly possible that this effect has more to do with tide height than light levels because more animals breed on both the full and new moons. The hypothesis that light levels may be a proxy for moon phase could be tested by assessing whether animals that approach beaches preferentially around full or new moons are the individuals whose circatidal activity is most affected by photic input. Light level changes may also be a proxy for changes in water depth, especially in estuaries such as Great Bay where the turbidity of the water significantly attenuates light levels (Chabot et al., 2008). Thus, water level changes and light levels may essentially be seen as redundant entraining signals, especially during the mating season when animals move into shallow water during high tide and thus they are exposed to more light during the daytime high tide. This hypothesis could be tested by attempting to entrain animals with tidal periods of LD cycles. Alternatively, it is possible that light may act more indirectly on the underlying locomotor oscillators. In the lab, lights-on and lights-off transitions can cause a large percentage of animals to express a type of "startle response" and begin to walk (Chabot et al., 2007; Figs. 2, 7, bottom panel). These kinds of acute increases in activity are known to induce phase shifts in the circadian rhythms of some mammals (Wickland and Turek, 1991; Gannon and Rea, 1995; Buxton et al., 2003). Thus, the activity itself can serve as a synchronizer of the underlying circadian clock. Lastly, tide magnitude varies considerably over the range of horseshoe crabs and some experience only very small microtides of a few cm (Ehlinger et al., 2003). Interestingly, while hydrostatic pressure changes entrain larval release in the crab *Rhithropanopeus harrisii* in tidal areas, in non-tidal areas solar day cues appear to synchronize this behavior and daily rhythms of larval release are common (Forward and Bourla, 2008). However, this does not seem to be the case in horseshoe crabs in microtidal environments since breeding among the populations appears to be asynchronous in these environments and thus not associated with LD cycles (Ehlinger et al., 2003). Interestingly, satiation may play a role: beach isopod behavior is also affected by LD cycles but primarily only if well-fed, when they avoid swimming during the day (Reid and Naylor 1989). It is worth noting that animals in our labs are generally not fed so hunger could influence the expression of their tidal rhythms and the effects of nutritional state on *Limulus* circatidal behavior could be assessed experimentally.

4 *Limulus* Clock(s)

4.1 Circalunidian clock hypothesis

While circatidal activity patterns have been documented in many intertidal species, the type of clock system that controls these rhythms is somewhat controversial (Palmer, 1995b; Palmer, 1997; Naylor, 1996, 1997). A long-standing question in the field of circatidal rhythms research is whether the locomotor activity of intertidal species is driven by (two) circalunidian, or (one) circatidal, clocks (with circadian clock influence). The circalunidian hypothesis was first proposed by Palmer and Williams (1986). This theory essentially proposes that the circatidal rhythms that are observed in intertidal animals 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 12.4h and can be invoked to explain the type of rhythm seen in Fig. 2. Alternatively, E. Naylor and colleagues hypothesize that intertidal animals have one 12.4h circatidal clock that drives behavioral rhythms and one circadian clock that modifies those rhythms (Naylor, 1958).

In general, our data fits the three criteria put forth by Pittendrigh and Daan (1976) to explain dual clock control and by Palmer (1997) as support of the circalunidian hypothesis: we have evidence of: 1) two separate rhythms that scan the day at different periodicities (Chabot et al., 2007); 2) "skipping" - sudden alternations of unimodal and bimodal patterns (Fig. 8) and; 3) "Splitting" - the separation of one component into two components (Chabot et al., 2007). It is difficult to model a single oscillator (or a circatidal oscillator modulated by a circadian oscillator) that could give rise to the data shown in these figures. The behavioral evidence supporting a dual oscillator model is also particularly solid in some fish (Erikkson, 1973).

This type of clock system (two-clocks) has been well established over the past four decades in mammals (Pittendrigh and Daan, 1976) and *Drosophila* (Stoleru et al., 2004) on the basis of behavioral and molecular evidence. While we are unaware of any corroborating molecular evidence, the behavioral results in *Limulus* are very similar to these species. Similar evidence supporting the (two-clock) circalunidian hypothesis in inter-tidal species has been seen in green crabs (all three criteria; Palmer, 1989; 2000) and the crab *Helice crassa* ("splitting"; Palmer and Williams, 1986). Therefore, at the present time, our working hypothesis is that horseshoe crabs possess two separate, ~24.8 hr clocks, each of which controls the expression of activity during one of the two high tide periods normally experienced by most animals in their natural habitat. However, these clocks appear to be easily "decoupled" from their behavioral output since "skipping" in the lab (Fig. 8) and the field (Barlow et al., 1983; Watson and Chabot, 2010) are not uncommon. This ability would allow a great deal of behavioral flexibility and would facilitate synchronization to tides in habitats in which there is only one tide/day (such as along the Gulf Coast, West of St. Joe Bay, FL; Rudloe, 1985) where only one clock would be coupled to behavior. In habitats where there are no tides, such as Indian River Lagoon, FL (Ehlinger et al., 2003), neither of the clocks would be coupled to behavior. In addition, the relative rarity of "splitting" in this species (2/33 animals; Chabot et al., 2007) suggests that when both clocks are expressed, they are strongly coupled to each other.

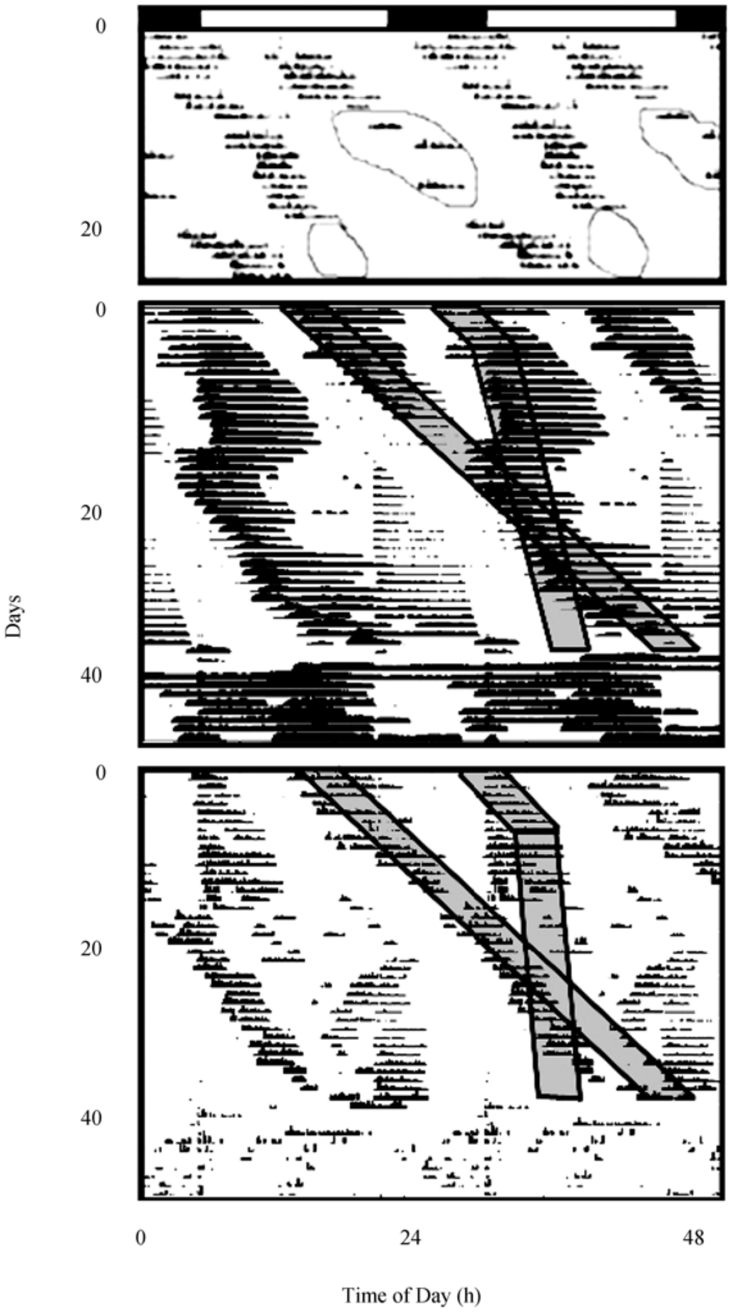

Fig. 8 Evidence for the presence of two circalunidian oscillators in *Limulus polyphemus* exposed to LD

Top Panels: free-running circatidal activity rhythm showing "skipping" of one of two components (circled). Bottom two panels: the effects of 12.4 and 12.1 cycles of water pressure on the locomotor activity patterns in *Limulus*. Rising water levels indicated by shaded boxes: First 8 days, two cycles/day delivered each at 12.4 h; next 30 days, two cycles/day - one at 12.4 h; other at 12.1 h. remaining 15 days - constant water levels. Note the different periods of each component. Note also the apparent drift of at least one of the components in the bottom panel when the 12.1 h tides were initiated.

4.2 Circatidal and circadian clock hypothesis

Some of our data may appear to support the presence of a circadian oscillator that modulates locomotion. In LD and DD, with otherwise constant conditions, some animals exhibit periodicities in the 24 h range (Chabot et al., 2004), but there are several possible explanations for these data, none of which requires the involvement of a circadian oscillator: 1) Foremost, it is likely that the circa-24 h rhythm was an artifact of the statistical analysis itself: Chi Square periodogram analyses often produce harmonics of underlying rhythms, so that, for example, animals expressing a 12.4 hour tidal rhythm will also appear to have a 24.8 hour circadian pattern (Van Dongen et al., 1999). This is especially true if one uses the Chi square periodogram instead of the Lomb-Scargle periodogram analysis (Ruf, 1999; Chabot et al., 2007); 2) Actogram 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); 3) In LD, as described above, it is also likely that direct responses to the LD cycle directly induced periodicities in the 24 h range thus masking endogenous rhythms. Most lab investigations of the locomotor rhythms of *Limulus* have been conducted in LD.

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 possible influence of the circadian clock on behavioral activity seems premature at this point especially since there is some evidence that tail movement is modulated in a circadian fashion (Powers and Barlow, 1985). However, our recent study has shown that the circadian eye rhythm of eye sensitivity, because it free-runs at very different periods that the circa-tidal rhythm of locomotion, is clearly controlled by a clock that is distinct from the clock(s) that control locomotion (Watson et al., 2008). In addition, while dozens of papers have been published documenting robust <u>circadian</u> rhythms of lateral eye changes, as far as we can determine, there has been no published indication of <u>circatidal</u> modulation of visual sensitivity. Whether these timing systems interact at all with each other remains to be elucidated.

5 Molecular Basis of Circadian and Circatidal Behavioral Rhythms in *Limulus*

The "period" protein, PER, is part of a feedback loop that is a central component of a circadian clock in *Drosophila* that drives behavioral locomotor rhythms and much of our basic understanding of the molecular biology of PER has come from work with this species (Konopka and Benzer, 1971; Bargiello et al., 1984; Hardin et al., 1990; Zweibel et al., 1991; Edery et al., 1994). PER is expressed with a ~24 hour rhythm, typically with the highest levels accumulating in cells at night and lower levels during the day (Zerr et al., 1990). Importantly, expression of homologues of this protein has been demonstrated in a variety of animals including humans and mice (Tei et al., 1997), butterflies (Regier et al., 1998), lancets (Schomerus et al., 2008) and several others. In many of these species, PER has been shown to oscillate with species-specific patterns that are phased to LD cycles. Thus, it appears that this protein, or at least the bioactive parts of it, is highly conserved throughout the animal kingdom.

We have preliminary evidence that a 71 kD protein that reacts with antibodies against *Drosophila* PER is expressed in both the protocerebrum and the subesophageal ganglion of the *Limulus* brain. We plan to test the hypothesis that the circadian clock is located in the protocerebrum by determining if PER varies by time of day in this part of the brain. While it is widely accepted that PER is an integral part of the molecular clock controlling circadian rhythms in many animals, its' role in clocks controlling circatidal behaviors is unknown Experiments are also currently underway to determine if PER is part of the molecular clock driving *Limulus* circatidal rhythms as well.

6 Conclusion

Time is a crucial factor to consider when addressing issues of behavior in horseshoe crabs. Since circadian rhythms have been demonstrated in virtually all organisms that have been studied, this is not dissimilar to all other organisms. However, the effects of time on horseshoe crabs are likely more profound because time of tide must also be taken into consideration. Over the past several years we have shown that "time of tide" is a crucial factor for predicting horseshoe crab behavior: they are generally active at high tides and less active during low tides. These changes in behavior are clearly driven by an endogenous timing system that may be best termed “circalunidian”. While the well-known *Limulus* circadian clock appears to have little, if any, influence on locomotion, time of day appears to have large effects on the behavior of *Limulus*: some individuals clearly prefer to be day- or night-active. Further, LD (and DL) transitions spur activity in some individuals although these effects appear primarily in the lab and may be primarily due to “instant on or off” photoperiodic transitions. The environmental factors that help to synchronize these behavioral rhythms are many and varied but tidal water pressure changes appear to be the primary cue with tidal temperature and currents playing lesser, but contributory, roles. Interestingly, photoperiod appears to influence activity patterns in a large percentage of animals either through the masking of circa-tidal rhythms or by the partial synchronization of those rhythms. Time of year is also a major factor when it comes to *Limulus* behavior and it appears that temperature is the key factor here: when water temperatures are above 12^{o}C, animals are active and most exhibit endogenous circatidal rhythms. When temperatures are below 12^{o}C, animals are much less active and do not express circatidal rhythms. *In situ*, the presence of circatidal rhythms of activity helps to synchronize breeding rhythms during a 1 month breeding season. Outside the breeding season, these rhythms appear to synchronize the animals to inundation of tidal flats where they forage during high tides. Finally, a 71 kD protein that reacts with antibodies against *Drosophila* PER is expressed in both the protocerebrum and the subesophageal ganglion of the *Limulus* brain. Experiments are underway to determine if this protein is part of the clocks that underlie the circadian rhythms of eye sensitivity or the circatidal rhythms of activity in *Limulus polyphemus*.

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