Activity rhythms expressed by juvenile American horseshoe crabs, Limulus polyphemus

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Abstract
The goals of this study were to determine if juvenile horseshoe crabs, Limulus polyphemus, express daily or tidal patterns of activity and how light and tidal cycles influence these patterns. When exposed to a light:dark cycle (n=24), 63% of juveniles exhibit daily patterns of locomotion and 25% of juveniles express circatidal patterns. When subsequently exposed to constant darkness, 17% express circadian rhythms of activity, 25% express a combination of circadian and circatidal patterns, and 46% express circatidal patterns of activity. When exposed to tidal cycles (n=42), 55% of juveniles express tidal patterns of activity, while the remainder exhibit either a daily pattern (17%) or no pattern (28%) of activity. Of those synchronized to the tidal cycle, 43% show entrainment by expressing circatidal activity when subsequently held at constant water depth. Overall, these results demonstrate that juvenile horseshoe crabs possess endogenous clocks influencing circatidal and circadian patterns of locomotion.

Keywords
Biology, Zoology, Psychology, Behavioral Sciences, Biological Sciences

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ACTIVITY RHYTHMS EXPRESSED BY JUVENILE AMERICAN HORSESHOE CRABS, *LIMULUS POLYPHEMUS*

BY

ELIZABETH ANNE DUBOFSKY

B.A., Boston University, Boston, MA. 2007

THESIS

Submitted to the University of New Hampshire

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In

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This thesis has been examined and approved.

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April 6, 2012
Date
DEDICATION

For my Aunts Lynne and Gloria.
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ABSTRACT

ENDOGENOUS RHYTHMS OF JUVENILE AMERICAN HORSESHOE CRABS,
*LIMULUS POLYPHEMUS*

by

Elizabeth Anne Dubofsky

University of New Hampshire, September, 2012

The goals of this study were to determine if juvenile horseshoe crabs, *Limulus polyphemus*, express daily or tidal patterns of activity and how light and tidal cycles influence these patterns. When exposed to a light:dark cycle (n=24), 63% of juveniles exhibit daily patterns of locomotion and 25% of juveniles express circatidal patterns. When subsequently exposed to constant darkness, 17% express circadian rhythms of activity, 25% express a combination of circadian and circatidal patterns, and 46% express circatidal patterns of activity. When exposed to tidal cycles (n=42), 55% of juveniles express tidal patterns of activity, while the remainder exhibit either a daily pattern (17%) or no pattern (28%) of activity. Of those synchronized to the tidal cycle, 43% show entrainment by expressing circatidal activity when subsequently held at constant water depth. Overall, these results demonstrate that juvenile horseshoe crabs possess endogenous clocks influencing circatidal and circadian patterns of locomotion.
INTRODUCTION

**Horseshoe Crabs Life History**

The American horseshoe crab, *Limulus polyphemus*, inhabits the Atlantic coast of the United States, its range extending from Maine through the Gulf of Mexico. These animals can be traced back over 450 million years and they have evolved unique behaviors (Shuster and Anderson 2003, Rudkin *et al.* 2008). One of the most striking behaviors occurs every spring and summer when hundreds of adults mate on beaches during high tide. Males either amplex (join) with a female or cluster around her (satellite males), attempting to fertilize eggs as she lays them in a pit. After the female lays her eggs, the amplexed pair leaves the beach while the satellite males remain, attempting to fertilize the eggs left behind (Brockmann and Penn 1992). The eggs develop for 3-4 weeks before hatching into trilobite larvae (Fig. 1).

After hatching, the larvae enter a six day planktonic stage at the end of which they molt and settle on the substrate (Shuster 1982). Juvenile horseshoe crabs inhabit intertidal zones near the breeding beaches moving into progressively deeper water as they age (Shuster 1982). After reaching maturity at 9-10 years (~17 molts, Sekiguchi *et al.* 1988), they inhabit deeper waters making frequent excursions to mudflats during high tide to feed and mate (Brockmann and Penn 1992, Lee 2010). Adults may live for an additional 10 years, an approximate total life span of 20 years (Walls *et al.* 2002).
Figure 1. Life cycle of the American horseshoe crabs, *Limulus polyphemus*. Adults lay eggs in the sand at high tide annually April through June. After 3-4 weeks, the eggs hatch into a non-tailed larval stage that spends 6 days in the water column. At the first molt, a tail begins to develop and the juveniles settle in mud and sand flats. As they age, they move progressively into deeper water until as adults they live in deeper water.
Horseshoe crabs are indirect deposit feeders and bioturbate the sediment as they move through it searching for prey (Lee 2010). They typically feed on insect larvae, polychaets, oligochaetes, crabs, bivalves and amphipods (Botton 2009). Prey is consumed by grasping food with chelae and using the other legs to carry it to the gnathobases and then moving adjacent pairs of legs out of phase, causing the gnathobases to grind the food and force it into the digestive system (Wyse and Dwyer 1973, Shuster 1982).

Major predators of horseshoe crabs include intertidal crabs, crustaceans, eels, fish, birds and humans (Shuster 1982, Walls et al. 2002, Chiu and Morton 2004). Of these predators, the largest pressure comes from humans who harvest horseshoe crabs for bait and biomedical industries. Currently, American eel (Anguliiia rostrada), whelk (Busycon spp.), and catfish (Ictaluridae spp.) fisheries use horseshoe crabs for bait (Berkson and Shuster 1999). Fishermen prefer gravid females, claiming that they attract more catch (Shuster et al. 2003). In the biomedical industry, a derivative from the horseshoe crabs’ blood, Limulus Amebocyte Lysate, is used to detect toxins and contaminants in medications and blood supplies. There are limits on the amount of blood that may be drawn from an individual crab, however blood loss and associated complications appear to cause up to 30% to die from the procedure (Leschen and Correia 2010).

One of the most common methods to avoid predation is via behavioral modification. A common behavior of horseshoe crabs is burying in soft sediments, such as mud. This behavior allows for predator avoidance (Meury and Gibson 1990) and increased foraging for prey while digging (Lee 2010). Once buried, horseshoe crabs are known to remain inactive if the mudflat becomes exposed which reduces exposure to air
and sunlight therefore buffering horseshoe crabs from changing temperatures that may be
detrimental (deFur 1988, Brockmann 2003). Since sand and mud rarely dry out during
low tides when exposed, it may also reduce desiccation (Shuster 1982, Shuster and
Sekiguchi 2003). Burying may also reduce predation pressure on juveniles and the
likelihood of being swept out to sea (Meury and Gibson 1990).

Activity Patterns Expressed in Early Life History

Despite the wealth of knowledge about adult horseshoe crabs, relatively little is
known about juveniles. After hatching, the larvae spend approximately 6 days in the
water column where they are positively phototactic and nocturnally active (Rudloe
1979). In the laboratory under both ambient and imposed LD cycles, larvae remain
nocturnally active. When larvae are kept in continuous low light, a population exhibits
more activity during the putative night than during the putative day. There is
significantly less activity during the putative night than during the night portion of a LD
cycle and increased activity during the putative day than during the light portion of a LD
cycle (Rudloe 1979). Additional studies have shown that larvae collected in the field and
brought into the laboratory will express a tidal rhythm of activity in constant conditions,
becoming active near the start of the expected ebb tide (Ehlinger and Tankersley 2006).
Moreover, larvae can be entrained to 12.4 h cycles of agitation and will continue to
express a circatidal rhythm of activity when the agitation cycles are terminated (Ehlinger
and Tankersley 2006). Therefore, larvae appear to have endogenous clocks controlling a
combination of daily and tidal activity patterns.
The end of the larval and beginning of the juvenile stage occurs with the first molt of the horseshoe crab. At this point, a telson is developed and the animal settles on the substrate (Rudloe 1981). After six molt cycles, the juvenile horseshoe crab begins a second year of growth during which there are three molt cycles. In the third year of growth there are two molts and in the fourth and all subsequent years, there is one molt per year (Sekiguchi et al. 1988). With each molt, the visual system develops more but is functional from the first instar, allowing animals to avoid obstacles and determine light levels (Meadors et al. 2001, Ridings et al. 2002).

Juvenile horseshoe crabs live in the intertidal zone usually in estuaries and marshes on soft sediments. They are generally inactive during the winter and molt during the summer (Rudloe 1981, Meury and Gibson 1990). Laboratory studies have shown that a simulated ebbing tide causes juvenile horseshoe crabs to follow the water and bury in the sand at the edge of the water. The animals only moved when covered with water, likely because the sand became semi-liquid and easier to move through (Meury and Gibson 1990).

Field studies in Florida with a tidal range of ~0.6 m, show that juvenile horseshoe crab activity peaks during the ebb tide two hours prior to low tide (Rudloe 1981) similar to laboratory studies by Meury and Gibson (1990). Studies of the juvenile horseshoe crab Tachypleus tridentatus in Japan showed similar trends of increased activity two hours before low tide (during ebb), however, no data were collected during high tides (Chiu and Morton 2004). When Florida juveniles were placed in subtidal enclosures, activity of juvenile horseshoe crabs was higher during the day than night (Rudloe 1981). However, this field experiment was not conducted at a location where juvenile
horseshoe crabs are normally found, but subtidally at 25 m and 60 m offshore. The activity was not continuously monitored, but sampled at six selected times relative to the tidal cycle. In Florida laboratory studies diurnal activity was also observed, but determining if the rhythm expressed was daily or circatidal was not possible due to missing data (Rudloe 1981). This experiment did not consider if an endogenous clock caused the activity pattern. Animals exposed to a semi-diurnal tidal regime of approximately 2 m, such as that in Little Sippewissett Marsh, Falmouth, MA, may show a different cycle than those acclimated to the diurnal tidal regime of 0.6 m in the Gulf of Mexico. Differences in activity patterns are seen in adults exposed to varying tidal regimes and magnitudes. Adults exposed to a semi-diurnal tidal cycle (two highs and two lows in 24.8 h) synchronize their mating to the high tides mating twice daily (Rudloe 1979, 1980, Cohen and Brockmann 1983). Adults exposed to a diurnal tidal cycle (one high and one low in 24.8 h) mate only once per day at high tide; horseshoe crabs in micro-tidal environments (tidal changes of several centimeters) express asynchronous mating (Cohen and Brockmann 1983, Ehlinger et al. 2003).

Two additional studies investigated activity patterns expressed by juvenile horseshoe crabs, but due to small sample size and low data acuity, no firm conclusions can be drawn (Casterlin and Reynolds 1979, Borst and Barlow 2002). Casterlin and Reynolds (1979) showed that when exposed to a light:dark cycle, juvenile horseshoe crabs (n = 10) from North Carolina were most active at night. However, the experiment only lasted three days and did not test if the rhythm was endogenous. The second study by Borst and Barlow (2002) tested animals from Massachusetts (n = 5) in constant darkness after four days of entrainment to a light:dark cycle entrainment. Only two of
five animals showed cyclic activity during the constant darkness and the resolution of the data collected (by hour) is not fine enough to determine if the animals were expressing a circatidal or circadian pattern of behavior. Results from both studies were interpreted as nocturnal but data presented show two bouts of activity per 24 h, indicating that there may be an alternate explanation for the activity pattern, an endogenous clocks producing a circatidal rhythm. A major goal of my thesis was to examine patterns of activity expressed by juvenile horseshoe crabs and determine if these patterns are under the influence of endogenous circadian and/or circatidal clocks.

**Types and Mechanisms of Endogenous Clocks**

Two types of biologically relevant clocks were examined in this study: daily and tidal. A daily clock is 24 h in length and synchronized to the external light:dark cycle. If this pattern of activity continues to be expressed under constant light conditions (constant low light or constant darkness), it is considered a circadian clock (~24 h long, Büning 1973). A tidal clock is 12.4 h in duration, the length of one high and one low tide, and activity is synchronized to the tidal cycle (Naylor 1958). A circatidal clock is ~12.4 h long and is expressed in the absence of tidal cues. However, a circatidal clock is not the only clock that may result in expression of a circatidal pattern of activity. A second type of clock that results in expression of a circatidal pattern of activity is a circalunidian clock (Palmer 1990). This clock is comprised of two clocks, each 24.8 h in length and 180° out of phase from the other. These clocks allow for two bouts of activity to be
expressed daily, causing the appearance of a circatidal pattern of locomotion (Palmer 1990).

Determining if a circatidal pattern of activity is controlled by a circatidal clock or a circalunidian clock is challenging since they both result in a circatidal tidal pattern of activity. Possible indicators of a circalunidian clock include: 1) different periodicities of rhythms, 2) “skipping” of bouts of activity or alternating between unimodal and bimodal activity patterns, 3) “splitting” of one activity bout into two, and 4) activity bouts of different durations (Palmer 1990, 1995a). While it has not been conclusively determined if circatidal or circalunidian clocks control activity patterns in any intertidal organisms, working hypotheses exist for many, including adult horseshoe crabs. The working hypothesis for adult horseshoe crabs is that of circalunidian clocks (Chabot and Watson 2010). However, the dual control hypothesis has been shown conclusively in fruit flies (Drosophila melanogaster, Helfrich-Forster 2001) and mammals (Pittendrigh and Daan 1976).

The primary indicator of an endogenous clock is the continuation of the same pattern of activity when held in constant conditions. Further, the organisms’ pattern should “drift”, starting successively earlier or later each day, depending on the length of the individual’s endogenous clock (Bünning 1973). This occurs because the organism’s endogenous clock is unlikely to be the exact length of a day or tidal cycle, but slightly shorter or longer (Bünning 1973). Lack of drift may indicate the influence of an uncontrolled exogenous cue. In constant conditions, expression of patterns controlled by endogenous circadian clocks typically persists longer than the expression of circatidal patterns controlled by endogenous clocks (Palmer 1995a, Chabot et al. 2008).
While the molecular basis of the endogenous circadian clock of adult horseshoe crabs is largely unknown, it is thought to be chemically and structurally similar to that of some well-studied insects, specifically *Drosophila spp.* (Price et al. 1998). Evidence for this stems from the fact that the molecular basis of endogenous clocks appears to be conserved across many taxa from insects to mammals (Price et al. 1998). However, the molecular basis of circatidal clocks is completely unstudied.

Circadian clocks are composed of three parts: clockwork, an input pathway and an output pathway. In this case, clockwork is defined as an oscillator encoded by genes and expressed as proteins in a predictable rhythm, the input pathway (sensory) allows entrainment and sets the internal cycle to the external patterns, and the output pathway is the expression of physiological rhythms (for example, wakefulness versus sleeping or patterns of locomotion; Sassone-Corsi 1998, Schoning and Steigher 2005). Specific neurons in the brain, such as the central/optic area in *Drosophila spp.*, have been determined to be “clock cells”, the portion of the brain that controls oscillations in behavior (Zordan et al. 2000). The input pathway, which allows entrainment by zeitgebers, requires a photoreceptor that may or may not be the compound eye of either *Drosophila* or horseshoe crabs. Extraocular photoreceptors may be sufficient to provide stimuli to the clock (Stanewsky et al. 1998, Saunders 1997, Giebultowicz 2000).

There are several proteins responsible for circadian oscillation of behavior. Two proteins, CLOCK (CLK) and CYCLE (CYC) heterodimerize and attach to the promoter region of the *period* and *timeless* genes (Kaushik et al. 2007). This activates the genes causing the production and accumulation of PERIOD (PER) and TIMELESS (TIM)
proteins (Kaushik et al. 2007). The PER and TIM proteins heterodimerize and enter the nucleus as a complex. Once in the nucleus, these proteins bind to and inactivate the CLK – CYC heterodimer, stopping the production of additional PER and TIM proteins (Kaushik et al. 2007). Light activates CRYPTOCHROME (CRY), which degrades TIM (Kistenpfenning et al. 2012). This allows for the degradation of the PER by DOUBLETIME (DBT) allowing CLK – CYC to bind to period and timeless starting the cycle over again (Price et al. 1998).

Circadian Clocks

Endogenous clocks controlling the timing of behaviors are ubiquitous in all phyla of life. These clocks were first discovered in Heliotrope (DeMairan 1729, Bünning 1960) when plants were observed to change the orientation of their leaves depending upon the time of day and location of the sun. Other phyla containing well-known clocks include Arthropoda, such as the locomotor clock in the fruit fly (Drosophila spp.) or fiddler crabs (Uca spp.), and Chordata, including wakefulness and feeding cycles in pigeons (Columba livia), sparrows (Passer domesticus), and humans (Bennett et al. 1957, Bünning 1973, Chabot and Menaker 1992, Veleri et al. 2003).

Barlow et al. (1977) discovered the endogenous circadian rhythm in horseshoe crabs. This circadian rhythm changes the sensitivity of the horseshoe crab eye, increasing it up to 1,000,000 times at night at the expense of decreasing spatial resolution (Barlow et al. 1980, Barlow and Powers 2003). This rhythm is controlled by a clock in the brain that communicates through efferent fibers to the eye. It was generally assumed that this same
clock influenced locomotion and that horseshoe crabs were primarily nocturnal animals. However, recent studies indicate that the circadian rhythm of eye sensitivity does not correlate with activity rhythms (Watson et al. 2008). An endogenous clock controlling circatidal activity rhythms partially drives locomotor activity patterns. However, some studies show adult horseshoe crabs tend to be more active at night while other studies indicate no preference for time of day (Chabot et al. 2004, 2007). I will build on these findings by investigating the possibility of endogenous circadian rhythms in juvenile horseshoe crabs despite the lack of evidence for one in adult horseshoe crabs.

**Circatidal Clocks**

Expressions of circatidal rhythms have been studied in many organisms. Initially, circatidal research focused on color change and oxygen consumption (Brown et al. 1953, Sandeen et al. 1954). One of the first locomotion studies examined the fiddler crab, *Uca pugnax* (Bennett et al. 1957). The number of minutes active per hour were recorded and averaged for the population of fiddler crabs held in constant conditions. The population showed two daily peaks of activity that drifted by 51 minutes each day. This activity pattern degraded after 7-8 days. The combination of the progression of the start of activity and the two daily peaks indicates a circatidal rhythm, while its expression in constant conditions indicates that it is controlled by an endogenous clock.

Subsequent to this research, the daily and tidal patterns of locomotion in green crabs, *Carcinus maenas*, were studied in great detail. The first study showed that they had a peak of activity at the nighttime high tide, but as with fiddler crabs, the pattern
deteriorated rapidly in constant conditions (3-4 days, Naylor 1958). The higher activity levels at night suggested to Naylor (1958) that this activity pattern was the result of a combination of both circatidal and circadian components. Naylor (1958, 1996, 1997) also concluded the circadian clock promotes activity while the circatidal clock inhibits activity.

In the crab *Sesarma reticulatum*, the same pattern of higher activity during the nighttime high tide, led to the theory of the circalunidian clock, composed of two clocks, each controlling one of the two bouts of activity. This circalunidian clock could explain some of the noise common with two peaks of activity daily including different periods between peaks, different durations of activity, a single peak spitting into two peaks, and a peak disappearing either temporarily or permanently (Palmer 1990, 1995a). A similar hypothesis is theorized to drive activity in fruit flies that have two clocks coupled 180° out of phase; one driving activity at dusk and one at dawn (Helfrich-Forster 2001).

Studies of adult horseshoe crabs have shown tendencies to express daily and circatidal patterns of locomotion (Chabot *et al.* 2004, 2007, 2008, Watson *et al.* 2008, 2009, reviewed in Chabot and Watson 2010). Some animals exhibit both these patterns of locomotion at the same time showing two peaks on their periodograms, one circadian and one circatidal (Chabot *et al.* 2004).

Overall, when placed into constant conditions, the majority of adult horseshoe crabs exhibit circatidal patterns of locomotion. When placed into constant conditions immediately after removal from their natural environment, 88% exhibit circatidal patterns of locomotion, but these patterns did not correlate to their native tidal cycle (Chabot *et al.* 2007). If subjected to a light cycle followed by constant darkness, 45% express circatidal
activity (Chabot et al. 2007). When exposed to light and tidal cycles simultaneously, all animals exhibit tidal patterns of locomotion. When placed in constant darkness and water depth, a majority of animals express circatidal patterns of locomotion (Chabot et al. 2008). This maintenance of circatidal rhythms in constant conditions indicates that adult horseshoe crabs have an endogenous clock controlling circatidal rhythms (Chabot et al. 2007, 2008). This endogenous circatidal clock likely influences normal behaviors that are best carried out at during high tides in their natural habitat. The two most common activities are mating and foraging for prey on mudflats that are only underwater at high tide (Rudloe 1980, Lee 2010).

Tidally synchronized mating behavior allows horseshoe crabs to choose nests at the high tide line, which provide the best environmental conditions for egg development (Penn and Brockmann 1994, Brockmann 2003). Use of mudflats as foraging grounds is supported by evidence from both tracking studies and measurement of horseshoe crab feeding pits (Watson et al. 2009, Lee 2010, Watson and Chabot 2010). During tracking studies, spawning was observed in May and June, but activity levels on mudflats remained high until the water temperature fell below 10.5°C. It was unclear how the adults were using the mudflats during high tide, however SCUBA studies indicated feeding-associated activities (Watson et al. 2009, Watson and Chabot 2010). A more in-depth study determined that horseshoe crabs forage on mudflats during high tides causing significant reduction in infaunal prey species for up to four weeks (Lee 2010, Lee 2012).

Despite evidence showing that an endogenous clock controls circatidal locomotion, there is large individual variability that has yet to be adequately explained.
Exogenous cues, or zeitgebers, that are known to influence the expression of tidal rhythms include inundation and hydrostatic pressure changes associated with depth changes (Watson et al. 2008, Chabot et al. 2011). Currents and large temperature changes are not very effective zeitgebers for entraining circatidal patterns of locomotion in adult horseshoe crabs. In addition to laboratory studies isolating these zeitgebers, field studies show animals held at the water surface express different patterns of locomotion (daily activity) from those held at the bottom of the estuary (tidal activity). This clearly indicates the importance of changes in water depth in the entrainment of tidal rhythms in horseshoe crabs. The second chapter of my thesis focuses on the cues that influence the expression of tidal rhythms by juvenile horseshoe crabs. My working hypothesis is that changes in water depth will be the most effective cue.

**Objectives**

The primary objective of the research presented in Chapter One was to determine if juvenile horseshoe crabs express a daily rhythm of locomotion that is under the control of an endogenous circadian clock. A second objective was to determine if juvenile horseshoe crabs would spontaneously express a circatidal rhythm of locomotion in the laboratory.

The primary objective of the research presented in Chapter Two was to determine if juvenile horseshoe crab locomotion could be entrained to two different types of artificial tides.
CHAPTER 1

EXPRESSION OF DAILY, CIRCADIAN, AND CIRCATIDAL PATTERNS OF LOCOMOTION BY JUVENILE HORSESHOE CRABS

Abstract

Adult American horseshoe crabs, *Limulus polyphemus*, possess endogenous circadian and circatidal clocks controlling visual sensitivity and locomotion, respectively. The goal of this study was to identify activity rhythms expressed by juvenile horseshoe crabs when exposed to a light:dark cycle followed by constant darkness. The locomotor activity expressed by juveniles was recorded using time-lapse video in the laboratory. Animals (n=24) were exposed to a 14:10 light:dark cycle for 10 days followed by 10 days of constant darkness. During the light:dark cycle, 63% of animals expressed daily patterns of activity and 25% showed circatidal locomotion (the remainder were arrhythmic). Of the 15 animals expressing a daily pattern of behavior, 47% expressed a significant preference \((P<0.05)\) for diurnal activity and 13% for nocturnal activity. Of the animals expressing a daily pattern of activity, the majority (66%) expressed a circadian pattern in constant darkness, but also expressed a secondary, circatidal, peak of activity. The remaining animals switched to a circatidal pattern of activity. Of the six animals expressing circatidal rhythms in the light:dark cycle, five continued to do so in constant darkness, while the remaining animal switched to a circadian pattern. Overall, these
results indicate that juvenile horseshoe crabs express both daily and tidal patterns of activity that are driven by endogenous clocks.

Introduction

Animals inhabiting or visiting the intertidal zone express a variety of activity patterns. The two most common patterns expressed are daily activity (synchronized to the light cycle) and tidal activity (synchronized to the tidal cycle). Three intertidal organisms whose activity patterns have been scrutinized are the green crab (*Carcinus maenas*), mole crab (*Emerita talpoida*) and fiddler crab (*Uca spp.*). All of these species exhibit a combination of tidal and daily patterns of activity. Within each species, there is wide individual variation attributable to varying selection pressures, native environments, reproductive status, and molt states (Bennett *et al.* 1957, Naylor 1958, Barnwell 1966, Forward *et al.* 2005). In addition to expressing these rhythms in their native environment, these species have been shown to express these activity patterns under constant conditions indicating that they have an endogenous component.

One species that inhabits and/or visits the intertidal zone is the American horseshoe crab, *Limulus polyphemus*. Adult horseshoe crabs are known to express both tidal and daily rhythms (Chabot *et al.* 2011). They have an endogenous rhythm of visual sensitivity; their eyes are up to 1,000,000 times more sensitive at night even when held in constant conditions (Barlow *et al.* 1980, Barlow 1983, Barlow and Powers 2003). However, the circadian clock controlling eye sensitivity does not control patterns of locomotion. Rather, an endogenous clock primarily controls locomotor activity patterns.
resulting in circatidal activity rhythms (Chabot et al. 2004, 2007, 2008, 2011, Watson et al. 2008, 2009, Chabot and Watson 2010, Watson and Chabot 2010). At the present time, there are no data demonstrating the presence of a circadian clock controlling locomotion, but many adult horseshoe crabs exposed to a light:dark (LD) cycle express a preference for either daytime or nighttime activity (Chabot et al. 2004, 2007, 2011). It is also possible that light modulates the circatidal clock (Watson et al. 2008) yielding a situation in which animals express more complex activity rhythms. For example, light might provide a redundant signal about tides because in a turbid estuary the amount of light reaching the bottom will correspond with the tidal cycle (Chabot et al. 2008). Despite evidence showing that light and an endogenous clock controlling circatidal rhythms influence the patterns of locomotion expressed by adult horseshoe crabs, one of the most consistent findings is that there is considerable variability in the types of patterns expressed by different animals. A frequently seen aspect of behavior in adult horseshoe crabs is masking, or the obscuring of an endogenous pattern of activity by environmental cues (Bregazzi and Naylor 1972, Chabot et al. 2008).

In addition to the studies of endogenous rhythms in adults, there are some studies on larval horseshoe crabs. Larvae spend approximately six days in the water column where they are positively phototactic and nocturnally active (Rudloe 1979). When collected in the field and brought into the laboratory, larvae express a circatidal rhythm of activity in constant conditions, becoming active near start of the expected ebb tide (Ehlinger and Tankersley 2006). Moreover, it was demonstrated that they can be entrained to 12.4 h cycles of agitation and will continue to express a circatidal rhythm of activity when the agitation cycles are terminated (Ehlinger and Tankersley 2006).
Therefore, larvae, like adults, appear to have an endogenous clock controlling circatidal rhythms that modulate their activity.

Despite the similar endogenous rhythms between these two life stages, ontogenetic studies of endogenous rhythms in other species have shown that not all stages express the same types of rhythms. Some species maintain the same pattern throughout all life stages while other organisms change the phase angle of their activity from crepuscular or diel as juveniles to nocturnal as adults (ampipods, *Orchestiidae tuberculata*, Kennedy et al. 2000; cane toads, *Bufo marinus*, Pizzatto et al. 2008). Some crabs, such as blue crab (*Callinectes sapidus*), have been shown to alter rhythms of vertical migration during four different stages; from no rhythm as zoeae, to diurnal as megalopae, nocturnal as juveniles, and circatidal as ovigerous adults (Forward et al. 2004).

Previous studies of juvenile horseshoe crabs have shown that a simulated ebbing tide causes animals to follow the water down the slope and bury in the sand at the edge of the water. The animals only moved when covered with water, expressing a preference for slack tide (Meury and Gibson 1990). However, this study did not present data for other phases of the tidal cycle. Field studies of juvenile horseshoe crabs show a peak in activity two hours prior to low tide during the ebb tide. However, when placed in subtidal enclosures in water deeper than where they are typically found, activity correlated to the light rather than the tidal cycle (Rudloe 1981). In a laboratory study by Rudloe (1981), juvenile horseshoe crabs exposed to constant water depth, ambient light, and ambient temperature expressed diurnal activity. However, the study did not examine if the rhythm of behavior was caused by an endogenous clock.
Studies of the juvenile horseshoe crab, *Tachypleus tridentatus*, in Japan show similar trends of increased activity two hours before low tide, however, no data was collected during high tides (Chiu and Morton 2004). Two more laboratory studies determined that juvenile American horseshoe crabs were nocturnal, but due to the low sample size and lack of data acuity, no conclusions can be drawn (Casterlin and Reynolds 1979, Borst and Barlow 2002). Casterlin and Reynolds (1979) tested animals in ambient light for a total of three days; the nature of the rhythm (endogenous vs. exogenous) was not examined. Further, the conditions in which and length of time the animals were held prior to testing was not addressed. This could impact the types of rhythms expressed by the animals since activity patterns may have degraded over an extended holding period (Palmer 1995b, Chabot et al. 2008). The study by Borst and Barlow (2002) tested for endogenous rhythms, but the resolution of the data (by hour) is not fine enough to determine expression of circatidal versus circadian patterns. Further, only two of five animals had rhythmic activity when exposed to constant conditions. In both studies, there are indications of a combination of both patterns of behavior in the data presented.

The primary goal of this study was to determine the types of activity rhythms expressed by juvenile horseshoe crabs when exposed to a light cycle. The secondary goal was to determine if these rhythms persist in constant conditions and therefore are under the control of endogenous clocks.
Materials and Methods

Collection and Care of Animals

Juvenile *L. polyphemus* (size range = 40 – 55 mm carapace width) were collected from Little Sippewissett Marsh, Falmouth, MA on four different dates (6/9/2010, 7/2/2010, 8/22/2010, and 9/1/2010) and transported in coolers to an indoor facility at University of New Hampshire, Durham, NH. Less than 24 h elapsed before they were placed in individual chambers and testing began. Activity patterns of 24 juvenile *L. polyphemus* were analyzed in 4 different trials, each lasting 20 days. Trials beginning on 6/9/2010 and 7/2/2010 tested five animals each and trials beginning on 8/22/2010 and 9/1/2010 tested seven animals each.

Prior to the start of a trial, all animals were fed to satiation using frozen *Spirulina* brine shrimp (Hikari Bio-Pure, Hikari, Hayward, CA) thawed in saltwater. Satiation was determined by cessation of food consumption based on the end of the stereotyped feeding behavior of rhythmic movements of the legs, as described by Wyse and Dwyer (1973).

Monitoring Animal Activity

In order to increase the visibility of animals at night, a 1 cm$^2$ piece of reflective tape (Nashua® Multi-purpose Foil Tape) was attached to the dorsal carapace of each horseshoe crab using cyanoacrylate-based glue (Krazy® glue). This did not cover any of their simple or complex eyes. Horseshoe crabs were then placed in individual 20 cm diameter plastic chambers filled with 3 cm of sand and 15 cm of 30 psu seawater held at
room temperature (~22°C). For the first ten days of the experiment animals were subjected to a summer light:dark cycle (LD, 14:10) using a modified dawn/dusk simulator to gradually increase and decrease incandescent light for 2 h at the beginning and end of each day (SunUp, Light Therapy Products, Plymouth, MA). Light levels were 163 ± 0.35 lux (average ± SEM) during the day and 54 lux at night. At the end of the initial ten-day period, animals were subjected to a ten-day period of constant darkness (DD). During DD, and the nights associated with LD, infrared lights illuminated the chambers.

Activity was recorded using black and white, infrared sensitive camera(s) (PC-222, Supercircuits, Austin, TX). Camera outputs were digitized (Canopus® ADVC-110, Grass Valley, San Francisco, CA), time-stamped and recorded on a computer (Macintosh, OS 10.5.6) using video capture software (Gawker, v. 0.8.3, Seattle, WA) which captured one frame every 30 s. In trials 1 and 2, a single camera was used. In trials 3 and 4, a multiplexer (Pelco Genex™ MX4009MD, 9 channel) was used to combine the output of four different cameras into a single video stream that was digitized as described above.

Videos were analyzed by eye in 5 min blocks. Any movement during a 5 minute block was recorded as a “1” while no movement was recorded as a “0”. These data were then plotted and analyzed further as actograms and Lomb-Scargle periodograms ($P < 0.001$) using the program ClockLab® (MatLab, Actimetrics, Evanston, IL, v. R2009a), as described in Chabot et al. (2008). Actograms were also visually inspected to confirm the results given by the periodograms, to reduce false peaks. Possible causes of false peaks are sudden environmental changes, such as “light-on, lights off” effects. These are readily
identified with visual inspection of an actogram and they tended to be reduced by using the dawn-dusk simulator. In general, both visual inspection and periodogram analyses were used to identify and quantify the types of rhythms expressed by the animals.

Significant differences in activity level during the day and night were determined for animals that expressed a daily rhythm in LD. Percent active per hour during light hours and dark hours was calculated by calendar day and analyzed using a non-parametric Wilcoxon matched-pairs signed-ranks test in InStat® (GraphPad Software, La Jolla, CA, v. 3.1a 2009).

To determine alpha, or the duration of the main bout of activity, best eye-fit lines were drawn through the “onset” and “offset” of active periods using a single-blind protocol.

**Results**

A total of 15 of 24 animals (63%) expressed a daily rhythm of activity when exposed to LD ($\tau = 23.90 \pm 0.35 \text{ h}$; mean ± SEM; Figs. 1 and 2). Seven of the 15 (47%) continued to express a ~24 hr (circadian) rhythm of activity in DD ($\tau = 23.38 \pm 0.30 \text{ h}$; Figs. 1 and 2), however all but one exhibited secondary peaks in the circatidal range ($\tau = 12.01 \pm 0.24 \text{ h}$, Fig. 2). In addition, eight of the 15 (53%) switched to circatidal rhythms of activity ($\tau = 15.02 \pm 1.83 \text{ h}$; Fig. 3). Six of the original 24 animals (25%) expressed circatidal rhythms of activity during LD ($\tau = 12.36 \pm 0.20 \text{ h}$), and five of these
Figure 1. Locomotor activity of a daily and circadian juvenile horseshoe crab. Image on the left is a double-plotted actogram; activity is indicated by black. The imposed light:dark cycle (LD) indicated at the top of the image. During the first ten days, horseshoe crabs were exposed to a 14:10 LD cycle and during the second ten days they were in constant darkness (DD). Images on the right are Lomb-Scargle periodograms for the same animal during either the LD period (top) or the DD period (bottom). Vertical scale is the relative strength of rhythmicity (Q(p)); horizontal axis shows length of periods in hours (10 - 30); the largest peak above the horizontal line of significance ($P < 0.001$) is indicated by a numerical value.
Figure 2. Locomotor activity of a horseshoe crab expressing daily activity in LD and a combination of circadian and circatidal activity in DD. Refer to Figure 1 for detailed description of figure components.
Figure 3. Locomotor activity recorded from a juvenile horseshoe crab expressing daily activity in LD and circatidal activity DD. Refer to Figure 1 for detailed description of figure components.
animals (80%) continued this same pattern of behavior when subjected to DD (τ = 12.48 ± 0.13 h). One animal switched from a circatidal pattern of behavior in LD to a circadian pattern in DD (20%, τ = 23.45, data not shown). Three of the 24 of the animals tested were arrhythmic.

Throughout this experiment, there were strong indications of masking. Two animals appeared to initially express a circatidal pattern of activity in LD, before synchronizing to the LD cycle (Fig. 4). Eight animals showed a preference for nighttime activity during the light cycle and then switched to a circatidal pattern in DD (Figs. 2 and 3). All of these animals appeared to have an underlying tendency for a circatidal rhythm that was masked by LD. This masking resulted in activity that appeared nocturnal, but was more likely the expression of either unimodal tidal activity (one bout at night advancing an hour each day) or unequal bimodal activity (one of the two bouts is longer or shows more activity than the other) rather than a true circadian clock.

Of the 15 animals expressing daily rhythms in LD, seven (47%) were significantly more active during the day and three (20%) during the night (Wilcoxon matched-pairs signed-ranks test, P < 0.02). The remaining five animals did not express significantly different activity levels between day and night. When diurnal animals during LD were exposed to DD, three expressed a circadian rhythm while four switched to a circatidal pattern of activity. Of the three nocturnal animals during LD, one remained circadian while two expressed circatidal patterns of behavior.

Activity duration (alpha) was calculated for 11 animals since the remaining animals did not have clear enough patterns to calculate alpha. During the LD portion of
Figure 4. Masking of locomotor activity of a juvenile horseshoe crab exposed to LD and DD. Refer to Figure 1 for detailed description of figure components. This animal initially expressed a primarily diurnal pattern of behavior, with some hint of an underlying tidal component. When not exposed to LD cues, this animal reverted to four bouts of activity daily, or a tidal rhythm.
the experiment, nocturnal animals had bouts of activity lasting 9.32 ± 2.20 h (n = 6). The one diurnal animal had an activity duration of 16.4 h, and the four circatidal animals expressed bouts of activity 14.94 ± 2.19 h long. During DD, alphas were 8.8 ± 0.69 h for animals expressing circadian rhythms (n = 7), and 5.57 ± 1.27 h (n = 4) for those expressing circatidal rhythms. The duration of activity for horseshoe crabs expressing daily patterns of activity was not significantly different between LD and DD conditions (LD: α = 9.32 ± 2.20 h; DD: α = 8.8 ± 0.69 h; P > 0.05, Mann-Whitney test). Tidal animals had significantly longer bouts of activity in LD than in DD (LD: α = 14.94 ± 2.19 h; DD: α = 5.57 ± 1.27 h; P = 0.019, Mann-Whitney test).

**Discussion**

In this study, the locomotor rhythms of juvenile horseshoe crabs were examined to determine the types of activity patterns expressed under summer LD as well as in DD. The majority (63%) of the animals expressed a daily rhythm of activity in LD. Of the 15 animals that expressed a daily pattern of activity in LD, seven expressed a nocturnal preference. Two previous studies conducted under ambient light (Casterlin and Reynolds 1979, Borst and Barlow 2002) indicated primarily nocturnal patterns of activity, as was seen in this study. In the laboratory, under a light cycle, larvae exhibited nocturnal patterns of activity (Ruldoe 1979, Ehlinger and Tankersley 2006). When adults expressed a daily pattern of activity, more were significantly active during the day than night, but the majority expressed no day/night preference (Chabot et al. 2007). The ability of all stages of horseshoe crabs to exhibit a daily pattern of activity indicates that the ability to determine the phase of the light cycle was evolutionarily significant. It is possible that it
is a vestigial cue on the activity patterns like that theorized in fiddler crabs (*Uca spp.*, Honegger 1973a).

A study of juvenile horseshoe crabs from Florida indicated that the juveniles were primarily diurnal under ambient light conditions, but data were not sufficient to determine if activity patterns were circatidal or daily (Rudloe 1981). The data from the present study were obtained at a resolution to resolve the question of daily versus circatidal, indicating that 29% of the animals expressed a circatidal rhythm when exposed to LD. When in DD, some of the juvenile horseshoe crabs that expressed a daily pattern of activity in LD switched to a tidal rhythm (8 of 15). A majority of the animals that continued to express a circadian pattern of activity in DD exhibited indications of a circatidal pattern of activity shown by a lesser bout of activity approximately 12 h after the primary activity. This resulted in a secondary peak on periodograms in the circatidal range as was seen in the previous studies by Casterlin and Reynolds (1979) and Borst and Barlow (2002). It is possible with an extended DD, the secondary peak would become more prevalent. Previous field studies of juveniles have shown tendencies for tidally based activities indicating a preference for low tide (Rudloe 1981, Chiu and Morton 2004). Juvenile horseshoe crabs may be more active during a specific tidal phase due to the availability of their prey (Palmer 1995b). Larval horseshoe crabs are also exhibit tidal patterns of vertical migration in their nests and emerge from their nest during the night (Rudloe 1979). In addition, larvae exhibit circatidal activity patterns under constant tidal conditions (Ehlinger and Tankersley 2006). When exposed to DD, adult horseshoe crabs exhibit a circatidal pattern of activity (Chabot et al. 2007). Given that all stages exhibit a tidal pattern of behavior when held in constant light conditions, it is likely that the daily
patterns of activity exhibited by the juveniles and the adults are due to masking of activity patterns by the light cycle (Chabot et al. 2007).

Masking of a circatidal activity pattern by a light cycle is not unusual; it has been shown in at least three other organisms. In fiddler crabs (Uca spp.), LD cycles have been shown to influence circatidal patterns of activity through synchronization of activity patterns with the light cycle and phase-shift experiments. When the light cycle was shifted, there was an immediate change in the pattern of behavior (Honegger 1973a). In C. maenas and Sesarma reticulatum, the circatidal pattern of activity was influenced by the light cycle with both organisms expressing highest activity during the nighttime high tide (Naylor 1958, Palmer 1990). Two juvenile horseshoe crabs in this study initially exhibited activity that started approximately an hour later each day indicative of a circatidal pattern of activity. When exposed to DD, many animals either switched from circadian to circatidal (8 of 15) or expressed a secondary peak of activity in the circatidal range (6 of 15). In combination, these results indicate masking of a circatidal activity pattern by the LD cycle. When exposed to LD, activity patterns may synchronize to the cycle. However, if the LD cycle is a masking cue, its removal should cause activity patterns to change (Honegger 1973a). If LD is not masking the activity pattern, then removal of the cue will not immediately influence the activity; it should continue the pattern from its current timing (Honegger 1973a).

The high prevalence of this change in activity patterns suggests that the presence of light and the absence of a tidal cycle may cause light to be interpreted as a secondary tidal zeitgeber. Light levels may provide a redundant tidal signal in turbid estuaries because the turbidity of water causes significant attenuation of light and as the depth of
The water varies with each phase of the tidal cycle, light levels at the benthos fluctuate correspondingly (Chabot et al. 2008, Watson et al. 2008). It is possible that the animals expressing nocturnal activity were not expressing a preference for night but rather a preference for a lower light level such as might occur during high tide. When exposed to constant tidal conditions and LD, the majority of adults exhibit daily patterns of activity indicating that light may have an influence, albeit weak, on an endogenous clock controlling a circatidal pattern (Watson et al. 2008, Chabot et al. 2011).

The cause of the variation of activity patterns seen in this study, ranging from daily or circadian to circatidal to arrhythmic, is not clear. However, there are several possible explanations. The arrhythmic activity could be due to exposure to only one cue, the light cycle. Juveniles in this study showed strong indications of masking by LD, and the arrhythmic activity could be an extreme version of masking. It is possible that increased eye-sensitivity of these animals at night was such that they did not experience large enough difference in illumination between day and night to synchronize their activity. In this case, the animal would perceive itself to be under constant conditions. Previous studies of larval horseshoe crabs have indicated that holding in constant low light causes a reduction in activity, leading to an arrhythmic pattern of activity (Rudloe 1979). The increased sensitivity of these animals could be due to their molt stage, since eyes continue to develop as the animal ages (Meadors et al. 2001). If these animals were one molt stage ahead of the others, it could influence their response to the light cycles.
The variation of activity expressed is not unusual for intertidal organisms. Three different species of crabs (C. maenas, Emerita talpoida, and Uca spp.) all exhibit daily and tidal patterns of activity, and within the species, exhibit a wide variety of patterns. Many studies of C. maenas use grouped data obscuring individual variation and allowing for broader conclusions, but still show variability in patterns expressed (Naylor 1958). Mole crabs (E. talpoida) use the tidal swash to migrate up and down the beach. The adults display significantly less variation in activity patterns than the megalopae and juveniles from the same beach (Forward et al. 2005). This is likely due to the variable selection pressures influencing different life stages. The most variation is seen in multiple species of fiddler crabs (Uca spp.). Activity patterns expressed include: circatidal, circadian, a combination of circatidal and circadian, and arrhythmic (Bennett et al. 1957, Barnwell 1966). Three possible explanations for this variation are the collection environment, reproductive state, and molt state (Barnwell 1966). Of these three, the only one applicable to the juvenile horseshoe crabs in this experiment is molt state. The molt state of the horseshoe crabs tested was unknown. None molted during the experiment, but length of time since the previous molt may have influenced the activity patterns.

In addition to variation in other crabs, adult horseshoe crabs express a wide range of activity patterns (Chabot and Watson 2010). These variations are likely due to other factors that influence activity in their natural environment. One factor that likely effects activity patterns is the availability of prey and satiation level. While the prey of juvenile horseshoe crabs is more available at high tides (Palmer 1995b), starvation may override the endogenous clock and change activity patterns. Other starved organisms show a change in activity patterns (Finger 1951, Browne and Evans 1960). It is possible that
satiation level feedbacks onto the endogenous clock. In addition to variation that may be caused by satiation level, there are variations in adult horseshoe crab activity patterns depending on the location and population from which they were collected. While the juveniles in this study were all collected in the same location, and likely from the same population, it is possible that some retain patterns of activity that are exhibited by other populations of horseshoe crabs. For example, adult horseshoe crabs exposed to different tidal cycles exhibit different patterns of mating. When exposed to a diurnal tidal cycle (one high and low per day), horseshoe crabs mate once per day (Rudloe 1979, Cohen and Brockmann 1983) while those exposed to a semi-diurnal tidal cycle (two highs and lows per day), mate twice per day (Rudloe 1985, Barlow et al. 1986). Adults that live in micro-tidal regions have asynchronous mating (Ehlinger et al. 2003). In addition to the synchronization of mating, changes in water depth caused by either the tidal cycle or wind triggers the emergence of the larvae (Rudloe 1979, Ehlinger et al. 2003). Because of the wide variation of activity patterns horseshoe crabs express depending on the environment, horseshoe crabs likely retain the ability to synchronize their activity with a wide variety of cues. Lack of the appropriate cue or enough cues may cause the horseshoe crab to express a variety of rhythms or no rhythm in attempts to "discover" the cue to which it wants to synchronize.

This study indicates that a component of activity patterns is attributable to an endogenous clock controlling circatidal and circadian rhythms. It is unclear if these activity patterns are caused by a combination of circadian and circatidal clocks or two circalunidian clocks. The working hypothesis in adult horseshoe crabs is that activity rhythms are driven by circalunidian clocks (Chabot and Watson 2010). Of the four
possible indicators of a circalunidian clock, the juveniles in this study exhibited three: two rhythms with different periodicities, "splitting" of one activity bout into two, and activity bouts of different durations (Palmer 1990, 1995a). In addition, the low number of juveniles that exhibit a truly circadian pattern in DD (7 of 24) indicates it is less likely that the pattern is a true circadian pattern. It may actually be the expression of one of the two circalunidian clocks. To determine if the second clock would be expressed, the DD portion of the study would need to be longer. Given these data, circalunidian clocks are the most parsimonious explanation for the activity patterns seen in juvenile horseshoe crabs, however further research must be conducted.
CHAPTER 2

ENTRAINMENT OF JUVENILE HORSESHOE CRAB LOCOMOTOR PATTERNS TO ARTIFICIAL TIDES

Abstract

Juvenile horseshoe crabs, *Limulus polyphemus*, express both circadian and circatidal rhythms of locomotion in the laboratory. Tidal rhythms appear to be under the influence of an endogenous clock, but the cues that synchronize this circatidal clock to natural tidal cycles are unknown. The goals of this study were to determine if juvenile horseshoe crabs were capable of synchronizing their activity to either changes in water depth or changes in water depth that included exposure to air at low tide (inundation cycles). The activity of juvenile horseshoe crabs was monitored in the laboratory using time-lapse videography and analyzed visually. Immediately after collection when exposed to water of a constant depth (n=17), 35% of the animals expressed a daily pattern of activity, primarily active at night, 12% expressed a tidal pattern of activity, 12% expressed a combination of daily and tidal activity, and 41% were arrhythmic. When
exposed to an artificial tidal cycle (n=25), 24% of the animals expressed a daily pattern of activity, 24% a tidal pattern of activity, 12% a combination of daily and tidal patterns of activity and 40% were arrhythmic. Upon termination of the tidal cycle, when exposed to constant water depth, 16% expressed a daily pattern of activity, 32% expressed a circatidal pattern of activity, 8% expressed a combination of daily and circatidal patterns of activity, and 44% were arrhythmic. When exposed to an inundation cycle (n=17), 6% expressed a daily pattern of activity, 71% expressed a tidal pattern of activity, 12% expressed a combination of daily and tidal patterns of activity, and 12% were arrhythmic. Of the tidal animals, 58% had a significantly different level of activity between ebb/low tide and flood/high tide (P<0.05). After the inundation cycle, when exposed to constant water depth, 18% expressed a daily pattern of activity, 41% expressed a circatidal pattern of activity, 6% expressed a combination of circatidal and daily patterns of activity, and 35% were arrhythmic. These results suggest that an inundation cycle is a stronger cue than water depth changes alone. In addition, it appears that activity patterns in juvenile horseshoe crabs are influenced primarily by an endogenous clock controlling circatidal rhythms, but are also influenced by the light cycle.

**Introduction**

The tidal cycle influences many aspects of intertidal life, such as locomotion and foraging activity. Ability to anticipate the changing tides allows intertidal animals to make optimal use of each phase of the tidal cycle (DeCoursey 1983, Golombek and Rosenstein 2010). To anticipate changing of the tides, organisms have evolved endogenous clocks that control circatidal activity rhythms with periods of ~12.4 h

Several species of crab, including green crabs (*Carcinus maenas*), fiddler crabs (*Uca spp.*), Dungeness crabs (*Cancer magister*) and *Sesarma reticulatum* express tidal patterns of activity (Bennett et al. 1957, Naylor 1958, Palmer 1990, Forward et al. 2005, Holsman et al. 2006, Vanagt et al. 2008). In addition, all of these organisms continue to express circatidal activity under constant tidal conditions, indicating it is under the control of an endogenous clock. This clock increases survival and fitness of intertidal organisms by allowing them to synchronize activity to and anticipate changing of the tides (Palmer 2000). Cues generally associated with tidal cycles include changes in water depth, hydrostatic pressure, current, turbulence, agitation, turbidity, temperature, salinity, and air exposure; any of these changes may serve as the entraining agent for an intertidal organism (Honegger 1973b, Palmer 1995b, Forward et al. 2005, Ehlinger and Tankersley 2006, Chabot et al. 2011). In addition, light levels may have an influence on the activity patterns as they significantly attenuate through turbid water, changing the levels which reach the benthos depending on the phase of the tidal cycle (Watson et al. 2008). In some organisms, water levels are the strongest entraining cue, while other organisms require multiple cues to entrain to a tidal cycle (Honegger 1973b, Chabot et al. 2011). Green crabs (*C. maenas*) express a tidal cycle entrained by a combination of changes in temperature, water depth, and exposure to air (Naylor et al. 1971) while fiddler crabs (*U. 
crenulata) showed increased entrainment when exposed to multiple tides cues rather than just one (Honegger 1973b).

While the biologically relevant rhythm is circatidal, it may be caused by two different molecular mechanisms, either a circatidal clock or two circalunidian clocks. A circatidal clock is \( \sim 12.4 \text{ h} \) long, the length of one high and one low tide. A circalunidian clock is \( \sim 24.8 \text{ h} \) long and the two clocks are coupled \( 180^\circ \) out of phase. This allows for two bouts of activity \( \sim 12.4 \text{ h} \) apart, producing a circatidal rhythm.

Adult American horseshoe crabs, *Limulus polyphemus*, frequently visit the intertidal zone and therefore must track the tidal cycle. These animals emerge from deeper waters during spring high tides to mate (Rudloe 1980, Cohen and Brockmann 1983, Brockmann 2003, Watson and Chabot 2010) but also frequent mudflats during most months of the year to forage for food (Watson et al. 2009, Lee 2010, Watson and Chabot 2010). Several studies have shown that adult horseshoe crabs have an endogenous oscillator modulating circatidal locomotor activities (e.g. Chabot et al. 2004, 2008, Watson et al. 2008). Further, adults can be entrained to artificial tidal cycles in the laboratory. When subjected to water depth changes that exposed them to air at low tide (inundation), the majority of subjects expressed a tidal pattern of locomotion synchronized to the imposed cycle (Chabot et al. 2008, 2011). Moreover, when the tides were stopped exposing the animals to water of a constant depth, the majority expressed a circatidal pattern of locomotion indicating their clock had entrained to the imposed tides (Chabot et al. 2011). In contrast, when exposed to smaller water depth changes that did not leave them exposed to air at low tide (0.2 m), fewer animals entrained to the tidal cycle and even fewer continued to express a tidal pattern of locomotion when the tides
were stopped. Exposure to an inundation cycle caused greater synchronization and entrainment than water depth changes alone, although depth changes were sufficient for entrainment of some individuals. Likewise, other cues associated with the tides, such as changes in temperature, salinity, and currents, influenced patterns of locomotion expressed by adults, but none were as effective as changes in water depth (Chabot et al. 2008, 2011).

In addition to tidal activity expressed by adult horseshoe crabs, a daily component is also expressed similar to other intertidal animals that express combinations of daily and tidal rhythms (e.g. *C. maenas*, Naylor 1958; *Excirolana chiltoni*, Ehright 1976; *Eurydice pulchra*, Reid 1988, Williams 1991; *S. reticulatum*, Palmer 1990). A combination of daily and tidal activity, is often expressed as one of two patterns: 1) an unimodal, circatidal pattern with an animal active during one tidal phase of the day or night (Palmer 1990, 1995a, Chabot and Watson 2010) or 2) an unequal, bimodal, circatidal pattern in which an animal exhibits two bouts of activity ~12.4 h apart; one activity bout is longer or contains more activity (Naylor 1958, 1996, 1997). These activity patterns could result from either a circadian clock modulating the output of a circatidal clock or two circalunidian clocks (24.8 h) 180° out of phase with one activity bout diminished or suppressed (Bregazzi and Naylor 1972, Palmer 1990, Chabot et al. 2008). The underlying mechanisms have not been resolved in adult horseshoe crabs that express these complex patterns of activity. In fact, the underlying mechanisms have not been resolved in any intertidal organisms, but many have working theories as to which mechanism is more likely. The working theory for adult horseshoe crabs is that activity is controlled by two coupled circalunidian clocks (Chabot and Watson 2010).
Another factor that might modulate an endogenous rhythm is hunger or feeding. Other species, such as the blowfly (*Phormia regina*), rat (*Rattus norvegicus*), and isopod (*E. pulchra*), have been shown to stop or reduce endogenous patterns of activity when satiated (Finger 1951, Browne and Evans 1960, Reid 1988). There is some evidence that this may also be a factor in adult horseshoe crab activity (Watson and Chabot 2010).

A number of studies have reported evidence that juvenile horseshoe crabs, like adults, express a variety of rhythms of activity. Two laboratory studies found that juvenile *L. polyphemus* were nocturnal (Casterlin and Reynolds 1979, Borst and Barlow 2002). However, neither study provided strong evidence that an endogenous clock drives the pattern. In both studies, data presented showed two bouts of activity, the one at night significantly larger than the one during the day. In a field study of juvenile horseshoe crab rhythms in Florida, Rudloe (1981) reported that the activity peaked two hours prior to low tide. When Meury and Gibson (1990) exposed animals to falling tides in the laboratory, the animals followed the falling water and buried in the sediment before extreme low tide. Therefore, as with adult *L. polyphemus*, juveniles appear capable of expressing both tidal and daily patterns of behavior, and, as demonstrated in Chapter One, these animals appear to be under the influence endogenous clocks controlling circatidal and circadian rhythms.

The major objective of this chapter was to determine if changes in water depth or inundation are capable of synchronizing and entraining the endogenous clock controlling tidal locomotion in juvenile horseshoe crabs.
Materials and Methods

Collection and Care of Animals

Juvenile American horseshoe crabs (*L. polyphemus*, 22 – 35 mm carapace width) were collected during the daytime low tide from Little Sippewissett Marsh, Falmouth, MA on two dates (8 on 8/7/2011 and 17 on 9/9/2011). They were transported in coolers to an indoor holding facility at the University of New Hampshire, Durham, NH. Animals were placed in individual testing chambers within 24 h. Between trials animals were kept in a communal tank filled with 5 cm sand and 25 psu seawater and exposed to a 14:10 light:dark cycle (LD).

Animals were fed to satiation once prior to the start of the trial using frozen *Spirulina* brine shrimp (Hikari Bio-Pure, Hikari, Hayward, CA) thawed in saltwater. Satiation was determined by observing the cessation of the stereotyped rhythmic movements of their legs, as described by Wyse and Dwyer (1973).

Experimental Set-Up

Horseshoe crabs were placed in individual 20 cm diameter plastic chambers filled with 3 cm of sand. These chambers were placed in larger tanks filled with 25 psu saltwater. Two mesh cut-outs (5 x 15 cm) in each chamber allowed aerated water in the large tanks to mix with the water in each chamber.
Two sets of larger tanks were used in these studies. The first set consisted of three 121 l garbage cans (66 x 55.9 x 72.4 cm) that held recording chambers, plus a fourth that served as a water reservoir. Two of the garbage cans held three recording chambers, while the third tank held two chambers. The second set-up consisted of a 263 l rectangular tank (93 x 51 x 55.5 cm) that held nine recording chambers connected to a water reservoir (Fig. 1).

Throughout each experiment, animals were exposed to a 14:10 LD cycle. A modified dawn/dusk simulator (SunUp, Light Therapy Products, Plymouth, MA) was used to gradually increase and decrease the light (100W, GE Reveal®, Schenectady, New York) for two hours at the beginning and end of each day. There was one light bulb over each garbage can and one over the larger holding tank. In addition, red lights (25W, Philips® Party & Deco, Andover, MA) continuously illuminated the chambers, one over each garbage can and two over the larger tank, exposing animals to dim red light at night. Since constant darkness was not necessary for this experiment, horseshoe crabs have shown decreased sensitivity to red light (Graham and Hartline 1935), and red light yielded better video than infrared lights alone, it was added to the set-up.

Semi-diurnal tides were created using pumps (Rio® Plus 800) powered via a timer (Amertac® TEO5W Indoor Weekly Digital Timer, Saddle River, NJ). Two sets of pumps were used to create the tides in the garbage cans. One set of pumps transferred water between the two tanks with three chambers each, creating low tide in one and high tide in the other
Figure 1. Diagram of tanks and recording chambers. Light grey indicates individual chambers and darker grey indicates larger water tanks. When creating tides in (A1) and (A2), water was between the two tanks, creating opposite tidal cycles. In tank (A3) and (B), water was pumped into and out of the reservoir.
(Fig. 1A, Tanks 1 and 2). The other set pumped water back and forth between the tank containing two chambers and the reservoir (Fig. 1A, Tank 3). The tides in the rectangular tank (Fig. 1B) were created by pumping the water from the tank into the reservoir (low tide) and then back into the tank (high tide). Tides advanced by 12 min each cycle, resulting in a 12.45 h cycle. Tides in trial 1 had a random start time (low tide at 1600), while tides in trials 2 – 5 replicated the tidal cycle in Little Sippewissett Marsh, Falmouth, MA. Water depth and tidal range varied by trial (Table 1). In trials 1, 2, and 3, water levels were 20 cm deep at low tide and increased to 65 cm deep at high tide. During trials 4 and 5 the surface of the sand was exposed to air during low tide and 68 cm deep at high tide (inundation).

A total of 25 animals were used in this study. During the water depth change study (trials 1 – 3), animals were tested immediately after collection. Eight animals were used in trials 1 and 2, and nine in trial 3. After each trial, animals were returned to a holding tank for seven days. During this time they were exposed to a 14:10 LD cycle and constant water depth. For the inundation study (Trials 4 and 5), a total of 17 animals were randomly chosen from the original 25 (8 for trial 4 and 9 for trial 5). No comparisons were made between experienced and non-experienced animals.

**Trial Length**

Trial 1 lasted for 20 days; the first ten days exposed animals to an artificial tidal cycle while the second ten days animals to constant water depth, halfway between high and low tide depths (Table 1). Trials 2 and 3 lasted for 30 days. During the first ten days animals
were exposed to water of a constant depth to determine what kind of rhythm they would express with no tidal cues. The remainder of trials 2 and 3 were similar to trial 1, ten days of artificial tides followed by ten days with a constant water depth. Trials 4 and 5 lasted for 20 days. During the first ten days, animals were exposed to an artificial inundation cycle, during which the surface of the sand was exposed to air at low tide. During the second ten days, animals were held in water of a constant depth halfway between high and low tide levels.

Data Collection and Analysis

To increase the visibility of animals at night, a 1 cm² piece of reflective tape (Nashua® Multi-purpose Foil Tape) was attached to the dorsal carapace of each horseshoe crab using cyanoacrylate-based glue (Krazy® glue). No simple or complex eyes were covered. All activity was recorded using analog video cameras. The output was digitized (Canopus® ADVC-55; ADVC-110) and recorded at a rate of one frame every 30 s using video capture software on Macintosh computers (Gawker, v. 0.8.3, Seattle, WA, Macintosh, OS 10.4.10; 10.5.8). Two types of cameras were used, Enforcer (EV-1323C12DW) and Supercircuits PC74WR. Both were infrared-sensitive, black and white cameras. The Enforcer camera was used because it was submersible, while the Supercircuits camera was only water resistant. Eight Enforcer cameras were used in each trial. The video streams were combined into a single stream using a multiplexer (Pelco Genex™ MX4009MD, 9 channel) and digitized.
Table 1. Water and tide cycle depth during each phase of the experiment.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Days 1-10</th>
<th>Days 10-20</th>
<th>Days 20-30</th>
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<tr>
<td>Trial 2</td>
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<td>Trial 3</td>
<td>Constant water depth (16cm)</td>
<td>Imposed tidal cycle (30cm)</td>
<td>Constant water depth (26cm)</td>
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<tr>
<td>Trial 4</td>
<td>Imposed inundation cycle (68cm)</td>
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<td>-</td>
</tr>
<tr>
<td>Trial 5</td>
<td>Imposed inundation cycle (51cm)</td>
<td>Constant water depth (26cm)</td>
<td>-</td>
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</table>
Videos were analyzed by eye in five minute blocks. Any movement during a five minute block was recorded as a “1” while no movement was recorded as a “0”. These data were plotted as actograms and Lomb-Scargle periodograms using ClockLab® (MatLab, Actimetrics, Evanston, IL, v. R2009a). Significance of rhythmicity was determined both visually and by periodograms ($P < 0.001$) to determine the primary component of rhythmicity during each experimental condition (Chabot et al. 2008).

Tracks of animal movement were made using EthoVision XT® (v. 8, Noldus Information Technology, Leesburg, VA) to track the organism through high or low tide and exported as an image (See Noldus et al. 2001).

Significant differences in activity levels during the ebb/low and flood/high tides were determined by calculating percent of time animals were active during each tidal phase and then comparing averages using InStat® (GraphPad Software, La Jolla, CA, v. 3.1a 2009). All data was analyzed using Wilcoxon matched-pairs signed-ranks test unless otherwise stated.

To determine the delay of onset of activity (phase angle) to imposed environmental cues (tidal cycle) and the duration of the main bout of activity (alpha), best eye-fit lines were drawn through the onset and offset of activity using a single-blind protocol.
Results

Of the 17 individuals subjected to 10 days of constant water depth and LD immediately after collection, six animals expressed a daily pattern of activity (Table 2, Fig. 2, \( \tau = 22.02 \pm 2.04 \) h, average \( \pm \) SEM). Two animals expressed a mix of daily and circatidal activity, and two displayed a circatidal pattern of activity (\( \tau = 13.02 \pm 0.96 \) h). Two additional animals were almost continuously active during this phase of the study and the five remaining animals were arrhythmic.

When exposed to water depth changes, animals were 20 cm underwater at low tide (\( n = 25 \)). Six animals displayed a daily pattern of activity (Fig. 2, \( \tau = 23.82 \pm 0.09 \) h), three expressed mix of daily and tidal patterns, and six expressed a tidal rhythm of activity (Figs. 3 and 4, \( \tau = 13.81 \pm 1.80 \) h). One animal was inactive and nine were arrhythmic.

During the inundation cycle, animals were exposed to air at low tides (\( n = 17 \)). Under these conditions, the majority (12 of 17) of animals expressed a tidal pattern of activity (\( \tau = 14.73 \pm 1.51 \) h, Figs. 5, 6, 7, and 8). One animal expressed a daily pattern of activity (\( \tau = 22.60 \) h) and two expressed a mixed daily and tidal pattern of activity. The remaining two animals were arrhythmic. Presence or absence of water did not impede ability of animals to move; some animals were most active during high tides and some during low tides (Figs. 7 and 8).

After exposure to tidal cycles, all animals were exposed to ten days of constant water depth, determining their ability to retain entrained activity patterns. During this
Table 2. Summary of results by animal. Gray indicates the phase was not performed.

<table>
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Figure 2. Locomotor activity recorded from a daily juvenile horseshoe crab exposed to constant water depth, a tidal cycle and then constant water depth, each for ten days. Image on the left is a double-plotted actogram; activity is indicated by black, with the imposed light:dark cycle indicated at the top of the image with vertical lines indicating night. Light gray bars indicate the flood and high tide. Images on the right are Lomb-Scargle periodograms for the same animal during either the constant water depth period (top), the tidal cycle (middle), or the constant water depth period (bottom). Vertical scale is the relative strength of rhythmicity (Q(p)); horizontal axis shows length of periods in hours (10 - 30); the largest peak above the horizontal line of significance (P < 0.001) is indicated by a numerical value.
Figure 3. Masked tidal activity from a juvenile horseshoe crab exposed to water depth changes and then constant water depth. Refer to Figure 2 for detailed description of figure components. This individual expressed a tidal pattern of behavior when exposed to a tidal cycle, however the two cycle were not synchronized, indicating that this individual was not entrained to the tidal rhythm to which it was exposed. This shows the tidal cycle that does not expose the individuals to air during low tide may be strong enough to be sensed but are not strong enough to entrain or may simply confuse the individual.
Figure 4. Tidal activity recorded from a juvenile horseshoe crab exposed to water depth changes followed by constant water depth. Refer to Figure 2 for detailed description of figure components. This individual expressed a clear tidal pattern of behavior after exposure to the tidal cycle ended, and there were some indications of a tidal pattern being expressed when exposed to the tidal cycle, but not enough to draw any definite conclusions. This individual entrained to the imposed tidal cycle, but did not express synchronized activity pattern until after the tidal cycle had ended.
Figure 5. Locomotor activity recorded from a juvenile horseshoe crab exposed to inundation followed by constant water depth. A 12.45 h inundation cycle (exposed to air at low tide) was imposed for ten days and then constant water depth for ten days. Refer to Figure 2 for detailed description of figure components. This animal expressed a clear tidal pattern of behavior during exposure to the inundation cycle and for four days after the cessation of the inundation cycle. This animal delayed its onset of activity 4.27 ± 0.25 h (n = 9 cycles) after flood tide had started and was most active during high tide.
Figure 6. Locomotor activity recorded from a juvenile horseshoe crab exposed to a 12.45 h inundation cycle and then constant water depth. Refer to Figure 2 for detailed description of figure components. During the inundation cycle the horseshoe crab had a period, phase angle, and alpha that closely mirrored the imposed inundation cycle. There was a significant difference between activity during flood/high tide and ebb/low tide ($P < 0.0001$, Wilcoxon signed-rank test). However, when the artificial tide cycle stopped, this animal significantly decreased its activity ($P = 0.0039$) and the tidal pattern of activity was intermittent.
Figure 7. Locomotor activity recorded from a juvenile horseshoe crab active at high tide. This animal was exposed to a 12.45 h inundation cycle followed by constant water depth, each for ten days. Refer to Figure 2 for detailed description of figure components. This animal showed tidal pattern of activity when exposed to the inundation cycle and a very weak tidal pattern of activity under constant water depth that was not synchronized to the previously imposed inundation cycle. This animal was mostly active during high tide. (A) and (B) are the tracks of the animal during low and high tide, respectively, and the time used is indicated on the actogram.
Figure 8. Locomotor activity of a juvenile horseshoe crab active during low tide. This animal was exposed to a 12.45 h inundation cycle followed by constant water depth, each for ten days. Refer to Figure 2 for detailed description of figure components. This animal showed a moderate tidal pattern of activity when exposed to the inundation cycle and a diurnal pattern of activity under conditions of constant water depth. This was one of the few animals that appeared to prefer to be active during the low tide portion of the imposed tide cycle. (A) and (B) are the tracks of the animal during low and high tide, respectively, and the time used is indicated on the actogram.
time, the most prevalent pattern of activity was circatidal (15 of 42, Figs. 4, 5, 6, and 7, $\tau = 12.18 \pm 0.23$ h); eight had been exposed to water depth changes and seven to the inundation cycle, all of which had expressed a tidal component of activity when exposed to tides. Three animals expressed a mix of circadian and circatidal activity and seven expressed a daily pattern of activity ($\tau = 23.27 \pm 0.45$ h). Eight had significantly decreased activity, resulting in no activity ($P < 0.05$). The remaining nine animals were arrhythmic.

After the cessation of the inundation cycle, overall activity significantly decreased in 11 of 12 animals that had expressed a tidal pattern under inundation ($P < 0.05$, Figs. 5, 6, 7, and 8) and in 2 of 2 animals that expressed a diurnal tidal pattern under inundation. A similar reduction in activity was seen in two of six tidal animals exposed to depth changes with no inundation (Fig. 3). Three additional animals exposed to inundation also displayed this decrease in activity after the cessation of the inundation; two went from being constantly active to a diurnal pattern and one went from diurnal activity to almost no activity. It appears that exposure to an inundation cycle may cause an increase in activity compared to activity levels under constant conditions and may change the patterns of activity expressed by juvenile horseshoe crabs.

During the inundation study, two of 17 animals were arrhythmic, while during the water depth change experiment, ten of 25 animals were arrhythmic. Six of 25 animals (24%) exposed to water depth changes expressed a tidal pattern of behavior (Fig. 4), while 12 of 17 (71%) expressed a tidal pattern of activity when exposed to inundation tides (Figs. 5, 6, 7, and 8). Further, when the tidal cycles stopped, ten of 25 animals
(40%) exposed to the water depth changes expressed a circatidal pattern (Fig. 4), while eight of 17 (47%) expressed a circatidal or mixed circatidal and circadian pattern after exposure to an inundation cycle (Figs. 5, 6, and 7). Exposure to an inundation cycle appeared to cause the circatidal pattern of activity to continue longer in atidal conditions (9 ± 1 d) than exposure to a tidal cycle (7.3 ± 0.9 d), but was not significantly different (P > 0.05, Mann-Whitney test).

When exposed to an inundation cycle, seven of 12 tidal animals were significantly more active (P < 0.05) during the flood/high tide than ebb/low tide (Figs. 3, 4, and 5). One animal exposed to water depth changes was also significantly more active during the flood/high tide when compared to the ebb/low tide.

Most animals exhibited no preference for day versus night (P > 0.05). During the initial constant water depth (n = 17), four animals were significantly more active at night and one during the day. During the water depth change, two animals expressed significant diurnal activity and three expressed nocturnal activity. Under the final constant water depth, daily animals were split between diurnal and nocturnal activity preference (n = 6). No animals exposed to inundation had a significant preference for day versus night.

Length of activity bouts (alpha) were calculated for all animals with clear enough patterns. During the initial constant water depth, nocturnal activity lasted 11.26 ± 0.53 h (n = 5). When exposed to water depth changes, nocturnal activity lasted 11.02 ± 0.28 h (n = 3). After cessation of the tidal cycle, nocturnal activity length shortened to 9.27 h (n = 1). The activity duration of the diurnal activity also mirrored the day length. During the
initial constant water depth, diurnal activity lasted 15.67 h (n = 1). When exposed to a tidal cycle, diurnal animals expressed an alpha of 11.59 ± 4.17 h (n = 3). Under constant water depth, diurnal activity lasted 13.60 h (n = 1). Tidal activity under the initial constant water depth lasted 5.63 h (n = 1). When exposed to water depth changes, tidal activity lasted 6.77 ± 0.35 h (n = 8). Under constant water depth, one animal expressed a circatidal pattern of activity showing two bouts of activity, each with a different duration (Fig. 2C; α = 2.73, 7.36; day, night, respectively).

Discussion

In both the water depth change and inundation cycle experiments, some animals entrained to the tidal cycle and continued to express a circatidal pattern of activity under constant water depth (Figs. 4 and 5). These data support the hypothesis that juvenile horseshoe crabs, like adult and larval horseshoe crabs, have endogenous clocks controlling circatidal rhythms (Chabot et al. 2008).

The majority of animals expressing a tidal rhythm of locomotion preferred to be active during flood and high tides (Fig. 6). Previous laboratory and field experiments have indicated a preference for ebb and low tide, opposite of what was seen in this experiment. One possible explanation for decreased activity during the low tide in this study is juvenile horseshoe crabs have a reduced ability to move when pore water drains away, as occurred in the inundation study (Meury and Gibson 1990). Juveniles in this study were capable of moving during low tide (Fig. 8). In their natural habitat, it is possible that juveniles inhabit areas where the pore water does not drain away, causing a
different activity pattern. The studies indicating that juveniles may prefer low tide occurred in Florida and Japan where juveniles inhabit wide expanses of sand directly exposed to the ocean. The juveniles tested in this study were collected in a tidal marsh that undergoes dramatically different water flow regimes than open beaches. During ebb tide in the marsh, water flowing into the ocean is limited to channels containing faster flowing water whereas on an open beach, water ebbs from the entire area. This difference in tidal flow likely changes pore water retention and currents to which the juvenile horseshoe crabs are exposed, causing expression of different activity patterns. Different populations of adults express different mating patterns, depending on the mating environment (Rudloe 1979, 1985, Cohen and Brockmann 1983, Barlow et al. 1986, Ehlinger et al. 2003). If adults are capable of expressing different types of rhythms, the juveniles must also retain this ability.

Juvenile horseshoe crabs may use activity during phases of the tidal cycle to remain in their optimal nursery habitat. Other organisms are active during specific phases of the tidal cycle to remain in their optimal environment. Both the snail (Olivella semistrriata) and mole crab (Emerita talpoida) use tides to reduce the probability of being stranded on the beach during low tide (Forward et al. 2005, Vanagt et al. 2008). In addition, Dungeness crabs (C. magister) use tidal currents to migrate into and out of littoral zones (Holsman et al. 2006).

Another evolutionary significant reason for tidally based activity is to decrease foraging time or avoid predators, even in their absence. The intertidal organisms that constitute the majority of juvenile horseshoe crabs’ prey exhibit a tidal pattern of activity, moving toward the surface during flood/high tide (Palmer 1995b). If the primary reason
for moving were to forage, then it would be more efficient for the horseshoe crabs to be active when their prey is closer to the surface. Avoidance of predators may have been evolutionarily selected; the patterns avoiding the most predators would be the one expressed. This has been seen in other marine organisms such as lobsters (*Homarus americanus*), even when the predators are not present. In the lab, lobsters are primarily nocturnal but have limited diurnal activity, the time when their natural predators hunt (Jury *et al.* 2005). However, the addition of predator cues to laboratory studies significantly constrains the activity of the lobsters to the night (Spanier *et al.* 1998).

Seabirds are one of the major predators of juvenile horseshoe crabs. During low tide, many seabirds forage in the marsh and juveniles are more vulnerable than adults (Walls *et al.* 2002). Activity during high tide could avoid these predators.

Two aspects of the study appeared to reduce arrhythmic activity: the timing and depth of the tidal cycle. When the start of the tidal cycle was randomized, 50% of animals were arrhythmic. Synchronizing the imposed tides with the natural tides decreased the number of arrhythmic activity patterns to 15% of animals. This may be due to the animals retaining the tidal rhythm from their natural environment. When exposed to water depth changes versus inundation, fewer animals expressed a defined pattern of activity (60% under water depth changes versus 88% under inundation cycle) and fewer still entrained to the tidal cycle (24% under water depth changes versus 71% under inundation cycle). This is likely due to the fact that inundation exposed the juveniles to more and stronger cues, including a larger water depth change (and therefore pressure change), a larger change in light levels, and air at low tide. The animals may have evolved to respond to
the combination of the tidal indicators rather than just one. More adults expressed tidal rhythms when exposed to inundation than changing water depth alone (Chabot et al. 2011). In addition, water depth changes may have been too weak a cue and caused confusion or conflict with the rhythm from their natural environment (Figs. 3 and 4). Inundation is more similar to their natural habitat in the marsh since the prime collecting area is out of water during low tide.

While it is clear that inundation causes a greater entrainment than water depth changes in both juveniles and adults, it is not clear what other cues may be necessary for juvenile horseshoe crabs to entrain to artificial tidal cycles. Adult horseshoe crabs retain their synchronization to a tidal cycle longer when exposed to several cues such as temperature and turbulence (Chabot et al. 2011), as do other intertidal organisms. In green crabs (C. maenas), a temperature cycle in combination with an inundation cycle produces a stronger circatidal pattern of activity than exposure to only an inundation cycle (Naylor et al. 1971). It is possible that the need for more than one tidal stimulus partially caused the wide variation in activity patterns expressed by juvenile horseshoe crabs. The wide variation in activity patterns of intertidal organisms is not that unusual. Three different species of crabs (C. maenas, E. talpoida, and Uca spp.) all exhibit daily and tidal patterns of activity and within species they exhibit a wide variety of patterns (Naylor 1958, Bennett et al. 1957, Barnwell 1966, Forward et al. 2005). The ability of these organisms to synchronize to different natal environments could cause expression of wide patterns in the laboratory.

It is possible that exposure to a light cycle in addition to the tidal cycle had an influence on activity patterns, via masking. Masking is a change in a pattern of behavior
caused by exposure to exogenous cues (Bregazzi and Naylor 1972). There was evidence of masking caused by the light cycle during this experiment (Fig. 4). Some of the horseshoe crabs were only active during the nighttime high tide, indicating modulation of the tidal activity patterns by the light cycle. While some animals still expressed a tidal component of behavior, masking in other individuals may have been strong enough such that only a daily rhythm of activity was expressed.

There was a significant reduction in the activity of animals expressing a tidal rhythm after the cessation of the tidal cycle (Figs. 5 and 6), but no change in the activity of animals that were not expressing a tidal pattern of activity (Fig. 2). Of the 18 animals that had a significant reduction in activity, 14 (78%) were tested in trials 4 and 5. One possible cause of this reduction in activity levels is the season. While these animals were not exposed to cues associated with changing seasons, it is possible that they possess a circannual clock that indicated winter was approaching and to reduce activity in preparation. Adults are known to reduce activity levels below certain temperatures; adults exposed to a winter photoperiod and summer temperature express a circatidal pattern of activity while those exposed to a winter photoperiod and temperatures show no activity (Schaller et al. 2010).

Exposure to a water depth halfway between high and low tide is a second possible cause for reduction in activity levels that may have confused the organisms (Chabot et al. 2008). In other organisms, circatidal activity patterns tend to degrade quickly when subjected to constant tidal conditions (Palmer 1995b). However, some adult horseshoe crabs have been shown to retain their circatidal pattern of activity for at least two weeks.
under constant conditions (Chabot et al. 2011). While the degradation was not unexpected, its rapid occurrence was unexpected. The water depth halfway between high and low tide may have caused enough conflicting cues that it resulted in no activity (Chabot et al. 2008).

While the reduction in activity levels after the end of tidal cycles could be due to the removal of a masking cue, it is unlikely in these circumstances. The first reason is because it is clear that circatidal patterns of activity are spontaneously expressed and therefore endogenous (Chapter 1). The endogenous nature of this rhythm makes it unlikely that the tidal cycle is masking the expression of a tidal rhythm. Secondly, when activity patterns are masked, the activity patterns match the imposed cue precisely; so the onset of the cue and activity are at the same time, while the off-set of both also co-occur (Bünning 1973). The alpha of these animals indicates that masking does not cause the circatidal activity, as it is not the same duration as the tidal phases.

This study indicates that there is a component of activity patterns attributable to an endogenous clock controlling circatidal rhythms. It is unclear if a circatidal clock or two circalunidian clocks causes these activity patterns. In adult horseshoe crabs, it is the working hypothesis that circalunidian clocks drives activity rhythms (Chabot and Watson 2010). In this study, neither the circalunidian nor combination of circatidal and circadian clocks was supported. Further investigations into the underlying mechanisms of the circatidal pattern of activity are required to elucidate the molecular basis of activity patterns.
CONCLUSIONS AND FUTURE RESEARCH

The results of these studies indicate that juvenile horseshoe crabs have endogenous clocks resulting in a combination of circatidal and circadian patterns of locomotion. Whether these patterns are caused by the combination of circadian and circatidal clocks or by circalunidian clocks is unclear. In LD at a constant water depth, most animals expressed a tidal pattern of activity indicating the presence of an endogenous clock controlling circatidal rhythms, while others exhibit a daily pattern of activity with some preference for being nocturnal. When subsequently exposed to constant darkness a portion of the animals continued to express a circadian rhythm of locomotion indicating the presence of a circadian clock. When exposed to artificial tides, the majority of animals synchronized their activity to the imposed tides with a preference for the flood/high tide. If exposed to air during the low tide portion of the tidal cycle (inundation cycle), activity was more strongly synchronized to the imposed tides and a greater percentage of animals expressed a tidal rhythm of activity. Interestingly, a number of animals also expressed activity patterns combining daily and tidal rhythms. The most common form manifested as activity during the nighttime high tides. This pattern could be the results of the two endogenous clocks interacting (circatidal and circadian or two circalunidian) or the masking effects of LD on the tendency to express a tidal rhythm.
In combination, expression of tidal and daily patterns of activity may have evolved to allow for activity during the times when chances of survival are the highest. During low tide and the day, predation pressures are increased and their prey is less active. In addition, higher temperatures and light levels during low tide and the day would increase thermal stress and the likelihood of desiccation. In the marsh where animals were obtained, they could get swept into the open ocean if they were active during the ebb tide. This might explain why many preferred to be active during the high and flood tides.

This series of experiments has opened many future areas of research on juvenile horseshoe crabs. For example, it would be interesting to determine if their lateral eyes and traditional photoreceptors are required for entrainment to LD. Recent evidence from our laboratory indicates the presence of the photosensitive pigment Cryptochrome, throughout their nervous system. Therefore, they may be capable of using extraocular photoreceptors to sense light and entrain their circadian rhythm. It would be interesting to expose juvenile horseshoe crabs to LD followed by LD with a decreased light level during the day. A similar experiment would be to allow the horseshoe crabs to synchronize to LD and subsequently expose the juveniles to low light for several days followed by constant darkness. Another aspect to explore further would be the locomotor rhythms of juvenile horseshoe crabs exposed to an inundation cycle in DD. It has been shown in adult horseshoe crabs that LD helps synchronize and maintain tidal rhythms; it would be interesting to see if this is also true in the juveniles. These experiments would help elucidate the effects of light on activity of juveniles and how the light cycle modulates the endogenous clock controlling circatidal locomotor activity.
It would also be interesting to determine the effects of chemical cues of predators on activity patterns. To conduct this, various predators of juvenile horseshoe crabs, such as green crabs (*Carcinus maenas*), could be kept in the reservoir tank, and the chemical cues from these organisms applied during specific phases of the tidal cycle by continuous recirculation of water. Finally, it would be interesting to conduct field studies continuously monitoring the activity of juvenile horseshoe crabs to determine if the conclusions from the laboratory studies can be applied to their native habitat.
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Abstract

Laboratory studies of juvenile American horseshoe crabs have demonstrated that they are capable of expressing both daily and tidal patterns of activity. However, the patterns normally expressed in their natural habitat are poorly understood. The goal of this study was to use time-lapse video and visual surveys to determine when the majority of juvenile horseshoe crabs are active. Video was recorded in Little Sippewissett Marsh, Falmouth, MA and Great Bay Estuary, NH. The longest segment of video confidently analyzed was 12 h, due to poor water quality and glare. Video indicated that juvenile horseshoe crabs were most active during high tide. Nine transects were monitored six times a day for five days resulting in a total of 18 data sets (data points missing due to severe weather). These data indicated that juvenile horseshoe crabs were most active at low tide and into the flood tide. While flood tide was the most active time, more data is necessary to determine if high or low tide preferred by more juveniles.
Introduction

While endogenous rhythms are difficult to study in the field due to the inability to maintain constant conditions, field studies are the only way to determine natural patterns of activity. These studies test if the hypothesis of endogenous rhythms of activity uncovered by laboratory studies are valid in the organism's natural habitat. In the laboratory, juvenile horseshoe crabs from Massachusetts expressed a combination of tidal and daily patterns of activity, which continued in constant conditions (Chapter 1 and 2). Together, these results show that juvenile horseshoe crabs possess endogenous clocks controlling circadian and circatidal activity rhythms. It is also clear from these laboratory studies that the tidal cycle has a stronger influence on activity than the light cycle. There are indications the light cycle masks activity patterns. When exposed to constant darkness, some animals maintain a circadian pattern of activity, but the majority expresses a circatidal activity pattern (Chapter 1). However, in their natural habitat, horseshoe crabs are constantly exposed to a light cycle. Little is known about activity patterns of juvenile horseshoe crabs in their natural habitat, including how the interaction of natural light and tidal cycles influence activity patterns.

Field studies of adult horseshoe crabs have shown that the majority of them are active during high tide with no preference for day versus night (Watson and Chabot 2010). Adults typically become active during the last two hours of the flood tide opposite of the tendency of juvenile horseshoe crabs in previous field studies. In Florida, juvenile activity peaked two hours prior to low tide (Rudloe 1981). However, this study only monitored activity at five times relative to the tidal cycle (before, during, and after low; several hours prior to and at high tide) and in different conditions from those in
Massachusetts. A field study of the juvenile horseshoe crab *Tachypleus tridentatus* in Japan showed similar trends of increased activity two hours before low tide, however, data were only collected for one hour, starting two hours prior to low tide (Chiu and Morton 2004). Based on data from my laboratory studies, juvenile horseshoe crabs in Massachusetts should express a combination of daily and tidal activity patterns with a higher prevalence of activity during the nighttime high tide.

In addition to studies of freely moving horseshoe crabs, studies of enclosed animals have also been conducted. When adult horseshoe crabs were studied *in situ*, the majority expressed circatidal rhythms, but there were some indications of a daily component. However, when *in situ* and not experiencing changes in water depth, adults tended to express a daily pattern of activity (Watson *et al.* 2009, Chabot *et al.* 2011). Therefore, while the majority of adults tend to express a circatidal rhythm, they are also capable of expressing a daily pattern (Chabot *et al.* 2011).

Rudloe (1981) found that the activity of juvenile horseshoe crabs in subtidal enclosures correlated more with the light cycle than the tidal cycle. However, these enclosures were at deeper water depths than normally inhabited by the animals, which could influence activity patterns. Furthermore, these animals were only exposed to a 0.6 m tidal cycle, while animals exposed to a 2 m tidal change may show different activity patterns. There is some evidence of this, as a previous study in Massachusetts indicated a nocturnal pattern of activity that may have masked a tidal rhythm (Borst and Barlow 2002). In addition, adult horseshoe crabs exposed to different tidal cycles exhibit different patterns of mating based on the tidal cycle. When exposed to a diurnal tidal cycle (one high and low per day), horseshoe crabs mate once per day while those
exposed to a semi-diurnal tidal cycle (two highs and lows per day), mate twice per day (Rudloe 1979, Cohen and Brockmann 1983, Rudloe 1985, Barlow et al. 1986). Adults inhabiting micro-tidal regions have asynchronous mating (Ehlinger et al. 2003).

Further laboratory studies of activity during ebb and low tide have shown that young-of-the-year horseshoe crabs cannot withstand strong currents (> 25 mm/s). When exposed to stronger currents, they bury in the substrate to reduce the likelihood of being swept into the open ocean (Meury and Gibson 1990). Therefore, it is adaptive for these small juvenile horseshoe crabs to determine the tidal cycle and reduce activity in preparation for the ebb tide. Larger juvenile horseshoe crabs can withstand a stronger current than young-of-the-year and may be active at a different time based on availability of prey and avoidance of predators (Meury and Gibson 1990).

Juvenile horseshoe crabs are selective feeders consuming insect larvae, polychaetes, oligochaetes, crabs, small bivalves, and amphipods (Wyse and Dwyer 1973, Shuster 1982, Chiu and Morton 2004). The availability of this prey is greater at high tide than at low tide (Palmer 1995) and the presence of water would make it easier for the juvenile horseshoe crabs to access prey (Meury and Gibson 1990). The majority of the predators on juveniles are seabirds and intertidal crabs (Shutster 1982, Walls et al. 2002). Shore birds and fiddler crabs (Uca spp.) are primarily diurnal visual predators and usually forage during low tide, while green crabs (Carcinus maenus) forage primarily at night and high tide (Bennett et al. 1957, Naylor 1958, Barnwell 1966, Stillman and Barnwell 2004). Therefore, depending on the dominant predator in a given area, horseshoe crabs might adjust their activity patterns so they are less active at times when their predators are more active.
The goal of this study was to determine the types of activity rhythms expressed by juvenile horseshoe crabs in the field.

Materials and Methods

Animals and Study Site

Juvenile American horseshoe crabs (*Limulus polyphemus*, 34 - 51 mm carapace width) were collected during the daytime low tide from Little Sippewissett Marsh, Falmouth, MA. Four 1 cm$^2$ pieces of reflective tape (Nashua® Multi-purpose Foil Tape) were attached to the dorsal carapace of each horseshoe crab using cyanoacrylate-based glue (Krazy® glue) to increase the visibility of animals at night. No complex or simple eyes were covered. These animals were used in studies during which animals were placed in an enclosure and videotaped.

Video

In Little Sippewissett Marsh (Fig. 1), a GardenCam (Brinno, Industry, CA) was attached to a 108 cm tripod and aimed at the ground of the marsh to record movement of juvenile horseshoe crabs in their natural environment (Trial 1, Table 1). Video was taken for one complete tidal cycle (12.4 h) on 7/28/2010. GardenCam videos had one image taken every minute, recorded to a USB drive, and stitched together as a video. In trial 1, no movement of juvenile horseshoe crabs was recorded; so pre-stocked enclosures were
Figure 1. Map of field site in Little Sippewissett Marsh, Falmouth, MA. The white square on the inset map indicates the location on Cape Cod, MA. The large map is of Little Sippewissett Marsh, Falmouth, MA. Diamonds indicate location of transects and the circle indicates location of video studies.
Table 1. Summary of trial components. Trial length is in the number of tidal cycles recorded. All lights were LED. Number of animals is how many horseshoe crabs were pre-stocked.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Length</th>
<th>Enclosure</th>
<th>Camera</th>
<th>Lights</th>
<th># Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>None</td>
<td>GardenCam</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>91.44 cm diameter cage</td>
<td>GardenCam</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>30.48 cm$^2$ cages with lid</td>
<td>GardenCam</td>
<td>6 flashlights</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>6 30.48 cm$^2$ cages</td>
<td>GardenCam</td>
<td>2 ropelights</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>6 30.48 cm$^2$ cages</td>
<td>PlantCam</td>
<td>4 ropelights</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>6 30.48 cm$^2$ cages</td>
<td>PlantCam</td>
<td>4 ropelights</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>6 30.48 cm$^2$ cages</td>
<td>PlantCam</td>
<td>4 ropelights</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>6 30.48 cm$^2$ cages</td>
<td>PC221</td>
<td>2 IR lights</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
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<td>1 30.48 cm$^2$ cage</td>
<td>GoPro</td>
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<td>1</td>
</tr>
<tr>
<td>10</td>
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<td>GoPro</td>
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<td>1</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>1 30.48 cm$^2$ cage</td>
<td>GardenCam</td>
<td>None</td>
<td>1</td>
</tr>
</tbody>
</table>
added for all future trials. In Trial 2, the GardenCam filmed a circular mesh cage (91.44 cm diameter, 2.54 cm$^2$ mesh) containing three horseshoe crabs. Video was taken for two complete tidal cycles (24.8 h) on 10/23/2010 (Table 1).

To identify movement of individual animals, all subsequent trials used cages (30.48 cm$^2$) built from 1.54 cm mesh fencing each containing one horseshoe crab. The cages were arranged in two rows of three, pushed approximately 5 cm into the substrate and secured with rebar and cable ties (Fig. 2). A PVC frame was constructed and affixed to the rebar at each outside corner to hold the camera.

In trial 3, the GardenCam and two red flashlights were attached to the crosspiece along with one red flashlight to each corner (Maxxima MF-37R Ultra Bright 6 Red LED Flashlight, Hauppauge, NY). Activity was recorded for two tidal cycles on 7/6/2011 (24.8 h). It was difficult to see the substrate during high tide due to the reflection of the flashlights on the water surface. Despite the ability to see the substrate during low tide, the cage lids obscured movement of the animals.

In trial 4 (and all subsequent trials), the cage lids were removed from the cages and the flashlights were replaced with two sets of solar-powered, red LED rope lights (Flipo Group, Inc., LaSalle, IL). The solar panels were attached to the top of the PVC frame and the rope lights were wrapped around the cages at the substrate. Activity was recorded for 2 tidal cycles on 7/25/2011 (24.8 h). The light provided by the rope lights was not sufficient to override the photocell turning off the camera at night.
Figure 2. Diagram of field set-up. This was used in both Little Sippewissett Marsh, Falmouth, MA and Great Bay Estuary, NH. (A) Birds-eye view of the set-up: six cages (30.48 x 30.48 cm, open squares), pieces of rebar (closed circles) and the corners of the camera frame (open circles). (B) Side view of the camera stand.
For the next trials, the GardenCam was replaced with a solar-powered PlantCam (solar panel: 12V, Moultrie, Alabaster, AL; camera: Wingscapes, Alabaster, AL) and two additional sets of rope lights for a total of 4 sets of rope lights. The PlantCam recorded one image every minute onto a SD card, images were imported into iMovie (v. 8.0.6) and stitched into a video. Videos were recorded on 9/9/2011 (Trial 5), 9/13/2011 (Trial 6), and 9/18/2011 (Trial 7) for 5.5, 5.0, and 1.5 tidal cycles (68.5, 63.5, 17.5 h), respectively, until the SD card was full. The PlantCam was used for two reasons: 1) it could be programmed to remain on constantly, as opposed to the GardenCam which shut down when light levels dropped below a predefined threshold and 2) the solar panel extended the recording duration. However, the PlantCam only recorded dark images as there was insufficient light at night.

In attempts to record video both at night and high tide, the set-up was moved to a cove at Fox Point, Great Bay Estuary, New Hampshire. An infrared-sensitive camera (Supercircuits PC221) and two infrared lights (Supercircuits IR25) were attached to the frame (Fig. 2). A cable was run from the frame to a computer (Macintosh, OS 10.4.11) where video output was digitized and recorded (Canopus® ADVC-55). Using video capture software (Gawker, v. 0.8.3, Seattle, WA), one frame was captured every 30 s, and time-stamped. Trial 8 recorded video for 12 tidal cycles (148.8 h) starting on 10/4/2011. While this video recorded during all times of day and tides, it was difficult to see what occurred during the flood, high, and ebb tides, due to poor water clarity in Great Bay Estuary.

To reduce distance from the camera and increase visibility of the juveniles, activity in one cage was recorded using GoPro with an extended battery (Woodman Labs,
Riverside, CA). One image was taken every 30 s for 0.5 tidal cycles on 11/10/2011 (5.5 h, Trial 9). The camera was placed on the substrate and attached to the interior of the cage using cable ties. This was repeated (Trial 10) on 11/16/2011 for 0.5 tidal cycles (5.75 h). Both trials began when the cage was exposed to air and continued through flood and part of high tide. In attempts to record more consecutive data, the GoPro was replaced with the GardenCam, in a waterproof case, placed on the substrate, and attached to the cage using hose clamps (Trial 11, 1/17/2011). Activity was recorded for 3 tidal cycles (37.5 h) but no useable data was recorded.

Videos were analyzed by eye for activity; any movement during a five minute period was recorded as a “1” while no movement was recorded as a “0”. When not confident in the ability to determine if there was movement or no movement, “-1” was recorded. These data were then plotted as actograms and Lomb-Scargle periodograms using ClockLab® (MatLab, Actimetrics, Evanston, IL, v. R2011a). Significance of rhythmicity was determined both visually and by periodograms ($P < 0.001$) to determine the primary component of rhythmicity during each experimental condition (Chabot et al. 2008).

Transects

Three transect sites in Little Sippewissett Marsh, Falmouth, MA were chosen based on water depths at low tide: one completely underwater at low tide (15.24 cm or deeper, 0.7574 m below high tide), one 2.54 cm underwater at low tide (0.885 m below...
high tide), and one exposed at low tide (0.91 m below high tide). At each site, three 10 x 10 m transects were marked. Transects were searched for active and trails of juvenile horseshoe crab. Active horseshoe crabs were defined as animals that could be seen moving through the substrate. Transects were checked eight times a day for five consecutive days: low, flood (low + 3 h), high, and ebb (low + 3 h). A total of 18 time points of data were collected. Transects at ten time points were not conducted due to severe weather. Due to arrival and departure times on the first and last day, only two time points were collected.

**Results and Discussion**

**Video**

Activity of juvenile horseshoe crabs in Little Sippewissett Marsh, Falmouth, MA was recorded over seven trials, covering 19 tidal cycles (9.5 days) In a cove at Fox Point, Great Bay Estuary, NH, data were obtained during 12 tidal cycles (6 days). From these data, only 63% ± 4% (average ± SEM) of hours recorded could be confidently analyzed. During flood tide, rising water caused increased reflection making it difficult to see the substrate, decreasing confidence in the ability to determine movement of animals. High tide also reduced confidence in recording of movement due to the low visibility caused by high amounts of suspended sediments and seaweed. Little to no data was collected at night due to reflection of artificial lights off of the substrate or inability to record anything but black. Of the data confidently analyzed, juvenile horseshoe crabs were active 4% ± 2% (~9 h) of the time recorded.
Of all the methods of recording movement, the GoPro camera provided the most reliable data in comparison to data provided from the GardenCam, PlantCam, or PC221 camera. This was because the GoPro was placed on the benthos circumventing the major issues of glare and poor water quality. However, the GoPro only recorded half a tidal cycle at a time. Two trials were run using this camera, one lasting 5.5 h and the other 5.75 h. The camera provided a side-view of the benthos and, when the horseshoe crab moved, a side view of it moving. While this worked better than any other method used, there were two major challenges. First, the camera’s field of view did not completely encompass the enclosure so while the video was useable for its entire length, there was not 100% confidence that all movement/non-movement was accurately recorded.

Secondly, this system is unable to take time-lapse video and only records still images without a time-stamp. While converting the photos into a movie is not a problem, the lack of a time-stamp limits the ability to determine when the animal was active. Since each image is a photograph, the individual file contains data on the “date modified” which was used as the time-stamp. This method was feasible for the quantity of data acquired in this study, but it is prohibitively time-intensive for a larger study. In addition, not enough continuous data was recorded to produce an actogram or periodogram, but it was noticeable that the animals became active immediately after being covered with water. The video started recording at extreme low tide and showed activity after minimal water coverage (depth of ~7.62 cm).

Five actograms and associated periodograms provided insight into the activity of individual horseshoe crabs in the field. Most of the video resulted in little data since there
was not enough confidence in viewing the video to ascertain if the animals were active or inactive. The collected data indicated that more activity occurred during high tide as opposed to low (Fig. 3). Due to the periodic nature of the data, the results from this portion of the study are limited in their ability to represent activity of juvenile horseshoe crabs from this part of the study.

**Transects**

The transect data provided more insight into the activity patterns and locations of the juvenile horseshoe crabs than the video data. The transects 2.54 cm underwater at low tide showed the most activity and trails. Four animals were active during the flood tide and three animals during low tide. In addition, trails were seen during all four parts of the tidal cycle (Table 2). On transects exposed during low tide, no active animals were observed; trails were seen during three of the four parts of the tidal cycle: flood, low, and ebb. On transects that were always underwater, no active juveniles were observed. Trails made by juvenile horseshoe crabs were seen at flood, ebb, and high tide (Table 2).

Areas of the marsh completely covered during low tide are generally found in the channels. These channels allow the water to flow from the back of the marsh to the ocean during ebb tide and back into the marsh during flood tide. As a result, currents in the channels are generally faster flowing. Juvenile horseshoe crabs have limited resistance to water currents such as those found in the channels in the marsh. When exposed to strong water currents, juvenile horseshoe crabs may be turned upside down and forced to swim in the water column (Meury and Gibson 1990). If the organisms cannot right themselves...
Figure 3. Typical actogram and periodogram of activity recorded in the field. The actogram (left) shows activity (dark bars), no activity (white spaces) and no data (horizontal grey lines). Grey blocks indicate environmental high tide. The periodogram (right) shows two significant peaks (above light grey line, $P < 0.001$), at 16.95 and 13 h.
Table 2. Count of active horseshoe crabs and their trails during each phase of the tidal cycle in three different areas of the marsh: deep water (always underwater), 2.54 cm of water (low tide, deeper water during high tide), and exposed (no water at low tide, deeper water during high tide). Water depth was taken during low tide.

<table>
<thead>
<tr>
<th>Elevation</th>
<th>Observation Type</th>
<th>Tide Cycle</th>
<th>Low</th>
<th>Flood</th>
<th>High</th>
<th>Ebb</th>
</tr>
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<tr>
<td>Deep water</td>
<td>Active</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Trails</td>
<td></td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2.54 cm of water</td>
<td>Active</td>
<td></td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Trails</td>
<td></td>
<td>15</td>
<td>33</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Exposed</td>
<td>Active</td>
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<td>0</td>
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</tr>
<tr>
<td></td>
<td>Trails</td>
<td></td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
and either exit the current or bury deep enough into the sand so that they are not swept up again, there is a high probability that they will be removed from the marsh. This would reduce the chance of survival for the juveniles as they are removed from their optimal environment and exposed to more predators. However, the presence of trails in the channels during the different phases of the tidal cycles indicates that the juvenile horseshoe crabs at least moved through this habitat. Observations of trails during the phases of the different tidal cycle indicate that it is unlikely that water level interfered with ability to count either active horseshoe crabs or their trails.

During low tide in exposed areas, the ability of horseshoe crabs to move through sand is limited as the pore water drains away. The pore water causes sand to become semi-liquid enabling the smallest animals to move through it (Meury and Gibson 1990). When there is no water present and the organisms are less able to bury into the sand, there is a higher risk of desiccation and predation. This may be one reason juvenile horseshoe crabs prefer areas that were always slightly damp or slightly underwater as opposed to those that were completely dry.

Meury and Gibson’s (1990) hypothesis that juvenile horseshoe crabs would need to confine their activity to slack tides to avoid currents present during other tidal cycle phases was opposite of the results in this study. The majority of the active animals in this study were observed during flood tide. This is likely due to the location of the horseshoe crabs in the marsh. The ability to be active during more phases of a tidal cycle, while avoiding currents that would remove them from the marsh, enables the horseshoe crabs to forage and find food more often increasing the growth rate and health of the animals. In
addition, their prey is more active during high tide (Palmer 1995) potentially reducing
time spent foraging. It also allows the crabs to bury into the benthos more easily and
rapidly, a quick way to avoid both crab and bird predators.

A previous study in Florida found that juvenile horseshoe crabs peaked in activity
level just before low tide (Rudloe 1978), however the current study produced conflicting
results, with the majority of juveniles active during flood and high tide. There are several
explanations for these conflicting results. The most likely reason is the variation in tidal
range between the two locations, from 0.6 m in Florida to 2 m in Little Sippewissett
Marsh, Falmouth, MA. Laboratory results of Massachusetts juvenile horseshoe crabs
have shown that in the absence of tidal cues their activity patterns tend to synchronize to
the light cycle. Given the limited tidal cycle in Florida and these laboratory results,
horseshoe crab synchronization to the light cycle supports the laboratory results.
However, previous laboratory studies that indicated the larger the hydrostatic pressure
change, the more animals synchronize activity patterns to the tidal cycle (Chapter Two).
These two laboratory results help to explain why the results from Florida and
Massachusetts horseshoe crabs vary. There is a large variation in the patterns expressed
by adults from these different locations, adult horseshoe crabs exposed to a diurnal tidal
cycle (one high and low per day) mate once per day while those exposed to a semi-
diurnal tidal cycle (two highs and lows per day), mate twice per day (Rudloe 1979, 1985,
Cohen and Brockmann 1983, Barlow et al. 1986).

A second explanation for the variation between the two populations of juvenile
horseshoe crabs is geography. In Florida, the nursery habitat is a homogenous sandy
beach devoid of channels (Rudloe 1981) while the habitat in Massachusetts is a
heterogeneous salt marsh with many channels of faster moving water (Fig. 1). These variations in the habitat could cause a change in activity patterns because of the differing effects of the ebbing tide; the minimal surface area for ebbing water causes stronger and faster currents in the marsh then currents from ebbing water on a continuous beach.

The activity during flood and high tide may protect juveniles in Massachusetts from nursery habitat removal, and the increased tidal range may cause tighter synchronization between the tidal cycle and activity. In addition, the tidal activity rhythms of the prey of juvenile horseshoe crabs provides greater support for their tidal activity pattern. These results are supported by laboratory findings that indicated movement during flood and high tide.

**Literature Cited**


