Developmental ecology of the American horseshoe crab *Limulus polyphemus*

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Abstract   During spawning events, horseshoe crab eggs are released from the female's oviducts, and fertilized by one or more males. Eggs are shaped by the female into discrete clutches deposited in nests at depths of 10–20 cm on intertidal estuarine beaches. Distinguishing between fresh eggs and the early developmental stages is obfuscated by the large amount of dense, opaque yolk. The first unambiguous confirmation of development is the formation of the rudimentary prosomatic appendages at the "limb bud" stage. Several days thereafter, the outer chorion is shed and the developing embryo expands and undergoes a series of molts within the clear inner egg membrane. The trilobite (first instar) stage thus attained may remain within the beach sediments for several more weeks, until hatching is facilitated by environmental factors such as hydration, agitation, and osmotic shock that accompany the infiltration of seawater into the nests. Trilobites exhibit endogenous circatidal swimming rhythms that are entrained by mechanical agitation, suggesting that peaks in larval swimming are timed to coincide with periods of high water and the inundation of the nests. Larval swimming is limited and does not appear to result in long-distance dispersal. The limited dispersal of the larvae has important implications for the population dynamics of relatively isolated populations. The rate of larval development is highly plastic and is influenced by temperature, salinity, dissolved oxygen, and the presence of pollutants. The broad environmental tolerances of horseshoe crab embryos and larvae are important in understanding their current geographic distribution and their evolutionary persistence [*Current Zoology* 56 (5): 550–562, 2010].

Key words   Horseshoe crab, *Limulus*, Development, Embryo, Larval dispersal

1   Introduction

The reproductive and developmental biology of horseshoe crabs is fascinating and unusual, and gives important insights into the reasons why this lineage has been so successful over evolutionary time. Horseshoe crabs are the only extant marine arthropods that have external fertilization without brooding of the eggs (Brusca and Brusca, 1990), and the only ones that migrate from offshore depths to the intertidal zone, where they spawn on estuarine beaches. In this paper, we provide a brief account of horseshoe crab spawning and an overview of embryonic development. We then discuss the behavior and physiology of horseshoe crab larvae, and the ways in which these attributes are integral to their survival across the wide range of estuarine environments in which they live. Next, we describe how an understanding of developmental ecology can aid fishery management practices through the use of egg density as an index of horseshoe crab intensity. To conclude, we discuss the role that developmental ecology may have had in explaining the long-term evolutionary resilience of horseshoe crabs.

1.1   Spawning

Horseshoe crabs typically come ashore as amplexed mated pairs; a male uses specially modified pedipalps ("claspers") to attach to the posterior of the opisthosoma of a female. At the time of spawning, the mated pairs are surrounded by multiple satellite males that milt as the female deposits her eggs (see reviews by Brockmann, 2003a; Brockmann and Smith, 2009). The mating system of horseshoe crabs has been extensively studied in recent decades, with emphasis on the role of lunar and tidal rhythms in spawning behavior (Rudloe, 1980; Barlow et al., 1986), the effects of body size (Brockmann, 1990; Loveland and Botton, 1992; Botton and Loveland, 1992) and eye and clasper condition (Duffy et al., 2006) on mating success, and the comparative fertilization success achieved by males utilizing alternative mating tactics (Brockmann et al., 1994, 2000; Brock-

mann, 2003b).

The ovarian network is highly branched and occupies a considerable portion of the prosoma (Makioka, 1988). The paired oviducts lead to two gonopores on the ventral surface that open on the genital papillae, which are prominent slits on the posterior surface of the genital operculum (immediately anterior to the book gills). Egg production varies as a function of female size; females > 260 mm prosoma width (PW) contain an average of 63,500 mature eggs but small females (< 200 mm PW) have about 14,500 eggs (Leschen et al., 2006). The eggs are released from the gonopores and fertilized by the sperm released from the amplexed and satellite males (Brockmann, 2003b). Extruded eggs are then shaped by the female into discrete clutches of loosely cemented eggs. On sandy beaches, egg clutches are typically deposited at depths of 15 cm (Weber and Carter, 2009) but in more gravelly or shelly substrates, eggs are deposited closer to the surface. The number of eggs per clutch is highly variable; Leschen et al. (2006) reported an average of 960 eggs/clutch for females from Cape Cod, Massachusetts. However, clutch size may be subject to latitudinal variation, as Weber and Carter (2009) reported an average of 5,786 eggs/clutch from Delaware Bay, Delaware (compared to an earlier estimate of 3,650 eggs/clutch from Shuster and Botton, 1985 for Delaware Bay, New Jersey) and 2,236 from Florida (Brockmann, 1990). Clutch size is not correlated with female size (Brockmann, 1996; Leschen et al., 2006), and overall fertilization success within the clutch is independent of the number of males participating in the spawning event (Brockmann et al., 1994). The enormous concentration of sperm (10^{10} ml^{-1}: Brown and Knouse, 1973) contributes to the high fertilization success in this species (Brockmann, 2003b; Loveland and Botton, unpublished).

1.2 Developmental biology

Horseshoe crab development has been the subject of several classic monographs, and the descriptions, light micrographs, and line drawings provided by authors such as Packard (1870, 1885), Kingsley (1882, 1883), and Munson (1898) are still valuable references today. The use of scanning and transmission electron microscopy enabled G. G. Brown and colleagues to describe fertilization and early development in great detail (e.g. Brown, 1976; Brown and Clapper, 1980; Bannon and Brown, 1980; Brown and Barnum, 1983; Barnum et al., 1985), and equally elegant studies of the development in the Japanese horseshoe crab *Tachypleus tridentatus* were conducted by K. Sekiguchi and colleagues (Se-

kiguchi et al., 1988a). The synopsis of horseshoe crab development provided below is largely drawn from reviews by Brown and Clapper (1981), Sekiguchi et al. (1988a), and Shuster and Sekiguchi (2003), and the nomenclature for the developmental stages follows these authors.

Unfertilized eggs (*Stage 0*) These are typically greenish-blue or greenish-gray, but some are pink. Unfertilized eggs are approximately 1.6–1.8 mm diameter and have a dry mass of 1.69×10^{-3} g (Shuster and Sekiguchi, 2003; Leschen et al., 2006). The egg surface is smooth, but immediately after release from the genital operculum, it may be slightly dented and is coated with an adhesive material that causes it to stick to other eggs and to sand grains. Unfertilized eggs have a large volume of yolk, surrounded by a tough outer chorion with many irregular pits when viewed under SEM (Sekiguchi et al., 1988a; Hajeb et al., 2009).

Fertilization and early granulation/degranulation (*Stages 1.1 through 1.8*) Horseshoe crabs have flagellated sperm that attach to the chorion in two stages. The initial attachment of the apical tip of the sperm head to the chorion is followed by the acrosome reaction and the penetration of the sperm acrosomal filament (Brown, 1976). There is no evidence for a predetermined site of sperm entry, or micropyle (Hajeb et al., 2009). A cortical reaction prevents polyspermy, and is evidenced by the appearance, fusion, and disappearance of a series of surface pits beginning about 3 min postfertilization and proceeding for about 30 min (Bannon and Brown, 1980; Barnum et al., 1985). At stage 1.8, about 1 day after fertilization, the egg surface has a series of shallow furrows.

Appearance of cleavage nuclei (*Stage 2*) *through germ disc formation* (*Stage 10*) The changes that occur within the egg from Stages 1 through 10 are not easily discerned even under a dissecting microscope because of the density and pigmentation of the yolk (Fig.1A). The use of the vital stain neutral red (1/20,000 to 1/400,000 in seawater adjusted to pH 5.8–6.0), has been recommended by Sekiguchi et al. (1988a). The eggs undergo superficial cleavage, and with careful examination under the dissecting microscope, cleavage nuclei can be found on the egg surface. As development progresses, these cells divide and the size of the blastomeres decreases, giving the overall impression that the egg surface is becoming smoother. The germ disk begins as a triangular-shaped indentation at Stage 7 and gradually enlarges as the embryo develops.

Germ Disk Expansion (*Stages 11 through 14*) The

germ disc continues to expand, and may be visible as a slightly uplifted area when the embryo is viewed laterally (Fig. 5.2 in Shuster and Sekiguchi, 2003). By stages 11 and 12, bands or furrows appear within the germ disk; these will eventually give rise to the prosomatic appendages (walking legs and chelicerae).

Limb Bud (*Stages 15-17*) Continued differentiation of the germ disk gives rise to the rudimentary prosomatic appendages (Fig. 1B). These appendages continue to elongate and become more clearly recognizable. Even at the limb bud stage, careful microscopic study is essential to distinguish this stage from early embryos or even from unfertilized eggs since their color and diameter are virtually identical.

First embryonic molt (*Stage 18*) The limb buds continue to grow, becoming noticeably pointed at their tip, and the embryo is becoming distinctly more flattened in this region (most easily noted in lateral view, see Fig. 5.2 in Shuster and Sekiguchi, 2003). The chelicerae are becoming more distinct, and the initial segmentation of the hepatopancreas is evident. The production of blood cells (amoebocytes) begins at Stage 18 (Coursey et al., 2003).

Second embryonic molt (*Stage 19*) The tips of the limb buds begin to develop a biramous claw-like shape; the lateral organs become prominent, and the embryo continues to flatten. Segmentation along the dorsal surface becomes evident.

Third embryonic molt (*Stages 20-1 and 20-2*) A clearly recognized transition occurs as the opaque chorion splits and the embryo can easily be seen, even in the field, with the unaided eye as it actively rotates within the clear inner egg membrane (Fig. 1C, D). The prosomatic appendages become fully segmented, and the book gills of the opisthosoma develop rapidly. Stage 20 embryos are nearly twice the diameter of fertilized eggs and early embryos. The lateral (compound) eyes and median ocelli (simple eyes) are visible on the dorsal surface.

Fourth embryonic molt and hatching (*Stage 21*) The embryo molts for a fourth time within the outer egg membrane, resulting in the first instar or *trilobite* larval stage (Fig. 1E). The book gills are larger than the previous stage and they move very rapidly. The animal greatly resembles the adult, except that the telson is lacking.

Post-embryonic growth and molting Trilobite larvae emerge from beach sediments (see *Section 2 Larval hatching*) and are briefly planktonic before settling to the benthos (Botton and Loveland, 2003). Shortly thereafter, molting to the second instar stage occurs, and the telson is formed (Fig.1F). The molting process is controlled by the hormone 20-hydroxyecdysone (Jegla, 1982). Horseshoe crabs continue to grow with many
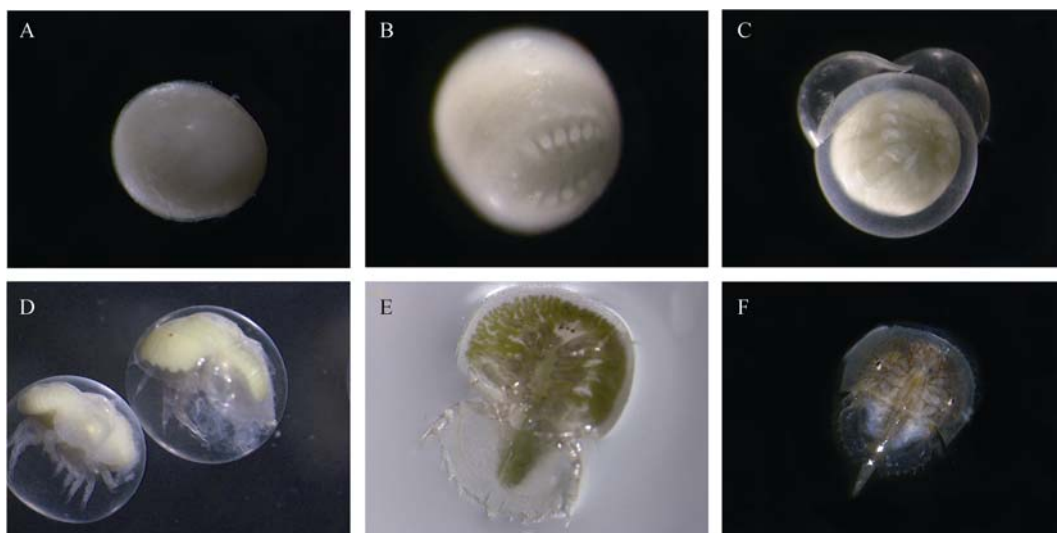

Fig. 1 Stages in the early development in *Limulus polyphemus*

A. An early embryo, approximately 1 week after fertilization. **B**. Stage 15 embryo (limb bud). **C**. Stage 20-1, just after the rupture of the chorion (the two semicircles at the top of the embryo). In routine processing of sediment samples to enumerate eggs (*section 4.3*), this is the first stage that would ordinarily be categorized as an embryo rather than an egg; **D**. Stage 20-2. Note the exoskeleton from the third embryonic molt is clearly visible below the opisthosoma of the embryo on the right. **E**. Trilobite (first instar) stage. The orientation of the larva with its ventral side upwards is typical and facilitates swimming behavior. **F**. Second instar stage. Note the presence of the telson (tail), which is absent from the trilobite. Photographs by L. Cusumano, C. Esposito, M. Malin, and M. Botton.

reaching the fourth instar by the end of their first summer (Botton et al., 2003a). It had been widely thought that juvenile horseshoe crabs were most abundant close to shore on intertidal sand bars (e.g. Botton et al., 2003a), but this assumption may reflect the lack of proper sampling gear to collect them subtidally (Burton et al., 2009; Tankersley and Boleman, unpublished data). Early instars feed on particulate organic material, but gradually consume benthic invertebrates such as polychaetes, crustaceans, and mollusks (Gaines et al., 2002).

Remarkably, *Limulus* requires about 9–11 years to attain sexual maturity (Sekiguchi et al., 1988b). Horseshoe crabs exhibit sexual size dimorphism as adults. The larger average prosoma width of females is most likely the consequence of differential maturity rather than a difference between sexes in the average molt increment (Smith et al., 2009a). Horseshoe crabs attain their average largest size in the central portion of their range (i.e., Georgia to the southern shores of Cape Cod), but are significantly smaller both north of Cape Cod and in the southernmost part of their range, the Gulf of Mexico (Shuster, 1979; Graham et al., 2009). Whether these differences relate to earlier maturation or differences in molt increment is not known.

1.3 Ecological importance of horseshoe crab eggs

Horseshoe crab eggs are widely recognized as an essential food for shorebirds passing through Delaware Bay during their Spring (northward) migration to the breeding grounds. Four species of long distance migrants-red knots *Calidris canutus rufa*, sanderlings *C. alba*, semipalmated sandpipers *C. pusilla*, and ruddy turnstones *Arenaria interpres* - comprise the vast majority of shorebirds that rely on horseshoe crab eggs, but numerous other species of shorebirds and gulls also feed on this resource (Botton et al., 2003b). The importance of this ecological relationship and its significance for the conservation biology of shorebirds has been the subject of intensive research since the 1980's (see reviews by Botton et al., 2003b; Mizrahi and Peters, 2009). The Atlantic States Marine Fisheries Commission management plan for horseshoe crabs explicitly recognized the need to maintain the *Limulus* population at a level that ensures sufficient eggs to meet the nutritional needs of migratory shorebirds in Delaware Bay (ASMFC, 1998). Because the shorebirds primarily feed on eggs in the upper 5 cm of the beach, a high density of spawning females is necessary to re-work the sediments and exhume the eggs because most egg clutches are deposited at 10–20 cm (Smith, 2007). The eggs that are lost to bird

predation probably comprise a relatively minor fraction of the overall egg input, and eggs on the sediment surface would probably desiccate and die even if there were no birds to consume them (Botton, 2009).

Horseshoe crab egg densities in surface sediments of other estuaries are generally much lower than in Delaware Bay, and while birds may opportunistically consume the eggs, it is likely that the eggs are too sparse and patchy to make a sufficiently reliable food source to sustain a large migratory population (Botton, 2009). Predation on horseshoe crab eggs and larvae by various crustaceans and surf zone fishes such as killifish *Fundulus heteroclitus* and Atlantic silversides *Menidia menidia* has also been reported (Botton, 2009), but has received relatively little study. The utilization of complex vegetative habitats by larvae and early benthic stage juveniles may be a consequence of increased predation risk in subtidal sandflats and non-vegetated areas (Tankersley and Boleman, unpublished data).

2 Larval Hatching

The use of the mid to upper-intertidal zone of sandy beaches by horseshoe crabs for nesting provides significant protection for developing eggs from aquatic predators. Moreover, deposition of clutches of eggs 10 cm–20 cm below the sediment surface reduces their exposure to harsh environmental conditions, including wave activity and extreme temperature and osmotic fluctuations (Rudloe, 1979; Shuster and Botton, 1985; Brockman, 1990; Botton et al., 1992; Botton et al., 1994; Penn and Brockmann, 1994; Weber and Carter, 2009). Thus, most embryos retained in the nests complete development and survive to the trilobite stage (Rudloe, 1979). Nevertheless, this nesting pattern imposes significant challenges for the emergence of larvae from the sediments, their entry into the water column, and their dispersal and transport away from the beach.

As outlined above (see *Section 1.2 Developmental Biology*), embryos complete development and molt to trilobite larvae in approximately 28 days, although the rate of development is temperature and salinity dependent (French, 1979; Jegla and Costlow, 1982; Ehlinger and Tankersley, 2004; see *Section 4.1 Environmental Physiology*). Larvae emerge from the nest and enter the water column when high-tides inundate the nests, typically during spring tides. Emergence may also be controlled by the light:dark cycle since peaks in larval abundance in the plankton occur at night (Rudloe, 1979; Ehlinger et al., 2003; Botton and Loveland, 2003). Larvae remain in the plankton for 7–10 days before

molting to the first juvenile instar and settling in shallow benthic, near-shore habitats (Rudloe, 1979; Shuster, 1982; Sekiguchi, 1988; Botton and Loveland, 2003; Burton et al., 2009). Like many aquatic species that lay eggs in terrestrial and semi-terrestrial areas, hatching is triggered by environmental cues that help to synchronize eclosion and emergence of larvae from the nests with conditions favorable for development and survival. Although hatching can occur in the absence of external stimuli (Jegla, 1979; Ehlinger and Tankersley, 2003), mechanical and osmotic processes associated with tidal inundation likely facilitate eclosion. When Stage 21 embryos were exposed to laboratory conditions that simulated submergence at high tide (i.e., hydration and agitation), most (65%–96%) hatched within 2 h of exposure to the simulated conditions and hatching levels were 2–4.5 × higher than those in control treatments (Ehlinger and Tankersley, 2003; Fig. 2). These results are consistent with field studies conducted in nearshore areas adjacent spawning beaches in Delaware Bay in which peaks in larval abundance in the plankton were correlated with periods of strong onshore winds and rough surf (> 30 cm wave height) (Botton and Loveland, 2003; Fig. 3).

These external mechanical processes may be aided by the movements and activity of the larvae and changes in the osmotic concentration within the egg. During development in the sediment, the perivitelline fluid inside the developing eggs becomes hyperosmotic to the ambient seawater and porewater (Ehlinger and Tankersley, 2003) (Fig. 4). Thus, when inundated, eggs experience a hypoosmotic shock (~ 70 mmol kg^{-1}), causing them to swell. As a consequence, the change in size and volume weakens the membrane causing it to rupture or making it more susceptible to mechanical disturbance.

Hatching in response to these exogenous cues associated with high water conditions (e.g., hydration, physical disturbance, hypoosmotic shock) is adaptive since it increases the likelihood that larvae hatch and emerge from the sediments when water levels reach the nest, thereby enhancing survival by preventing larvae from being stranded on the beach. The same responses to environmental changes might be equally advantageous in habitats that lack significant tidal changes, such as shallow water coastal lagoons and embayments. In these areas, water levels are generally determined by freshwater runoff and wind forcing. Consequently, the
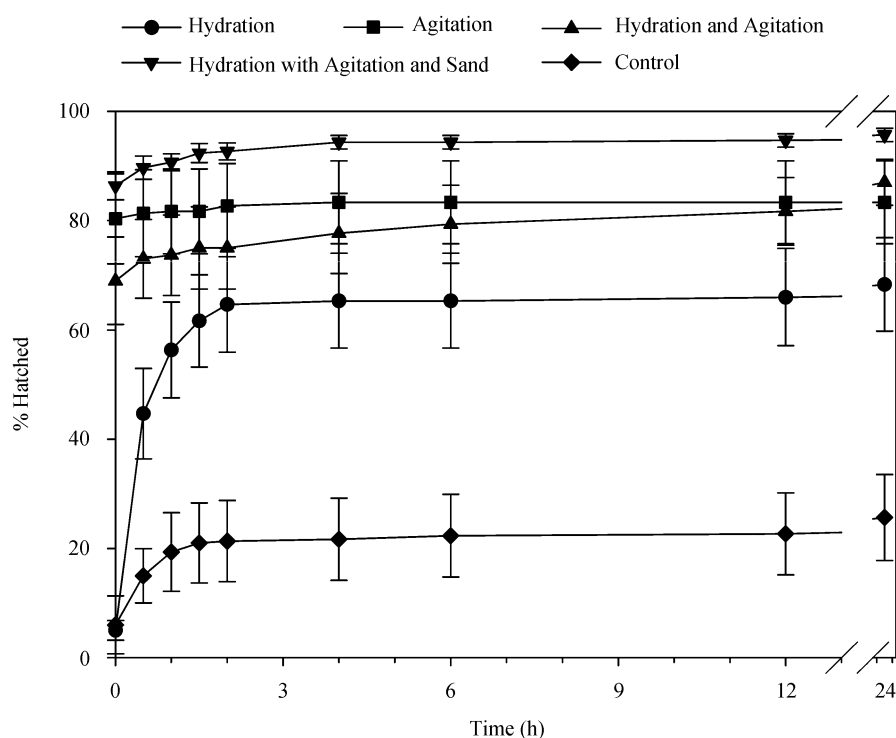

Fig. 2 Percentage ($\bar{X} \pm SE$) of hatched larvae (out of 30 embryos) over 24 h following exposure to one of the four hydration and agitation treatments ($n = 10$)

Embryos in control conditions were maintained in rearing conditions (moist paper towels) and were not inundated with seawater or agitated by shaking. (Figure modified from Ehlinger and Tankersley, 2003).
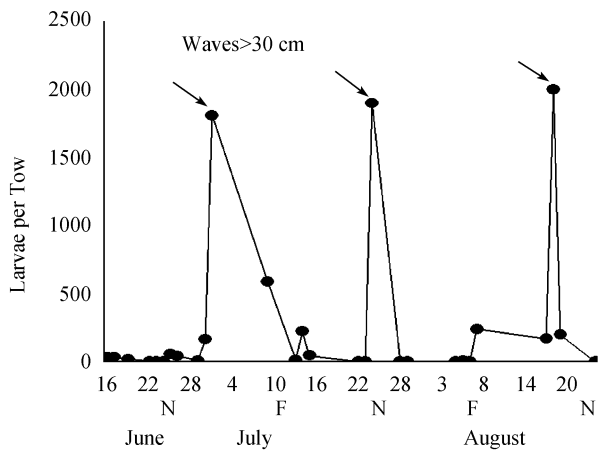
Fig. 3 Abundance of horseshoe crab trilobite larvae in nearshore plankton samples taken adjacent to Delaware Bay, NJ, beach from June– August 1998

Arrows indicate samples taken on days when wave heights > 30 cm. N= new moon, F = full moon. Figure modified from Botton and Loveland, 2003.
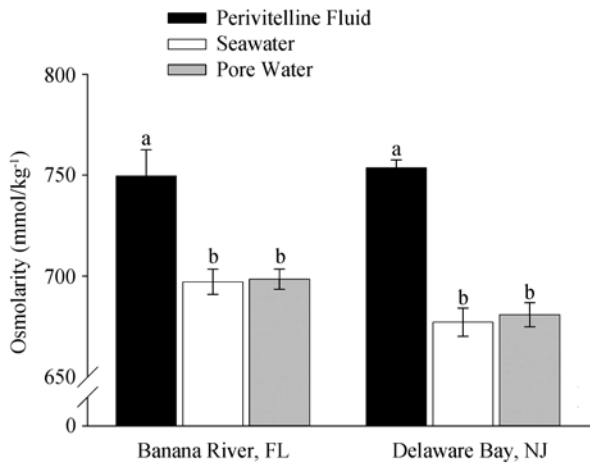

Fig. 4 Osmolarity ($\bar{X} \pm$ SE) of the perivitelline fluid within the eggs and the surrounding seawater and pore water collected in the Banana River (FL) and Delaware Bay (NJ)

Bars with the same letters are not significantly different at P < 0.05, $n = 50$. Figure modified from Ehlinger and Tankersley, 2003.

frequency of shoreline inundation is unpredictable and aperiodic. Indirect evidence that environmental cues associated with inundation trigger hatching comes from plankton surveys conducted in microtidal areas of Indian River Lagoon, FL (Ehlinger et al., 2003; Ehlinger and Tankersley, 2009). Spatial and temporal patterns of larval abundance were more closely associated with changes in water level than adult spawning activity, resulting in an apparent decoupling between peaks in spawning and larval abundance (Ehlinger et al., 2003; Ehlinger and Tankersley, 2007). Finally, in both tidal

and non-tidal areas, episodic hatching and emergence of large numbers of larvae might also serve to overwhelm aquatic predators locally, as has been suggested for other marine organisms with synchronized release of gametes and offspring.

Although temporal patterns in trilobite hatching and abundance in the plankton appear to be best explained by physical disturbances and osmotic changes experienced by developing embryos during periods of high water, larvae are typically more abundant in plankton samples collected at night (Rudloe, 1979; Ehlinger et al., 2003; Botton and Loveland, 2003). This apparent diel pattern in larval hatching might be a consequence of the phase relationships between the light:dark cycle and the dominant constituents of the tides. For example, during the summer spawning period in Delaware Bay (May –August), night tides are higher than day tides during new and full moons (spring tides). Thus, water levels might only reach nests in the mid- and upper-intertidal during nocturnal high tides. Behavioral responses of larvae to light may also contribute to diel differences in trilobite abundance. Rudloe (1979) reported that larvae swim more actively in darkness and are positively phototactic to dim light. Indirect evidence for the nocturnal emergence and presence of larvae in the plankton comes from feeding studies of Atlantic silversides (Spraker and Austin, 1997). Comparisons of the feeding periodicity of *Menidia menidia* collected over a 24-h period revealed that *Limulus* larvae were common in the gut contents of fish collected at night, but were relatively rare in the diets of individuals collected during the day.

3 Behavior and Dispersal of Post-hatch Trilobite Larvae

The ecology of *Limulus* larvae after they leave the nest and enter the plankton is poorly understood. Like many planktonic larvae, trilobites are weak swimmers relative to the velocities of horizontal currents in estuaries and intertidal sand and mud flats (0.04 –0.15 m s⁻1; Hidu and Haskin, 1971; Botton and Loveland, 2003). Although they cannot make headway against these currents for horizontal movement, they can move vertically to take advantage of tidal flow moving in the appropriate direction. Transport of newly-hatched trilobite larvae away from the natal beaches and adult nesting areas appears to be facilitated by a tidal rhythm in swimming activity and vertical migration (Ehlinger and Tankersley, 2006). Coupling of vertical migrations to cycles in the direction of tidal-stream flow, a behavioral process referred to as selective tidal-stream transport, is com-

monly used by estuarine organisms for unidirectional transport (see review by Forward and Tankersley, 2001). Ebb-phased vertical migrations are used for seaward transport away from adult populations and export to coastal areas; flood-phased migrations are used for up-estuary transport and recruitment to settlement habitats. When placed under constant laboratory conditions, newly-hatched larvae from areas with semi-diurnal tides exhibited an endogenous rhythm with a free-running period that matched the tides at the collection site (~12.5 h) (Ehlinger and Tankersley, 2006). Maximum swimming activity occurred 1.5–2.0 h after high tide (i.e., during falling tide) and periods of quiescence occurred during the expected time of the rising tide. Thus, the endogenous rhythm exhibited by trilobites is appropriately timed to facilitate unidirectional horizontal advection seaward and away from the beach by ebb currents (i.e., ebb-tide transport; Forward and Tankersley, 2001). Although a similar circatidal rhythm has been well-documented in adult horseshoe crabs (Chabot et al., 2004, 2007, 2008, 2010; Watson et al., 2008; for review see Chabot and Watson, 2010), the synchrony of the active phase is advanced by ~ 2 h (i.e., before high tide) compared to the rhythm of exhibited by larvae.

Laboratory experiments indicate the circatidal rhythm of trilobites can be entrained prior to hatching by cycles of mechanical agitation, similar to those experienced by developing embryos during periods of tidal inundation (Ehlinger and Tankersley, 2006). Thus, physical disturbance serves as both a trigger for hatching and a cue for entraining (i.e., zeitgeber) the vertical swimming activity of larvae to local tidal conditions.

Despite possessing behaviors that prevent stranding and promote seaward transport, evidence from field studies suggest that larvae remain in shallow, nearshore habitats adjacent to spawning beaches (Rudloe, 1979; Shuster, 1982; Sekiguchi, 1988; Botton and Loveland, 2003; Burton et al., 2009). For example, Botton and Loveland (2003) reported that trilobite larvae were 10 –100 × more abundant in plankton samples collected near the shoreline than they were in tows 100 –200 m offshore. Similar results have been recorded in shallow lagoons (e.g., Indian River Lagoon, FL) which lack significant tidal currents. In these areas, trilobite larvae are rarely collected in the plankton and are primarily found in structured benthic habitats and vegetation (i.e., sea grass and drift algae) near spawning beaches (Ehlinger et al., 2003; Ehlinger and Tankersley, 2009; Tankersley and Boleman, unpublished data). Since field data suggests that larvae are not transported great distances from

the natal beach, the ebb-phased tidal rhythm in activity and larval swimming exhibited in by newly hatched trilobite larvae may be involved in position maintenance in older larvae. Increased activity (e.g., burrowing in the substrate or positive rheotaxis) during the falling tide would prevent larvae from being transported down-estuary or to deeper water. Following molting, juveniles also appear to stay within nearshore, subtidal habitats where they undergo several molts before migrating to deeper water (Rudloe, 1981; Shuster, 1982; Burton et al., 2009). Given the short duration of the post-hatch trilobite stage (7 –10 days) and their tendency to remain in intertidal flats and nearshore benthic habitats, their potential for long-distance dispersal and transport, even within the same estuary, is limited.

4   Effect of Environmental Factors on Survival and Development

4.1   Environmental physiology

Although the tolerance and physiological responses of older juvenile and adult horseshoe crabs to natural fluctuations in environmental conditions (i.e., temperature, salinity, oxygen concentration) has been studied extensively (see review by Towle and Henry, 2003), less is known about the ability of early life history stages to cope with conditions characteristic of intertidal and shallow subtidal estuarine habits. Because horseshoe crabs nest in semi-terrestrial regions of sandy beaches, developing embryos and larvae are exposed to fluctuations in environmental conditions that are more extreme and variable than those experienced by juvenile and adult crabs. Consequently, *L. polyphemus* embryos and larvae are remarkably hardy and able to tolerant the rapid and often harsh environmental conditions of intertidal areas.

Studies of salinity tolerance indicate the optimal conditions for embryonic development are between 20–30 PSU (Jegla and Costlow, 1982; Laughlin, 1983; Sugita, 1988; Ehlinger and Tankersley, 2004). Outside of this range, development time (time to hatching) increases. Although most studies have focused on the effects of lower salinity conditions (<35 PSU) on development and survival, Ehlinger and Tankersley (2004) examined the effects of more extreme salinities, i.e., those typically experienced by crabs inhabiting shallow water lagoons and embayments, on time to hatching and time to metamorphosis to the juvenile stage. Embryos completed development at salinities below 60 PSU, but hatching was delayed in more hypersaline conditions (50 and 60 PSU). Larvae were also more tolerant of

salinity extremes than developing eggs and survived salinities from 10 to 70 PSU, but the duration of the trilobite stage increased with increasing salinity (Ehlinger and Tankersley, 2004).

Most studies indicate that the optimal temperatures for embryonic development are between 25–30°C (Jegla and Costlow, 1982; Laughlin, 1983), although this range may differ slightly with latitude (Ehlinger and Tankersley, 2004). Eggs and larvae are more sensitive to high temperature than to high salinities. Ehlinger and Tankersley (2004) reported that temperatures >35 °C are lethal to embryos and adversely affect the growth and development of larvae. Nevertheless, tolerance of extreme high temperatures appears to increase slightly with age (Fraenkel, 1960; Jegla and Costlow, 1982; Laughlin, 1983). Reynolds and Casterlin (1979) reported that juveniles were able to tolerate temperatures as high as 40°C. Both embryos and larvae are tolerant to cold temperatures. Jegla (1982) reported that trilobite embryos and larvae are capable of surviving extended periods (up to 8 months) when stored at temperatures between 13 –15°C. Under natural conditions, this cold temperature tolerance enables embryos and larvae to overwinter in the nests and complete development the following spring (Botton et al., 1992).

Trilobite larvae are relatively insensitive to hypoxia and anoxia, but their tolerance of low O_2 conditions declines as they approach metamorphosis. Bademan (2009) reported that > 75% of newly-hatched trilobite larvae survived exposure to anoxia for 168 h. Yet, median survival times (LT_{50}'s) for pre-metamorphic larvae and juveniles were 108 h and 18.6 h, respectively. A similar decrease in the tolerance of hypoxia also occurs as larvae age. This ontogenetic shift might be related to the ability of larval and juvenile crabs to regulate oxygen uptake under declining oxygen conditions. Palumbi and Johnson (1982) and Bademan (2009) reported that the critical oxygen tension (P_c) at which post-hatch larvae are able to sustain O_2 uptake was ~ 5 kPa. P_c values increased to 9.65 kPa and 12.8 kPa in pre-metamorphic larvae and juveniles, respectively (Bademan, 2009). This gradual shift in O_2 tolerance during early development may be associated with the likelihood of each life history stage encountering hypoxic or anoxic conditions, rather than a consequence of the transition from pelagic to benthic habitats that accompanies metamorphosis. While buried in nests, embryos and larvae may experience low oxygen conditions and larval trilobites that hatch while still in the sediment may remain trapped there and exposed to hypoxia for extended periods of time (Rudloe, 1979; Botton et al., 1988; Botton et al., 1992; Penn and Brockmann, 1994; Jackson et al., 2008). Because planktonic larvae and newly metamorphosed juveniles are typically found in nearshore subtidal habitats, they may be less likely to be exposed to extended periods of hypoxia or anoxia than larvae buried in nests below the sediment surface (Botton et al., 2003; Botton and Loveland, 2003).

Compensatory mechanisms utilized by *Limulus* trilobites and juveniles in response to low oxygen conditions include increases in heart rate and book gill ventilation rates (Bademan, 2009). Ventilation and heart rates of post-hatch larvae are relatively stable at O_2 levels above 10% O_2 saturation (~2.12 kPa). As with survival, pre-metamorphic larvae and juvenile crabs are more sensitive to low O_2 than recently hatched larvae and begin increasing their ventilation and heart rates at ~ 20% O_2 saturation.

4.2 Effects of environmental pollutants

Horseshoe crabs spawn across a wide cross-section of habitats, ranging from relatively pristine to estuaries impacted by industrial, agricultural and municipal wastewater. Based on laboratory bioassays, Botton et al. (1998 a, b) and Botton (2000) found that stage 20 embryos and trilobite larvae were highly tolerant of pollution by metals, including cadmium, mercury, copper, zinc, and tributyltin compared with larvae of other marine arthropods, and that acute toxicity from these metals would require levels exceeding those normally found in even the most severely polluted U.S. estuaries (Botton and Itow, 2009). Bioassays using oil (Laughlin and Neff, 1977; Strobel and Brenowitz, 1981), PCB's and related compounds (Neff and Giam, 1977) and insecticides (Weis and Ma, 1987) also demonstrated that *Limulus* embryos and larvae are highly pollution-tolerant. Botton and Itow (2009) speculated that stress proteins (e.g. HSP70 and HSP90), which presumably evolved to enable embryos and larvae to survive the temperature and osmotic stresses of the intertidal zone (Botton et al., 2006; Greene et al., accepted), could also be important in pollution tolerance.

Some laboratory studies have indicated that sublethal levels of toxins can lengthen embryonic development and/or intermolt duration (Strobel and Brenowitz, 1981; Botton et al., 1998b), induce a variety of developmental anomalies (Itow et al., 1998a), and interfere with regeneration (Itow et al., 1998b). In field studies, the frequency of abnormal *Limulus* embryos from estuaries near New York City (Jamaica Bay, New York and Sandy Hook Bay, New Jersey) was no higher than it was from

lower Delaware Bay (Botton and Itow, 2009), suggesting that water quality in the more urban locations was not seriously impacting horseshoe crabs. Interestingly, embryos of the Japanese horseshoe crab *Tachypleus tridentatus* had a much higher rate of developmental abnormalities when collected from polluted waters (Botton and Itow, 2009), but the exact composition of these waters is not known.

Because horseshoe crab eggs are the primary food for migratory shorebirds in Delaware Bay (Botton et al., 1994; Mizrahi and Peters, 2009), there is ongoing concern about the potential trophic transfer of contaminants. However, Burger et al. (2003) concluded that the current concentrations of metals in horseshoe crab eggs were unlikely to have adverse effects upon shorebirds during the 2-3 wk of feeding in Delaware Bay.

4.3 Importance of understanding *Limulus* developmental schedule when collecting data on egg abundance

Life history factors of *Limulus* are not well documented. Species of horseshoe crabs are long lived; however, little is known about survival rates between sequential developmental stages. For example, little is known regarding the behavior and molting cycle of juvenile horseshoe crabs once they leave the intertidal zone. Recent interest in egg deposition by *Limulus*, particularly in Delaware Bay, has been largely focused on the link between egg abundance and nutritional value to migratory shorebirds (Mizrahi and Peters, 2009).

At the beginning of the spawning season, in late April or early May, the beach is empty of horseshoe crab eggs. Newly arriving spawning horseshoe crabs deposit their eggs in relatively undisturbed sediments. However, since the spawning season may extend into July, or even August, successive waves of spawning crabs begin to compete for sediments that gradually saturate with eggs. In the process of successive deposition of eggs, inevitably some eggs that were laid earlier will be displaced by the burrowing activity of the newly arrived females (Smith, 2007). At this time, one may observe fresh eggs lying on the surface of the beach sediments. These eggs are either consumed by migratory shorebirds, or they desiccate, or the eggs are washed into the offshore waters and become food for near shore fish and crabs (Botton, 2009). In addition, previously deposited egg clutches may become fragmented and dispersed among the sediments, often working their way toward the surface. Nevertheless, a significant portion of egg clutches reside at depths of 10-20 cm in the sand (Weber and Carter, 2009), where development proceeds.

Quantitative sampling of horseshoe crabs is best achieved by driving a series of replicate cores into the sediments to depths of 25 cm or more (Pooler et al., 2003). In routine workup of these samples, eggs are generally assessed as either "live eggs", or "dead eggs" if they have begun to decay or are coated with fungi. The problem with simply assuming that all eggs are "live eggs" is that this also assumes that they are "freshly laid eggs." However, as noted in the earlier description of *Limulus* development (see *section 1.2*), very close microscopic examination is necessary to distinguish recently fertilized eggs from early embryos, and there is virtually no way to visually distinguish between unfertilized and newly fertilized eggs. Given the temporal aspects of egg deposition by successive waves of spawning females, a field sample of eggs on a particular date may actually consist of an assemblage of eggs that are in varying stages of development. Thus, there is a strong possibility that eggs collected in the successive weeks may have been deposited in an earlier week and are not "freshly laid eggs." A sample of eggs on a particular date may not reflect the actual age distribution of the eggs because of asynchronous developmental rate, and environmental factors such as temperature and density.

To accurately assess the transition of eggs into embryos, and embryos into "trilobite" larvae, one must apply a developmental schedule to the field data. Culturing a sub-sample of about 100 eggs from the core sample for 30 – 40 days provides a schedule which is specific to the time that the eggs were initially deposited. We have found that correcting raw field data, using culture data, may have a profound effect on our understanding of the pattern of spawning, as well as estimating the life table factors of early development in horseshoe crabs (Loveland and Botton, in preparation). For example, temporal patterns of egg abundance may appear to be quite random when one plots raw field data. But, after applying corrections from developmental schedules obtained by culturing field eggs, coherent patterns emerge, reflecting lunar periodicity in Delaware Bay; these corrections generate an orderly progression of eggs developing into embryos, and embryos metamorphosing into larvae.

Most of the data available on egg density has been collected without regard to developmental schedules, because much of the current interest in horseshoe crab egg density centers on the availability of eggs to migratory shorebirds (Mizrahi and Peters, 2009). A rather

massive decrease in horseshoe crabs in the Delaware Bay region during the decade of the 1990's (e.g. Smith et al., 2009b) resulted in significant loss of horseshoe crab eggs which are a necessary resource for migratory shorebirds. Eggs consumed by shorebirds would probably be lost to the population anyway since its unlikely they would remain viable and complete development near the sediment surface. However, in order for horseshoe crab eggs to be abundant on the surface, it requires rather high densities of eggs to be present at depth, where normal development proceeds. When eggs are diminished at depth, it follows that eggs are also not abundant on the surface. Thus, there is a correlation between the density of horseshoe crabs spawning on the beaches of Delaware Bay, and the amount of food resources available to migratory shorebirds (Smith, 2007). Studies of the latter may have trumped the interest in horseshoe crab populations, particularly investigations on early life factors. We feel that more emphasis should be placed on the demographics and early life history of *Limulus* per se, which would also yield data on resources necessary for the management of migratory shorebirds.

5 Developmental Ecology, Fishery Management, and Evolutionary Success: Some Concluding Remarks

An understanding of horseshoe crab developmental ecology can contribute to the sound scientific management of *Limulus* populations. Based on DNA microsatellites, King et al. (2005) suggested that the species should be subdivided into Gulf of Maine, middle Atlantic, southeast Atlantic, Florida-Atlantic, and Florida-Gulf regions for management purposes. Because the larvae do not disperse widely from their natal beaches, there is probably limited connectivity among different spawning areas (Botton and Loveland, 2003; see section 3 *Behavior and Dispersal of Post-hatch Trilobite Larvae*). Thus, the most vulnerable populations of horseshoe crabs to potential over-exploitation may well be the smaller, more geographically isolated populations at the northern and southern fringes of its range. Unlike many marine bivalves and decapods that have planktonic larvae capable of long-distance dispersal, *Limulus* larvae tend to concentrate in nursery habitats near the breeding beaches. Should there be depletions of smaller, isolated populations of horseshoe crabs, it is highly unlikely that there would be significant levels of recruitment to these populations through larval settlement.

Most of the focus on horseshoe crab management has centered on the large middle Atlantic population, because of the relationship between *Limulus* eggs and migratory shorebirds in Delaware Bay (e.g. ASMFC, 1998; Mizrahi and Peters, 2009). Adult horseshoe crabs in the middle Atlantic disperse and intermingle on the continental shelf (King et al., 2005). While not minimizing the ecological importance of the Delaware Bay population, we suggest that the limited estuary-shelf movements of adult crabs in locations such as northern New England (e.g. Moore and Perrin, 2007; James-Pirri, 2010; Schaller et al., 2010; Watson and Chabot, 2010), coupled with limited larval dispersal, puts these populations at greater risk of local extinction.

Horseshoe crabs (xiphosurids) are well-known living fossils that date to the Late Ordovician, some 445 million years ago (Rudkin and Young, 2009). The versatility of the adult body plan in facilitating activities such as burrowing, feeding, and the aeration of respiratory structures may help explain its geological longevity (Shuster and Anderson, 2003). Moreover, adults can thrive over a wide range of temperatures, salinities, and sediment types and can feed on an assortment of foods (Fisher, 1984; Shuster and Anderson, 2003; Rudkin and Young, 2009). We suggest that the role of developmental ecology in understanding the evolutionary success of horseshoe crabs has been underappreciated. The adult body plan may have served them admirably over time, but the durability of horseshoe crabs implies that their embryos and larvae must have also been able to survive whatever environmental challenges confronted them. It is worth noting that we do not know when intertidal spawning and/or the trilobite larvae first evolved, as no fossil of a horseshoe crab embryo or larva has been found. This is not surprising, as even adult horseshoe crabs rarely fossilize because of the non-mineralized chitinous exoskeleton (Shuster and Anderson, 2003; Rudkin and Young, 2009). The horseshoe crab developmental schedule is highly plastic and the larvae can survive in the sediments for many months until hatching when conditions are favorable. As we have emphasized in this review, these embryos are eurythermal, euryhaline, and extremely tolerant of variable water quality. In our view, these traits have played a major role in the long-term evolutionary success of the horseshoe crab lineage.

References

ASMFC, 1998. Interstate fishery management plan for horseshoe crab. Fishery Management Report No. 32. Washington, DC: Atlantic States Marine Fisheries Commission.

Bademan ML, 2009. Tolerance and physiological responses of larval and juvenile horseshoe crabs *Limulus polyphemus* to hypoxia and anoxia. MS Thesis, Florida Institute of Technology, Melbourne, Florida, 1−58.

Bannon GA, Brown GG, 1980. Vesicle involvement in the egg cortical reaction of the horseshoe crab *Limulus polyphemus* L. Develop. Biol. 76: 418−427.

Barlow RB, Powers MK, Howard H, Kass L, 1986. Migration of *Limulus* for mating: Relation to lunar phase, tide height, and sunlight. Biol Bull 171: 310−329.

Barnum SR, Clapper DL, Brown GG, 1985. Modifications of the fertilized egg surface following the cortical reaction in *Limulus polyphemus* L. as viewed with the scanning electron microscope. J. Morphol. 183: 185−198.

Botton ML, 2000. Toxicity of cadmium and mercury to horseshoe crab *Limulus polyphemus* embryos and larvae. Bull. Environ. Contamin. Toxicol. 64: 137−143.

Botton ML, 2009. The ecological importance of horseshoe crabs in estuarine and coastal communities: A review and speculative summary. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 45−63.

Botton ML, Johnson K, Helleby L, 1998a. Effects of copper and zinc on embryos and larvae of the horseshoe crab *Limulus polyphemus*. Arch. Environ. Contamin. Toxicol. 35: 25−32.

Botton ML, Hodge M, Gonzalez TI, 1998b. High tolerance to tributyltin in embryos and larvae of the horseshoe crab *Limulus polyphemus*. Estuaries 21: 340−346.

Botton ML, Itow T, 2009. The effects of water quality on horseshoe crab embryos and larvae. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 439−463.

Botton ML, Loveland RE, 1992. Body size, morphological constraints, and mated pair formation in four populations of horseshoe crabs *Limulus polyphemus* along a geographic cline. Mar. Biol. 112: 409−415.

Botton ML, Loveland RE, 2003. Abundance and dispersal potential of horseshoe crab *Limulus polyphemus* larvae in the Delaware estuary. Estuaries 26: 1472−1479.

Botton ML, Loveland RE, Jacobsen TR, 1988. Beach erosion and geochemical factors: Influence on spawning success of horseshoe crabs *Limulus polyphemus* in Delaware Bay. Mar. Biol. 99: 325−332.

Botton ML, Loveland RE, Jacobsen TR, 1992. Overwintering by trilobite larvae of the horseshoe crab *Limulus polyphemus* on a sandy beach of Delaware Bay (New Jersey, USA). Mar. Ecol. Prog. Ser. 88: 289−292.

Botton ML, Loveland RE, Jacobsen TR, 1994. Site selection by mi-

gratory shorebirds in Delaware Bay, and its relationship to beach characteristics and abundance of horseshoe crab *Limulus polyphemus* eggs. Auk 111: 605−616.

Botton ML, Loveland RE, Tiwari A, 2003a. Distribution, abundance, and survivorship of young of the year in a commercially exploited population of horseshoe crabs *Limulus polyphemus*. Mar. Ecol. Prog. Ser. 265: 175−184.

Botton ML, Harrington BA, Tsipoura N, Mizrahi DS, 2003b. Synchronies in migration: Shorebirds, horseshoe crabs, and Delaware Bay. In: Shuster CN Jr, Barlow RB, Brockmann HJ ed. The American Horseshoe Crab. Cambridge, Massachusetts: Harvard University Press, 6−32.

Botton ML, Pogorzelska M, Smoral L, Shehata A, Hamilton MG, 2006. Thermal biology of horseshoe crab embryos and larvae: A role for heat shock proteins. J. Exp. Mar. Biol. Ecol. 336: 65−73.

Brockmann HJ, 1990. Mating behavior of horseshoe crabs *Limulus polyphemus*. Behaviour 114: 206−220.

Brockmann HJ, 1996. Satellite male groups in horseshoe crabs *Limulus polyphemus*. Ethology 102: 1−21.

Brockmann HJ, 2003a. Nesting behavior: A shoreline phenomenon. In: Shuster CN Jr, Barlow RB, Brockmann HJ ed. The American Horseshoe Crab. Cambridge, Massachusetts: Harvard University Press, 33−49.

Brockmann HJ, 2003b. Male competition and satellite behavior. In: Shuster CN Jr, Barlow RB, Brockmann HJ ed. The American Horseshoe Crab. Cambridge, Massachusetts: Harvard University Press, 50−82.

Brockmann HJ, Colson T, Potts W, 1994. Sperm competition in horseshoe crabs *Limulus polyphemus*. Behav. Ecol. Sociobiol. 35: 153−160.

Brockmann HJ, Nguyen C, Potts W, 2000. Paternity in horseshoe crabs when spawning in multiple-male groups. Anim. Behav. 60: 837−849.

Brockmann HJ, Smith MD, 2009. Reproductive competition and sexual selection in horseshoe crabs. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 199−221.

Brown GG, 1976. Scanning electron microscopical and other observations of sperm fertilization redactions in *Limulus polyphemus* L. (Merostomata: Xiphosura) J. Cell Sci. 22: 547−562.

Brown GG, Barnum SR, 1983. Postfertilization changes in the horseshoe crab *Limulus polyphemus* L. Biol. Bull. 164: 163−175.

Brown GG, Clapper DL, 1980. Cortical reaction in inseminated eggs of the horseshoe crab *Limulus polyphemus* L. Develop. Biol. 76: 410−417.

Brown GG, Clapper DL, 1981. Procedures for maintaining adults, collecting gametes, and culturing embryos and juveniles of the horseshoe crab *Limulus polyphemus* L. In: Hinegardner R, Atz J, Fayet R ed. Laboratory Animal Management, Marine Invertebrates. Washington DC: National Academy Press, 268−290.

Brown GG, Knouse JR, 1973. Effects of sperm concentration, sperm aging and other variables on fertilization in the horseshoe crab *Limulus polyphemus* L. Biol. Bull. 144: 462−470

Brusca RC, Brusca GJ, 1990. Invertebrates. Sunderland, Massachusetts: Sinauer Associates.

Burger J, Dixon C, Shukla T, Tsipoura N, Jensen H et al., 2003. Metals in horseshoe crabs from Delaware Bay. Arch. Environ. Contamin. Toxicol. 44: 36−42.

Burton WH, Kelley FS, Franks EA, 2009. Distribution of juvenile

horseshoe crabs in subtidal habitats of Delaware Bay using a suc-tion-dredge sampling device. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 285−293.

Chabot CC, Kent J, Watson WH, 2004. Daily, circadian and tidal rhythms of locomotory activity in the horseshoe crab *Limulus polyphemus*. Biol. Bull. 207: 72−75.

Chabot CC, Betournay SH, Braley N, Watson WH, 2007. Circadian and circatidal rhythms of locomotion in the horseshoe crab *Limulus polyphemus*. J. Exp. Mar. Biol. Ecol. 345: 79−89.

Chabot CC, Skinner SJ, Watson WH, 2008. Locomotion rhythms expressed by the horseshoe crab *Limulus polyphemus*: 1. Synchronization by artificial tides. Biol. Bull 215: 34−45.

Chabot CC, Watson WH, 2010. Circatidal rhythms of locomotion in the American horseshoe crab *Limulus polyphemus*: Underlying mechanisms and cues that influence them. Curr. Zool. 56: 499−517.

Coursey Y, Ahmad N, McGee BM, Steimel N, Kimble M, 2003. Amoebocyte production begins at stage 18 during embryogenesis in *Limulus polyphemus*, the American horseshoe crab. Biol. Bull. 204: 21−27.

Duffy EE, Penn DJ, Botton ML, Brockmann HJ, Loveland RE, 2006. Eye and clasper damage influence male mating tactics in the horseshoe crab *Limulus polyphemus*. J. Ethol. 24: 67−74.

Ehlinger GS, Tankersley RA, 2003. Larval hatching in the horseshoe crab *Limulus polyphemus*: Facilitation by environmental cues. J. Exp. Mar. Biol. Ecol. 292: 199−212.

Ehlinger GS, Tankersley RA, 2004. Survival and development of horseshoe crab *Limulus polyphemus* embryos and larvae in hyper-saline conditions. Biol. Bull. 206: 87−94.

Ehlinger GS, Tankersley RA, 2006. Endogenous rhythms and en-trainment cues of larval activity in the horseshoe crab *Limulus polyphemus*. J. Exp. Mar. Biol. Ecol. 292337: 205−214.

Ehlinger GS, Tankersley RA, 2007. Reproductive ecology of the American horseshoe crab *Limulus polyphemus* in the Indian River Lagoon: An overview. Florida Scientist 70: 449−463.

Ehlinger GS, Tankersley RA, 2009. Ecology of horseshoe crabs in microtidal lagoons. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 149−162.

Ehlinger GS, Tankersley RA, Bush MB, 2003. Spatial and temporal patterns of spawning and larval hatching by the horseshoe crab *Limulus polyphemus* in a microtidal coastal lagoon. Estuaries 26: 631−640.

Fisher DC, 1984. The Xiphosurida: Archetypes of bradytely? In: Eldredge N, Stanley SM ed. Living Fossils. New York: Springer-Verlag, 196−213.

Forward RB Jr, Tankersley RA, 2001. Selective tidal stream transport of marine animals. Oceanogr. Mar. Biol. Ann. Rev. 39: 305−353.

Fraenkel G, 1960. Lethal high temperature for three marine inverte-brates: *Limulus polyphemus*, *Littorina littorea*, and *Pagurus longicarpus*. Oikos 11: 171−182.

French KA, 1979. Laboratory culture of embryonic and juvenile *Limulus*. In: Cohen E ed. Biomedical Applications of the Horse-shoe Crab (Limulidae). New York: Alan R. Liss, 61−71.

Gaines EF, Carmichael RH, Grady SP, Valiela I, 2002. Stable isotopic evidence for changing nutritional sources of juvenile horseshoe crabs. Biol. Bull. 203: 228−230.

Graham LJ, Botton ML, Hata D, Loveland RE, Murphy BR, 2009.

Prosomal-width-to-weight relationships in American horseshoe crabs *Limulus polyphemus*: Examining conversion factors used to estimate landings. Fish. Bull. 107: 235−243.

Greene MP, Hamilton MG, Botton ML, accepted. Levels of heat shock proteins (HSP70 and HSP90) in horseshoe crab *Limulus polyphe-mus* embryos exposed to osmotic stress. Mar. Biol.

Hajeb P, Christianus A, Zadeh SS, Saad CR, 2009. Sperm attachment on the egg of Malaysian king crab *Carcinoscorpius rotundicauda*. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conser-vation of Horseshoe Crabs. New York: Springer, 237−247.

Hidu H, Haskin HH, 1971. Setting of the American oyster related to environmental factors and larval behavior. Proc. Nat. Shellfisheries Assoc. 61: 35−50.

Itow T, Loveland RE, Botton ML, 1998a. Developmental abnormali-ties in horseshoe crab embryos caused by heavy metals. Arch. En-viron. Contamin. Toxicol. 35: 33−40.

Itow T, Igarashi T, Botton ML, Loveland RE, 1998b. Heavy metals inhibit limb regeneration in horseshoe crab larvae. Arch. Environ. Contamin. Toxicol. 35: 457−463.

Jackson NL, Smith DR, Nordstrom KF, 2008. Physical and chemical changes in the foreshore of an estuarine beach: Implications for viability and development of horseshoe crab *Limulus polyphemus* eggs. Mar. Ecol. Prog. Ser. 355: 209−218.

James-Pirri MJ, 2010. Seasonal movement of the American horseshoe crab *Limulus polyphemus* in a semi-enclosed bay on Cape Cod, Massachusetts (USA) as determined by acoustic telemetry. Curr. Zool. 56: 575−586.

Jegla TC, 1979. The *Limulus* bioassay for ecdysteriods. Biol. Bull. 156: 103−114.

Jegla TC, 1982. A review of the molting physiology of the trilobite larva of *Limulus*. In: Bonaventura J, Bonaventura C, Tesh S ed. Physiology and Biology of Horseshoe Crabs. New York: Alan R. Liss, 83−101.

Jegla TC, Costlow JD, 1982. Temperature and salinity effects on de-velopmental and early posthatch stages of *Limulus*. In: Bonaven-tura J, Bonaventura C, Tesh S ed. Physiology and Biology of Horseshoe Crabs. New York: Alan R. Liss, 103−113.

King TL, Eackles MS, Spidle AP, Brockmann HJ, 2005. Regional differentiation and sex-biased dispersal among populations of the horseshoe crab *Limulus polyphemus*. Trans. Am. Fish. Soc. 134: 441−465.

Kingsley JS, 1892. The embryology of *Limulus*. J. Morphol. 7: 35−66.

Kingsley JS, 1893. The embryology of *Limulus*. J. Morphol. 8: 195−268.

Laughlin RB Jr, Neff JM, 1977. Interactive effects of temperature, salinity shock, and chronic exposure to no. 2 fuel oil on survival, development rate and respiration of the horseshoe crab *Limulus polyphemus*. In: Wolff DA ed. Fate and Effects of Petroleum Hy-drocarbons in Marine Organisms and Ecosystems. Oxford: Per-gammon Press, 182−191.

Leschen AS, Grady SP, Valiea I, 2006. Fecundity and spawning of the Atlantic horseshoe crab *Limulus polyphemus* in Pleasant Bay, Cape Cod, Massachusetts, USA. Mar. Ecol. 27: 54−65.

Loveland RE, Botton ML, 1992. Size dimorphism and the mating system in horseshoe crabs (*Limulus polyphemus* L.). Anim. Behav. 44: 907−916.

Laughlin R, 1983. The effects of temperature and salinity on larval growth of the horseshoe crab *Limulus polyphemus*. Biol. Bull. 164: 93−103.

Makioka T, 1988. Internal morphology. In: Sekiguchi K ed. Biology of Horseshoe Crabs. Tokyo, Japan: Science House Co Ltd, 104−132.

Mizrahi DS, Peters KA, 2009. Relationships between sandpipers and horseshoe crab in Delaware Bay: A synthesis. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 65−87.

Moore S, Perrin S, 2007. Seasonal movement and resource-use patterns of resident horseshoe crab *Limulus polyphemus* populations in a Maine, USA estuary. Est. Coasts 30: 1016−1026.

Munson JP, 1898. The ovarian egg of *Limulus*. J. Morphol. 15: 111−207.

Neff JM, Giam CS, 1977. Effects of Arochlor 1016 and Halowax 1099 on juvenile horseshoe crabs *Limulus polyphemus*. In: Vernberg FJ, Calabrese A, Thurberg FP, Vernberg WB ed. Physiological Responses of Marine Biota to Pollutants. New York: Academic Press, 21−35.

Packard AS, 1870. The embryology of *Limulus polyphemus*. Am. Nat. 4: 498−502.

Packard AS, 1885. On the embryology of *Limulus polyphemus*. III. Am. Nat. 19: 722−727.

Palumbi SR, Johnson BA, 1982. A note on the influence of life history stage on metabolic adaptation: The responses of *Limulus* eggs and larvae to hypoxia. In: Bonaventura J, Bonaventura C, Tesh S ed. Physiology and Biology of Horseshoe Crabs. New York: Alan R. Liss, 115−124.

Penn D, Brockmann HJ, 1994. Nest-site selection in the horseshoe crab *Limulus polyphemus*. Biol. Bull. 187: 373−84.

Pooler PS, Smith DR, Loveland RE, Botton ML, Michels SF, 2003. Assessment of sampling methods to estimate horseshoe crab *Limulus polyphemus* egg density in Delaware Bay. Fish. Bull. 101: 698−703.

Reynolds WW, Casterlin ME, 1979. Themoregulatory behavior and diel activity of *Limulus polyphemus*. In: Cohen E ed. Biomedical Applications of the Horseshoe Crab (Limulidae). New York: Alan R. Liss, 47−59.

Rudkin DM,Young GA, 2009. Horseshoe crabs: An ancient ancestry revealed. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 25−44.

Rudloe A, 1979. Locomotor and light responses of larvae of the horseshoe crab *Limulus polyphemus* (L.). Biol. Bull. 157: 494−505.

Rudloe A, 1980. The breeding behavior and patterns of movement of horseshoe crabs *Limulus polyphemus* in the vicinity of breeding beaches in Apalachee Bay, Florida. Estuaries 3: 177−183.

Rudloe A, 1981. Aspects of the biology of juvenile horseshoe crabs *Limulus polyphemus*. Bull. Mar. Sci. 31: 125−133.

Schaller SY, Watson WH III, Chabot CC, 2010. Seasonal movements of horseshoe crabs *Limulus polyphemus* in the Great Bay Estuary, New Hampshire (USA). Curr. Zool. 56: 587−598

Sekiguchi K, 1988. Biology of Horseshoe Crabs. Tokyo, Japan: Science House Co Ltd.

Sekiguchi K, Yamamichi Y, Seshimo H, Sugita H, 1988a. Normal development. In: Sekiguchi K ed. Biology of Horseshoe Crabs. Tokyo, Japan: Science House Co Ltd, 133−224.

Sekiguchi K, Seshimo H, Sugita H, 1988b. Post-embryonic development of the horseshoe crab. Biol. Bull. 174: 337−345.

Shuster CN Jr, 1979. Distribution of the American horseshoe "crab," *Limulus polyphemus* (L.). In: Cohen E ed. Biomedical Applications of the Horseshoe Crab (Limulidae). New York: Alan R. Liss, 3−26.

Shuster CN Jr, 1982. A pictorial review of the natural history and ecology of the horseshoe crab *Limulus polyphemus*, with reference to other Limulidae. In: Bonaventura J, Bonaventura C, Tesh S ed. Physiology and Biology of Horseshoe Crabs. New York: Alan R. Liss, 1−52.

Shuster CN Jr, Botton ML, 1985. A contribution to the population biology of horseshoe crabs *Limulus polyphemus* in Delaware Bay. Estuaries 8: 363−372.

Shuster CN Jr, Anderson LI, 2003. A history of skeletal structure: Clues to relationships among species. In: Shuster CN Jr, Barlow RB, Brockman HJ ed. The American Horseshoe Crab. Cambridge, Massachusetts: Harvard University Press, 54−188.

Shuster CN Jr, Sekiguchi K, 2003. Growing up takes about ten years and eighteen stages. In: Shuster CN Jr, Barlow RB, Brockmann HJ ed. The American horseshoe crab. Cambridge. Harvard University Press, 103−133.

Smith DR, 2007. Effect of horseshoe crab spawning density on nest disturbance and exhumation of eggs: A simulation study. Est. Coasts 30: 287−295.

Smith DR, Mandt MT, MacDonald PDM, 2009a. Proximate causes of sexual size dimorphism in horseshoe crabs *Limulus polyphemus* of the Delaware Bay. J. Shellfish Res. 28: 405−417.

Smith DR, Millard MJ, Carmichael RH, 2009b. Comparative status and assessment of *Limulus polyphemus* with emphasis on the New England and Delaware Bay populations. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 361−386.

Spraker H, Austin HM, 1997. Diel feeding periodicity of Atlantic silverside *Menidia menidia* in the York River, Chesapeake Bay, Virginia. J. Elisha Mitchell Sci. Soc. 113: 171−182.

Strobel CJ, Brenowitz AH, 1981. Effects of Bunker C oil on juvenile horseshoe crabs *Limulus polyphemus*. Estuaries 4: 157−159.

Sugita H, 1988. Environmental adaptations of embryos. In: Sekiguchi K ed. Biology of Horseshoe Crabs. Tokyo, Japan: Science House Co. Ltd, 195−224.

Towle DW, Henry RP, 2003. Coping with environmental changes: Physiological challenges. In: Shuster CN Jr, Barlow RB, Brockmann HJ ed. The American Horseshoe Crab. Cambridge, Massachusetts: Harvard University Press, 224−244.

Watson, WH, Bedford L, Chabot CC, 2008. Dissociation between circadian rhythms of visual sensitivity and circatidal rhythms of locomotion in the American horseshoe crab *Limulus polyphemus*. Biol. Bull. 215: 46−56.

Watson WH III, Chabot CC, 2010. High resolution tracking of horseshoe crab *Limulus polyphemus* movements in an estuary with a fixed array ultrasonic telemetry. Curr. Zool. 56: 599−610.

Weber RG, Carter DB, 2009. Distribution and development of *Limulus* egg clusters on intertidal beaches in Delaware Bay. In: Tanacredi JT, Botton ML, Smith DR ed. Biology and Conservation of Horseshoe Crabs. New York: Springer, 249−266.

Weis JS, Ma A, 1987. Effects of the pesticide diflubenzuron on larval horseshoe crabs *Limulus polyphemus*. Bull. Environ. Contamin. Toxicol. 39: 224−228.