Effects of habitat size and temperature on success and growth of larval horseshoe crabs (*Limulus polyphemus*)

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Abstract
The horseshoe crab (*Limulus polyphemus*) is an important component of the marine ecosystem and is frequently used in biomedical research as well as other laboratory studies. When this research is conducted, a large number of horseshoe crabs are often contained in a limited amount of space, and therefore experience overcrowded conditions. These conditions often lead to the spread of various etiologies and result in developmental issues for the horseshoe crabs. Through this research, the effect of habitat size and temperature on the success of growth and development of larval and juvenile horseshoe crab was examined. After exposing the horseshoe crabs to two different temperatures (18°C and 24°C) and two different habitat sizes (large and small), it was determined that horseshoe crabs are most successful in smaller habitats and warmer temperatures. This research suggests that at least for horseshoe crab populations in Long Island Sound increasing water temperatures alone may not adversely affect larval success.

Introduction
Considered to be a living fossil, the horseshoe crab (*Limulus polyphemus*) has been an important component of the marine ecosystem for the last 360 million years (Dunlap 1999). There are four extant species of horseshoe crab, however only *Limulus polyphemus* is found along the eastern coast of North America. While adults and juveniles of this species can thrive in subtidal benthic habitats, embryonic and larval horseshoe crabs are typically located in the intertidal zone (Ehlinger and Tankersley 2004).

The breeding behavior of *Limulus polyphemus* is unique when compared to that of other marine arthropods (Laughlin 1983). Typically occurring during late May and early June, horseshoe crabs will synchronize nesting to occur a few hours each day in correlation with the new and full-moon high tides (Brockmann 1990). When horseshoe crabs mate, females, with males attached to them, travel up onto a beach and deposit their eggs within the sand by creating a sediment-water slurry. Once they are deposited, the female moves over the nest, pulling the male horseshoe crab directly over the eggs. He then releases free-swimming sperm that fertilizes the deposited eggs (Brockmann 1990). The eggs themselves are soft and covered in a jelly-like substance. The surfaces of the eggs harden after exposure to seawater and are then pushed down further into the sediment by the female (Shuster et al. 2003). Most nests are approximately 15 to 20 cm deep (Shuster et al. 2003). The depth of the eggs typically depends on the size of the female (Shuster et al. 2003). From that point, embryonic development occurs.

As described by Patten (1912), *Limulus* goes through fifteen different embryonic stages. The first part of the body to experience growth is the opisthosoma, or the posterior portion. The prosoma, which is the fused anterior portion of the body, grows secondarily. Both segments grow around the egg until finally their ends meet along the axial ridge and eventually fuse (Patten 1912). Embryonic development occurs rapidly. The time period from fertilization to hatching of *Limulus polyphemus* is approximately 14 days, at which time the horseshoe crab has gone through four molts already. *Limulus* hatchlings – known as trilobite larvae – do not resemble the adult animal. The larva has an immovable terminal spine covered by a mid-piece rather than a telson (Shuster et al. 2003). Trilobite larvae metamorphose into what is known as the first-tailed stage. During this stage, a telson develops, however it is still fused to the body (Shuster et al 2003). What triggers the transformation from trilobite larva to the first-tailed stage is still not fully understood.

During the developmental period, eggs growing on estuarine beaches are potentially vulnerable to thermal stress, especially during the trilobite larva stage. To determine the effect that increased temperatures have on the developmental process, Botton et al. (2006) examined the survival rate of both embryos and horseshoe crabs in the trilobite larva stage that had been exposed to heat shock. In most marine organisms, proteins, known as Hsp's, help organisms deal with any experienced heat stress by assisting to restore the folds of proteins that have been damaged by increased temperatures and other environmental stressors. In the study performed by Button et al. (2006), they compared the Hsp70 protein between animals that had experienced heat shock and those that hadn't. They determined that the levels of Hsp70 in organisms that had experienced the heat shock were slightly elevated compared to those in the control group. Therefore, in order to thrive successfully in temperature stressful environments, horseshoe crabs appear to maintain high baseline levels of proteins that combat cellular stress, for example Hsp70 (Botton et al. 2006). A study performed by Laughlin (1983) also examined the effect that temperature, as well as salinity, has on the development of horseshoe crabs. Through this study it was found that the lowest success occurred in combinations of low temperature and low salinity (Laughlin 1983).

Horseshoe crab larvae have been utilized in laboratory research for many years. Often during the occurrence of laboratory experiments many organisms will be held in a limited amount of space, causing overcrowded conditions to occur. Often in these overcrowded conditions, both infectious and noninfectious etiologies have the potential to develop and spread rapidly throughout the community (Lewbart 2012). Organisms in these conditions will also frequently experience poor water quality and high levels of ammonia. Aside from that, horseshoe crabs have also been reported to experience issues during the molting process of the shell, legs, and telson (Lewbart 2012). Puncture wounds, fractures, and damage to the exoskeleton have also been reported. Previous studies have examined both juvenile and adult horseshoe crabs in order to determine the effects of crowding on behavior, morphology, and reproduction (Tamar 1968; Lewbart 2012). A 1968 study performed at Woods Hole examined the hyperglycemic response of the horseshoe crab (*Xiphosura polyphemus*) in response to various stimuli. It was determined that even crowded conditions can cause the horseshoe crab to exhibit a hyperglycemic response (Tamar 1968).

This research investigates the combined effects of crowding and temperature on the growth, development, and survival of embryonic and larval horseshoe crabs in order to determine how such factors might combine to negatively affect horseshoe crabs held at aquariums and other facilities as well as to examine the potential effects of climate change on the development of horseshoe crab eggs and larvae in nature. It was originally hypothesized that horseshoe crabs would thrive more successfully at higher temperatures and under less crowded conditions.

Figure 1: Location of egg collection at Long Warf Beach located in New Haven, CT.

Materials and Methods

Horseshoe crab eggs were legally collected from Long Wharf Beach located in New Haven, CT (Figure 1). Eggs were collected by locating two nests on the upper portions of the beach and digging until eggs were spotted within the sediment. The collected eggs were taken back to the laboratory and counted. 600 eggs were separated equally (150 eggs each) into four experimental chambers consisting of two different sizes, large (16 x 11.5 x 7 cm) and small (13 x 9 x 4.5 cm). Each chamber was modified using mesh in order to allow water to pass freely through both the bottom and the sides of the container, which prevented anoxic conditions from occurring. One large and one small container were placed into a tank maintained at a temperature of 24°C, while the other two containers were placed into a tank maintained at a temperature of 18°C. Artificial seawater with a salinity of 27 ppt was used in both of these tanks.

The containers were examined daily in order to document any changes, such as development or death, as well as to feed. Once the eggs began to hatch into trilobite larva, the number of hatchlings was counted and the date of hatching was recorded and compared among containers. Hatchlings were then fed a mixed, proprietary diet. Horseshoe crabs were left to feed for 45 minutes, after which point any excess food was removed and the containers were placed back into their respective tanks. Once molting began to occur, the number of horseshoe crabs that transitioned from trilobite larva to the first tailed stage was counted and the date of the transition was recorded. This information was once again compared among the containers. Any horseshoe crabs that had reached the first tailed stage were separated into a different container and fed a mixed diet. Once again, horseshoe crabs were allowed to feed for 45 minutes, at which point they were placed back into the containers and put back into the proper tanks.

Results

The data collected was analyzed and the significance was determined by performing a two-way ANOVA through the statistical program SPSS

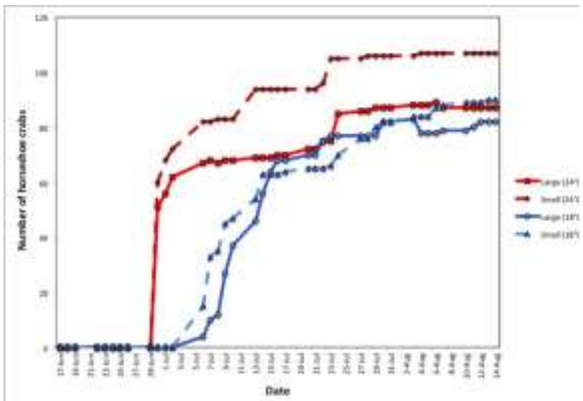

Figure 2: Hatching results of *Limulus polyphemus* for each of the containers between June 17, 2015 and August 14, 2015

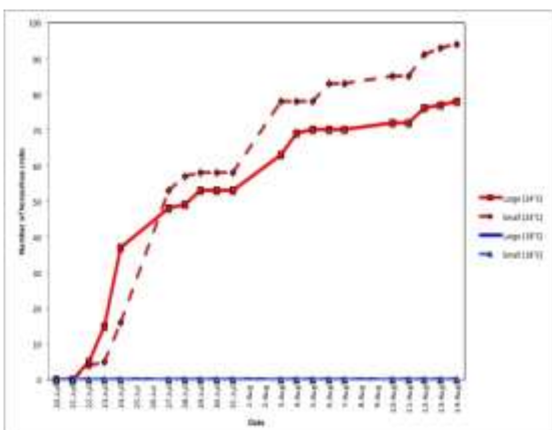

Figure 3: Molting results of *Limulus polyphemus* for the large and small containers kept in a water temperature of 24°C

As shown in Figure 2, horseshoe crabs in both large and small chambers maintained in the warmer water temperature of 24°C displayed an increased rate of hatching compared to those horseshoe crabs maintained in the cooler water temperature of 18°C. Of the horseshoe crabs maintained in the water held at 24°C, those raised in the smaller habitat size displayed an increased rate of development compared to those in the larger habitat.

This same trend was displayed in the cooler water conditions of 18°C (Figure 2). However, from day 29 (July 15, 2015) until day 43 (July 29, 2015) there was a reversal of this trend where horseshoe crabs in the larger habitat size experienced more hatching than those in the smaller habitat size. However, the difference between the amounts of horseshoe crabs that had hatched was always less than 10 individuals.

As depicted in Figure 3, during the duration of the study none of the horseshoe crabs maintained in the cooler water temperature of 18°C experienced molting. All horseshoe crabs held in water at 24°C did molt from trilobite larva to the first tailed stage. Similarly, those horseshoe crabs developing in the smaller habitat size also experienced increased rates of molting.

It was determined that temperature was a significant influence on the rate of hatching ($p \leq 0.001$) as well as the rate of molting ($p \leq 0.001$). Although habitat size was shown to not be significant ($p \leq 0.112$), it did exert a secondary influence on both rates of hatching and molting.

Discussion

In general, horseshoe crab development occurred most successfully in the warmer water temperature conditions of 24°C for both habitat sizes, although crowding appears to exert a secondary influence on development. Faster development at higher temperatures is not an unusual occurrence in marine animals and can most likely be contributed to an increase in metabolic rate that occurs with warmer temperatures. This is consistent with the work of Rudloe (1981) who determined that under conditions of lower temperature, the metabolism of *Limulus polyphemus* declines, often leading to periods of increased dormancy.

Horseshoe crab eggs maintained in water at 18°C in the small chambers showed a similar hatching rate to those maintained at 24°C in the larger chamber. On the other hand, those in the 18°C large chamber displayed the lowest hatching rate. This indicates that crowding, as well as temperature, has an influence on hatching.

In Long Island Sound, horseshoe crabs mate and lay eggs primarily in late May to early June when water temperatures range between 14°C and 16°C. The research presented here suggests that this temperature range is not optimal for rapid development of the eggs. However, eggs are also laid in clusters of 80,000 or more eggs so it is possible that crowding effects in the nest play an important role in horseshoe crab development, at least in more northern populations of horseshoe crabs.

The molting data suggest that the metabolic costs associated with higher temperatures do not negatively affect horseshoe crab juveniles but rather continue to benefit them, at least in laboratory studies where food supply is dependable.

The information gained from this study is provocative and suggests that at least for horseshoe crab populations in Long Island Sound, increasing water temperatures resulting from global warming, within reason, may not adversely affect larval and juvenile horseshoe crab development. Other factors associated with increased temperatures, such as increases in sediment anoxia, may counteract any benefit as sediment hypoxia and anoxia have been shown to negatively affect egg development in horseshoe crabs (Shuster, et al. 2003). The study also suggests that crowding may not have the same negative effects on horseshoe crab eggs and trilobite larvae as its does on later stage larvae, juvenile, and adults in zoos and aquariums.

Conclusion

This research has shown that embryonic and larval horseshoe crabs develop most successfully in warmer water temperatures and smaller habitat sizes when exposed to crowded conditions. Although this research suggests that increasing water temperatures due to global warming may be beneficial to northern populations of horseshoe crabs, secondary effects of warming temperatures would be detrimental to their development.

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