Optimizing Rearing Protocols for *Limulus polyphemus* Larvae and Juveniles – The Effects of Tank Conditions on Oxygen Metabolism.

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Abstract

Horseshoe crabs are invertebrates that have existed for millions of years. Their blood is used in the medical field as a test for endotoxins; it is the most efficient and reliable test to date. Scientists have not been able to synthetically generate the exact formula for the blood, and therefore the horseshoe crab population must be restored in order to ensure that the blood does not disappear with the population. The purpose of this work was to gain a better understanding of the best protocols to use for rearing *Limulus* eggs, larvae, and juveniles in captive settings. The goal of this study was to determine the effects of temperature on metabolic rate and rate of development of larvae and first-stage instars of *Limulus polyphemus.* It was found that the larval stage had a greater metabolic demand for oxygen then the preceding juvenile stage, and that growth rates were likely higher in warmer temperatures.

Introduction

Limulus polyphemus, the North American horseshoe crab, is one of the earth's living fossils; their lineage extends back over 350 million years (Avise, et al. 1994). Horseshoe crabs appeared on earth millions of years before the dinosaurs and have existed for millions of years after their demise (Avise, et al. 1994). Until recently, horseshoe crabs were found in great abundance worldwide, with different species primarily concentrated on the east coast of North America and the islands around the Philippines. The largest concentration of *Limulus polyphemus* in the world today is found within Delaware Bay (Odell, et al. 2005).

The importance of the horseshoe crab to both the natural world and to humans should not be under-estimated. Many trans-Arctic migratory shorebirds, like the endangered red knot, depend on horseshoe crab eggs as food along their migration route (Odell, et al. 2005). Furthermore, pharmaceutical companies rely on horseshoe crab blood for its ability to detect bacterial endotoxins. Their blood has been found to coagulate immediately when it comes in contact with bacterial endotoxins, therefore stopping the Brownian motion of the bacteria particle (Odell, et al. 2005). The use of the Limulus blood is referred to as the Limulus amebocyte lysate (LAL) assay. Researchers have discovered that using LAL assay it takes regularly 45 minutes to discover any endotoxins, whereas the second fastest test would take up to 48 hours. LAL is used to test all medicinal products including: medicines, intravenous fluids and disposable equipment among others (Odell, et al. 2005).

Despite their importance, the global abundance of the horseshoe crab has decreased dramatically over the years as a result of pollution, coastal habitat loss, accidental harvesting as by-catch and overharvesting as part of the bait fishery (Scharding, et al. 1998). This has resulted in a rise of conservation efforts worldwide in response to their increasingly low numbers (Scharding, et al. 1998).

Aquaculture, the farming of aquatic organisms, including fish, mollusks, crustaceans, and aquatic plants implies some form of intervention in the culturing process. The cultivation of marine organisms under tightly controlled conditions is one increasingly utilized means of assisting in marine species conservation efforts. To date, a laboratory group from UNH led by Dr. Carmela Cuomo has developed protocols for the consistent successful captive breeding of adult horseshoe crabs and has also been successful with their systems and protocols for the hatching of Limulus eggs and the growth and development of early stage larvae. Numerous questions remain regarding how to optimize these systems for maximum larval growth and development. Work by Havens (2011) has recently determined that early larval stage *Limulus* has a greater metabolic demand for O_2 per unit mass than juvenile stages under very similar laboratory conditions. This study was undertaken in an effort to expand on Havens (2011) work.

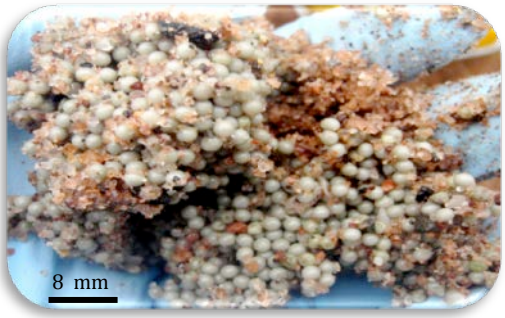

Figure 1. Horseshoe Crab Eggs

Materials and Methods

Eggs (Fig. 1) were obtained from a known spawning area of *Limulus polyphemus* located within Long Wharf Park, in New Haven, CT. Only one nest was used for this experiment in order to insure that the eggs were the same age and stage.

The eggs were brought to the lab, placed in seawater, counted, and placed in bowls in numbers of 100, 500, and 1000. The containers were set up in the lab, given unique identifiers, and aerated. Over 50,000 eggs were counted and utilized for a variety of experiments. For this particular work 600 horseshoe crab trilobite larvae and first instar juveniles that developed from the collected eggs were used. These crabs were isolated in floating systems (100 trilobite larvae and 100 juveniles in each of two containers per tank) and placed in one of three 190 L recirculating, aerated, temperature-controlled tanks (Fig. 2).

Each tank was set to a different temperature: 17^0C, 22^0C, and 27^0C; all tanks were maintained at the same salinity of 27 ppt. All crabs were patted dry and weighed prior to the initiation of the experiment.

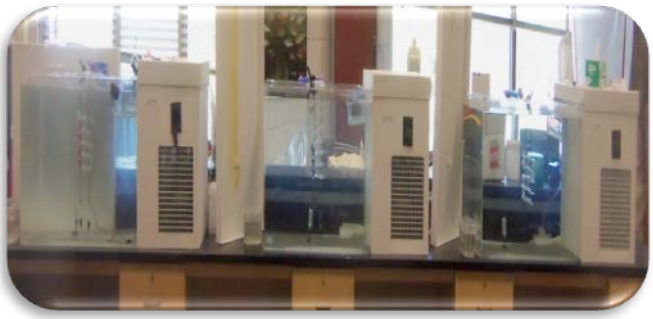

Figure 2. Three Tank Set-Up

At the start of the experimental trial, crabs were placed in 1.5 ml respirometer chambers and allowed to acclimate for two hours (Fig. 3). Every half hour throughout the acclimation period, the chambers were flushed with oxygen-saturated seawater. At the conclusion of the acclimation period, the chambers were sealed for a two hour trial period. At the end of that period, water samples were taken from each chamber using a glass syringe. All water samples were then measured for dissolved oxygen concentration using a dissolved oxygen meter that had been calibrated prior to the start of the experimental trial (Fig. 4). The amount of oxygen used by the crabs during the trial period was used to determine metabolic rate, the amount of oxygen used per unit mass over time.

Figure 3. Juvenile horseshoe crabs during trials in mini-respirometers.

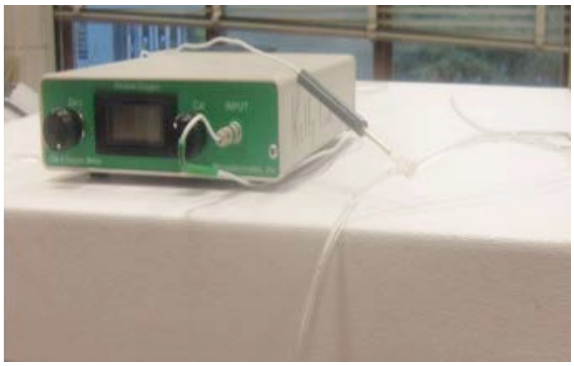

Figure 4. Oxygen Reader

Results and Discussion

The metabolic rate of larvae varied in each treatment (Fig. 5). In Tank 1, at 17^0C, the larvae consistently used the least amount of oxygen. This is likely due to the fact that enzymatic activity, an important component of metabolism, is slower at lower temperatures. Tank 2, at 22^0C, had the highest amount of metabolic activity. Tank 3, at 27^0C, showed less overall metabolic activity then Tank 2. It must be noted that for Tank 3, the final trial on 7 Aug., was performed on newly hatched larvae. The organisms used for the previous trials had molted into the next stage and therefore had to be replaced.

Tanks 1 and 2 showed similar levels of positive growth (Fig. 6). Larvae in Tank 3 also are believed to have grown since they successfully molted; however, since the organisms had to be replaced, the growth rate could not be calculated. The lower metabolic rate noted in Tank 3 – despite successful growth and molting – may be due to the crabs shifting their energy budget by reducing movement in order to continue devoting energy to growth; however, movement was not quantified in this study.

There was less variation in the Stage 1 results, with a constant difference noted throughout the tanks. As with the larval trials, Tank 1 had the lowest metabolic activity. The differences between Tanks 2 and 3 are quite small, with Tank 3 showing only a slight increase. The overall data shows that the Stage 1 crabs have lower metabolic demand per gram than their larval counterparts. Curiously, all of the Stage 1 animals displayed negative growth (loss of weight) during the six-day trial. The reason for this is unclear. It may be that the crabs were not receiving sufficient food; however, all of them did

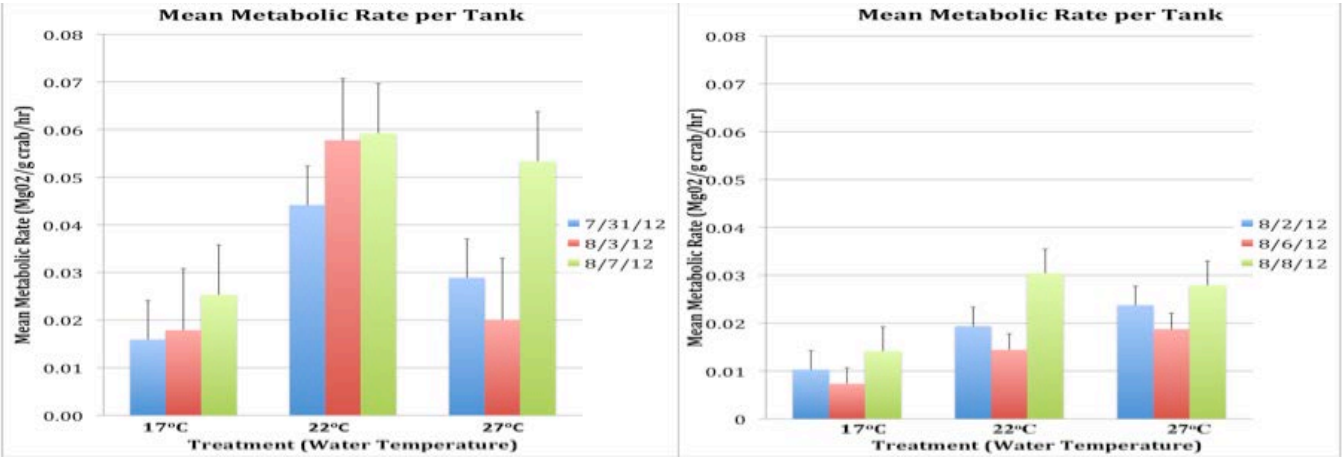

Figure 5. Mean Metabolic Rate of Larvae (Right) and Mean Metabolic Rate of Stage 1 (Left)

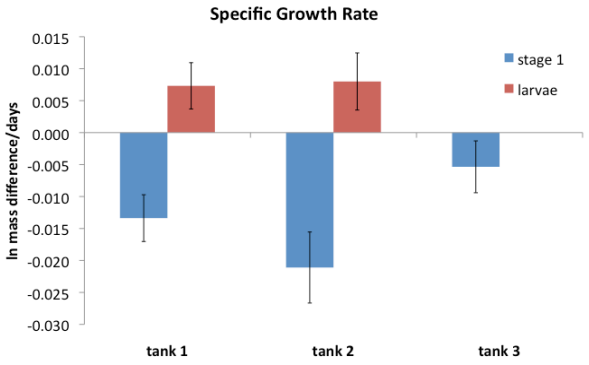

Figure 6. Specific Growth Rate of Both Stages

survive and molted into another stage. In an aquaculture/captive rearing setting, the warm conditions in Tank 3 would be preferred because both stages grew the fastest, with no mortality, and no apparent difficulty in maintaining metabolic rate.

Conclusions and Future Studies

For future studies, it would be valuable to track growth throughout all of the life stages, to see possibly whether the metabolic costs and growth rates vary in animals that are either newly molted or about to molt.

Growth and metabolic rates should also be studied in later stages to examine longer-term trends. Further, it would be valuable to try even higher temperatures in order to find the temperature threshold where growth and metabolic
rate is impaired. Horseshoe crabs can be found throughout the Gulf of Mexico and thus it is not surprising that they can tolerate, and even benefit from, higher temperatures. Paradoxically, increasing ocean temperatures due to climate change may in fact increase their northern range and could lead to an increase in population. However, increasing temperatures may also lead to existing southern habitats becoming too warm. Additionally, it is unknown if the increase in temperature could have an effect on food supplies or other elements of physiology.

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Biography

Martha Perez is currently a senior at the University of New Haven majoring in Marine Biology. She hopes to continue her education in graduate school and aspires to pursue a career in aquaculture.