THESIS APPROVAL SHEET

Title of Thesis: Growing Horseshoe Crabs for Conservation and Environmental Education

Name of Candidate: Jessica Baniak jbaniak1@umbc.edu

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Thesis and Abstract Approved:

Signed by:
Dr. J Sook Chung
32908D5B5BD64E7...

Dr. J Sook Chung

chung@umces.edu

Professor

Dept. Marine Biotechnology

4/17/2025 | 12:54 PM EDT

NOTE: *The Approval Sheet with the original signature must accompany the thesis or dissertation. No terminal punctuation is to be used.

Jessica Baniak

701 E Pratt St, Baltimore, MD 21202
(301) 538-4336 | jkbaniak@gmail.com

Education:

University of Maryland Baltimore County - Baltimore, MD
M.S., Marine-Estuarine Environmental Science (MEES), Cumulative GPA: 4.0,
Expected May 2025

University of Maryland Baltimore County - Baltimore, MD
B.S., Biological Sciences, Minor in Environmental Science.
Magna Cum Laude, Cumulative GPA: 3.85, May 2023

Relevant Experience:

Graduate Research Assistant, Institution of Marine and Environmental Technology (IMET), Baltimore, MD August 2023-Current.

- Modifying/Adapting aquaculture procedures for juvenile horseshoe crabs under differing conditions.
- Coordinating with the Maryland Department of Natural Resources for Horseshoe Crabs in the Classroom to create educational curriculum.
- Participating in multiple outreach activities including presentations and tabling events to spread awareness of research and encourage engagement with the marine sciences.

Aquaculture Intern, Morgan State Patuxent Environmental Aquatic Research Laboratory (PEARL), St. Leonard, MD, June 2022-August 2022.

- Maintained, counted, and cultured oysters as part of the aquaculture team working in the hatchery.
- Conducted research projects on heat tolerance in soft-shell clams and oyster anesthesia under Dr. Ming Liu.

Environmental Advocacy Intern, Maryland Pesticide Education Network, Remote September 2021-December 2021.

- Researched background knowledge, webpage design, interacted with government, private sector, and nonprofit organizations.

Volunteer:

Gaithersburg Student Union, Gaithersburg, MD, September 2018- May 2019.

- Engaged in community service projects for the City of Gaithersburg, visiting retirement homes, working at soup kitchens, attending town hall meetings, giving out holiday presents, etc.

Awards:

2025 Outstanding Graduate Student Award in Marine Biotechnology

Graduate Research Support:

2023 $60,161, Interdisciplinary Consortium for Applied Research in the Environment (ICARE) NSF Research Training Grant: 08/27/2023 - 05/21/2025.

2023 $7,000, Kansai Gaidai: Merit Scholarship.

2023 $1,000, Grad Alumni Scholarship.

Select Presentations and Outreach Events:

Professional Presentations:

Jessica Baniak. Growing Horseshoe Crabs for Conservation and Environmental Outreach. National Shellfish Association. March 6th-10th.

Jessica Baniak. Growing Horseshoe Crabs for Conservation and Environmental Outreach. MEES Colloquium. October 25th-26th, 2024.

Jessica Baniak. Horseshoe Crabs in the Classroom Training for the Maryland Department of Natural Resources. August 22nd, 2024.

Jessica Baniak. Growing Horseshoe Crabs for Conservation and Environmental Outreach. Green Eggs and Sand Training for the Maryland, Delaware, and New Jersey Departments of Natural Resources. June 8th-9th, 2024.

Jessica Baniak. Growing Horseshoe Crabs for Conservation and Environmental Outreach. CNMS Graduate Research Fest. April 12th, 2024.

Jessica Baniak. Horseshoe Crab Temperature Experiments for Conservation. MEES Colloquium. March 22nd-23rd, 2024.

Jessica Baniak. Horseshoe Crab Temperature Experiments for Conservation. ICARE Conavigator Event. February 23rd, 2024.

Jessica Baniak. Pushing the Limits: Heat Tolerance in Soft-Shell Clams. 2022 Chesapeake Watershed Forum. November 4th-6th, 2022.

Jessica Baniak. Pushing the Limits: Heat Tolerance in Soft-Shell Clams. PEARL

Intern Trainee Presentations, St. Leonard, MD. August 4th, 2022.

Jessica Baniak. The Peaceful Sleep: More Efficient Ways to Sedate Oysters for Sampling. PEARL Intern Trainee Presentations, St. Leonard, MD. August 4th, 2022.

Outreach Events:

Outreach with "Horseshoe Crabs in the Classroom" by the Maryland Department of Natural Resources. Assisted MDNR with 6-10 public schools across Maryland from 2023-2025. My roles included rewriting the directions for the program and assisting with a workshop for all teachers, visiting all the schools throughout the year to check in on the program, sending and analyzing surveys taken by the teachers about the program, and updating the program based on teacher feedback.

Women in STEM Event at Baltimore Highlands Elementary School. March 14th, 2025.

Presentation for Howard Community College Undergraduate Students at the Institute of Marine and Environmental Technology. February 27th, 2025.

Tabling for STEM Unbound at the Institute of Marine and Environmental Technology. October 24th, 2024.

Tabling for Urban Conservation Day at Masonville Cove. September 14th, 2024.

Horseshoe Crab Release Event for Horseshoe crabs in the Classroom at the MAEOE youth summit at Sandy Point State, MD. May 30th, 2024.

Tabling for Institute of Marine and Environmental Technology Open House. May 4th, 2024.

Manuscripts in Preparation:

1. Growing Horseshoe Crabs for Conservation and Environmental Education.
2. Literature Review of Culture Conditions for all Species of Horseshoe Crabs over the Past 40 years (1980-2025).
3. Updating/Adapting Growth Conditions for the Atlantic Horseshoe Crab Aquaculture: A Study in Temperature Effects.
4. Applied Research Case Study: Integrating Marine Organisms into Classroom Curriculum with Researcher Support.

Title of Document: GROWING HORSESHOE CRABS FOR CONSERVATION AND ENVIRONMENTAL EDUCATION

Jessica Kristen Baniak, M.S., 2025

Directed By: Professor, J. Sook Chung, Marine-Estuarine and Environmental Sciences

The Atlantic horseshoe crab, *Limulus poylphemus*, is classified as vulnerable by the International Union for Conservation of Nature (IUCN) due to overharvesting, habitat loss from coastal development, and climate change effects. Since horseshoe crabs have a 18–22 year lifespan and reach sexual maturity in approximately 10 years, conservation efforts have been focused on reintroductions programs that raise juveniles to be released in under a year. This research updates/adapts aquaculture procedures for *L. polyphemus* using temperature treatments to produce the largest horseshoe crabs most likely to survive in the wild. Furthermore, I am partnered with the Maryland Department of Natural Resources on a program called “Horseshoe Crabs in the Classroom” where we work with elementary, middle, and high school public teachers across the state of Maryland to raise horseshoe crabs in their respective schools. This collaboration integrates horseshoe crab aquaculture techniques into real-world programs that benefit the community.

GROWING HORSESHOE CRABS FOR CONSERVATION AND ENVIRONMENTAL EDUCATION

By

Jessica K. Baniak

Thesis submitted to the Faculty of the Graduate School of the University of Maryland, Baltimore County, in partial fulfillment of the requirements for the degree of

Master of Science

2025

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Chapter 1: Literature Review of Horseshoe Crab Culture Conditions over the Past Forty Years

1.1 Overview of Horseshoe Crabs

Horseshoe crabs belong to the order Xiphosura and the phylum Arthropoda. There are two subfamilies named Limulidae and Tachypleinae (Sekiguchi & Sugita, 1980). This historic creature has lived for approximately 450 million years, first appearing in the Devonian period of the Paleozoic era (Rudkin & Young, 2009). Over time, they have proven to be ideal creatures to study evolutionary stasis due to their fossil records displaying similar characteristics to modern forms with little morphological changes over their 200 million years on earth (Bicknell et al., 2022; Sekiguchi & Sugita, 1980).

Figure 1. ***Image of L. polyphemus.*** *This recently deceased horseshoe crab was grown from eggs provided by the Maryland Department of Natural Resources. (A) Top of the carapace. The body divisions and compound eyes are shown. (B) Underneath the carapace. Appendages, moth, and book*

gills are shown.

Horseshoe crabs are divided into three sections: the prosoma, the opisthosoma, and the telson (Figure 1). They have a primary set of compound eyes with accompanying simple eyes. They have six sets of appendages with five sets of legs and one set of chelicerae at the dorsal side. These chelicerae direct food into their mouths, located in the center of their body. Horseshoe crabs bend at the hinge and use their telsons (tails) to steer movement and right themselves when flipped. Their book gills are located above the telson and are used for breathing and propulsion swimming.

Horseshoe crabs experience sexual dimorphism with differences in size, genital pores, and walking legs. Females horseshoe crabs average a female-to-male size ratio of approximately 1.3, with the hypothesis that larger female size is due to fecundity advantages (Brockmann & Smith, 2009; M. D. Smith & Brockmann, 2014). This additional size is also caused by females undergoing one additional molt/year of growth compared to their male counterparts. The genital pores for males are firm and pointed, while female pores are broad and convex. The pedipalps, or first ambulatory legs, have different tips depending on male or female, with males having a 'boxing glove' or grasping appendage to hold onto the female during mating.

Horseshoe crabs are found worldwide with four species surviving to modern times. The species include *Limulus polyphemus, Carinoscorpius rotundicauda, Tachypleus gigas,* and *Tachypleus tridentatus*. The American Horseshoe crab, *L.*

polyphemus, inhabits the coast of North America from Maine to the Yucatan peninsula (S. A. Smith & Berkson, 2005). The latter three species are found in Southeast Asia from India to Japan, including the East Indies and the Philippines (John et al., 2018).

Tachypleus tridentatus, the tri-spine horseshoe crab, is found in the east and southeast coastal areas of Asia, including the coasts of southern Japan, China, Taiwan, northern Vietnam, the Philippines, and the islands of Borneo and Java (Vestbo et al., 2018). *Carinoscorpius rotundicauda*, the mangrove horseshoe crab, is found in south and southeast Asia, including the coasts of eastern India and Bangladesh, coasts of Malaysia, Thailand, Cambodia, and Southern Vietnam (Vestbo et al., 2018). *Tachypleus gigas*, the southern horseshoe crab, is found in similar habitats to *C. rotundicauda* in south and southeast Asia, including eastern India, the coasts of Malaysia, Thailand, the east coast of the Thai-Malay Peninsula, and southern Vietnam (Vestbo et al., 2018). While *T. gigas* and *C. rotundicauda* share similar ranges, *C. rotundicauda* are associated with mangroves and mudflats, while *T. gigas* inhabit sandier sediments on exposed beaches (Noor Jawahir et al., 2017).

1.2 Horseshoe Crab Life Cycle

Horseshoe crabs have an 18-22 year lifespan with reported environmental tolerance ranges of -5-35 °C and 5-30 ppt (S. A. Smith & Berkson, 2005). Horseshoe crabs are arthropods with growth categorized by molts and instar stages. Depending on the species, they reach sexual maturity in about 10 years and 16-17 instars for

L.polyphemus and 13-14 years or 17-18 instars for *T. tridentatus* (Gaines et al., 2002; Sekiguchi et al., 1988; S. A. Smith & Berkson, 2005; P. Xu et al., 2021).

Horseshoe crabs are integral to the ecosystem and have diverse connections to other species. For instance. *L. polyphemus* eggs serve as a source of nutrients at critical stopover locations for migratory shorebirds, like the Red Knot, on the Atlantic coast (Gillings et al., 2007). Furthermore, adult horseshoe crabs serve as bioturbators for the sediment-water nutrient exchanges (Liao et al., 2019; Shuster & Sekiguchi, 2009). Horseshoe crabs are considered keystone species in the Delaware Bay and serve many purposes that are often overlooked.

1.2a Spawning and Growth

Horseshoe crabs are external fertilizers, with one female laying eggs in the sand and competing males attempting to fertilize those eggs. Most horseshoe crabs inhabit estuarine habitats and coastal areas with a focus on 0.4 km^2 of the intertidal zone identified as nursery/spawning area (Ehlinger et al., 2003; Meilana et al., 2021). Three species: *L. polyphemus, T. gigas,* and *T. tridentatus* lay their eggs in sandy beaches, and *C. rotundicauda* lay their eggs in mangrove muddy sand areas (Fairuz-Fozi et al., 2018). Spawning typically occurs surrounding the new and full moons (M. D. Smith & Brockmann, 2014). Males that are younger and in better condition attach directly to a female horseshoe crab out at sea and stay paired until they reach the spawning habitat, while older males, known as satellite males, roam the shoreline for mating pairs (Brockmann, 2002).

Horseshoe crabs develop in different habitats throughout their lives. For instance, *L. polyphemus* develops within beach sediments for about three to four weeks, reaching the trilobite stage/ first instar larval stage. The second through fourth instar juveniles are typically found on tidal flats. Juveniles migrate to deeper waters by late summer/early fall (M. Botton et al., 2003). Adult horseshoe crabs live in the deeper ocean until they return to the beaches to spawn, although little is known about their exact whereabouts in the deeper ocean (Bopp et al., 2021).

That first summer serves as a pivotal time for the horseshoe crabs, with studies reporting only about 0.001% survival for *L. polyphemus* through the first summer (M. Botton et al., 2003; Carmichael et al., 2003; S. A. Smith & Berkson, 2005). While juveniles have a high tolerance of contaminants like heavy metals, oil, and organic compounds (M. L. Botton & Itow, 2009), their survival is dependent on the environment they are exposed to. Survival during these first few months highly depends on the temperature and salinity ranges of the juveniles' areas (M. Botton et al., 2003; M. L. Botton et al., 2006; Laughlin, 1983).

1.2b Feed

Horseshoe crabs are dietary generalists that feed on mollusks, arthropods, polychaetes, and organic materials (M. L. Botton, 2009; M. L. Botton & Haskin, H., 1984). As horseshoe crabs grow, their diet shifts, with the prey size being the most influential factor (Carmichael et al., 2009; Gaines et al., 2002). First instars live off the yolk from their eggs (Gaines et al., 2002). Instars two and three survive off

benthic and suspended matter. Instars five through eleven experience a shift in diet to polychaetes and more solid foods (Gaines et al., 2002). For older juveniles, mollusks comprise about ¼ of the diet (Gaines et al., 2002). One study found that bivalves contributed over 87% of the food items to their diet (M. L. Botton & Ropes, 1989).

1.3 Threats to Horseshoe Crabs: Harvesting, Industry, and Environmental Concerns

All species of horseshoe crab have been decreasing in number for several decades (Fakir Mohan University et al., 2021; D. R. Smith et al., 2023; P. Xu et al., 2021). One example depicts an almost 10-fold decrease in the density of *L. polyphemus* eggs between the 1980s and 2000s in New Jersey (M. Botton et al., 2003). The International Union for Conservation of Nature (IUCN) classifies *L. polyphemus* as vulnerable due to overharvesting, habitat loss from coastal development, and climate change effects (D. R. Smith et al., 2023). Among the other three species, *T. tridentus* is classified as endangered, and *T. gigas* and *C. rotundicauda* are listed as data deficient.

The IUCN creates extinction risk assessments on species of interest by appointed red-list authorities who are specialists in the species of interest. The ranking encompasses 9 categories from not evaluated to extinct. The vulnerable classification indicates that the species is at a high risk of extinction. The endangered classification is the next step up, being described as a species at a very high risk of

extinction. A data-deficient classification means that the IUCN has not collected enough data to classify the species into any category.

Across the species, common threats include biomedical harvesting, environmental pollution, and degradation of spawning grounds and habitat. Excluding the previously listed factors, *L. polyphemus* is specifically impacted by the commercial eel and whelk bait industry with a heightened threat to female crabs (Krisfalusi-Gannon et al., 2018). The other three species are affected by harvesting egg-bearing females for culinary purposes (Faridah et al., 2015; John et al., 2018; Sheikh et al., 2019; P. Xu et al., 2021). While Asian horseshoe crabs are not used in the bait industry (Faridah et al., 2015), there have been examples of the export of Asian horseshoe crabs to the United States for use in their bait industries (John et al., 2018).

1.3a Biomedical Harvesting

For the biomedical industry, *L. polyphemus* have been harvested from Delaware Bay for LAL production since 1982 (Kreamer & Michels, 2009). *T. tridentatus* has been harvested for TAL production since the 1980s (P. Xu et al., 2021). The amoebocyte collected from horseshoe crab hemolymph is used to create *Limulus* amoebocyte lysate (LAL) or *Tachypleus* amebocyte lysate (TAL), which creates assays approved by the FDA. These assays are sensitive to the detection of endotoxins produced by gram-negative bacteria (Gauvry, 2015; Tinker-Kulberg, Dellinger, Brady, Robertson, Levy, et al., 2020). For biomedical industries, most drug

testing requires the routine use of these assays (Piehler et al., 2020), which creates a high demand for horseshoe crab blood.

A steadily increased demand by biomedical industries for LAL and TAL poses a threat to horseshoe crab populations due to the mortality associated with the bleeding process. Atlantic States Marine Fisheries Commission (ASMFC) estimates approximately 15% bleeding-related mortality, which could be higher considering the potential death from trauma or fatigue after release (Anderson et al., 2013; Krisfalusi-Gannon et al., 2018). In the United States, there is a restriction of approximately 30% of the estimated blood volume of an individual horseshoe crab allowed to be extracted, leading to a preference for bleeding larger adult females (James-Pirri et al., 2012). For the Asian horseshoe crabs, there are instances of 100% mortality in China due to complete blood extraction from *T. tridentatus* and imported *T. gigas* from Vietnam (Gauvry, 2015). The current rate of mortality and demand for LAL and TAL are likely to reach unsustainable levels soon (Krisfalusi-Gannon et al., 2018). While there are alternatives to LAL and TAL assays, the use of horseshoe crab blood persists in many countries, including the United States.

1.3b Whelk and Eel Bait Industry

L. polyphemus have been mass-harvested since the 1800s. From 1850-1920s, it was reported by the ASMFC that approximately 1.5-2 million horseshoe crabs were harvested annually for use as fertilizer and livestock feed. However, as interest in horseshoe crab use for fertilizers declined, the industry shifted focus to horseshoe

crab harvest for use as bait for the American eel and whelk fisheries. While bait harvest continues, it was reported in 2022 that the coastwide bait landings were 570,988 compared to the coastwide quota of 1.59 million horseshoe crabs (Horseshoe Crab: commercial and recreational fisheries, 2025). Bait technology has advanced, and fewer horseshoe crabs are needed, but there are still concerns about overfishing by environmental groups (Berkson & Shuster, 1999).

The horseshoe crab industry is a special case as it is centered around specific locations and times of the year, leading to periods when they are especially vulnerable to over-exploitation. For instance, the majority of *L. polyphemus* harvest is conducted in the Delaware Bay region. It is known that artisanal invertebrate fisheries can lead to potential over-exploitation due to small-scale operations targeting patches of specific species and overharvesting from these locations (Defeo et al., 2009). Horseshoe crabs fall into this category as they are located in well-known patches during certain times of the year. Furthermore, even if fisheries are not targeting specific horseshoe crabs, the incidental mortality can affect those surrounding the target of interest.

1.3c Environmental Concerns

The loss of breeding habitat is occurring more frequently, negatively affecting all species of horseshoe crabs. For example, *L. polyphemus* have preferred breeding habitats on slightly sloped beaches, which are commonly used for real estate and development (Nordstrom, 2004). Sandy beach ecosystems experience increased

threats from smaller-scale anthropologic sources to overarching global problems. Sandy beaches and intertidal zones are essential for ecosystem services like the breakdown of organic materials and pollutants, water filtration, biodiversity maintenance, and a nursery for juvenile creatures (Defeo et al., 2009). With increasing human coastal populations, the amount of beach infrastructure and activities have become an increasing threat to these areas (Defeo et al., 2009). Urban infrastructure expansion into coastal regions has put a strain on the Asian horseshoe crabs, with certain areas like Japan and Peninsular Malaysia having their breeding habitats almost completely lost (M. L. Botton, 2002).

Furthermore, sandy beaches are also experiencing natural phenomena, like erosion, at elevated levels compared to the past. In 2018, 24% of sandy beaches worldwide eroded at rates exceeding 0.5 m/yr. This erosion targets the main spawning ground and essential habitat for juvenile horseshoe crabs. Furthermore, the majority of sandy shorelines in marine protected areas are eroding (Luijendijk et al., 2018). Even in the protected areas meant to combat anthropogenic challenges, there are still threats of erosion.

1.3d Fishery Management

With declining horseshoe crab populations, the horseshoe crab management board of ASMFC approved a horseshoe crab fishery management plan for *L. polyphemus* in 1988, currently in use in the United States. For *L. polyphemus*, conservation actions include harvest regulations, designating protected areas,

systematic monitoring, and educational awareness programs (D. R. Smith et al., 2023). However, Asian horseshoe crabs have fewer regulations due to the complexity of environmental priorities of different host countries in the horseshoe crab ranges (John et al., 2018).

The Asian horseshoe crab is legally protected in Bangladesh, Indonesia, Singapore, Vietnam, India, and specific provinces/regions in China and Japan. However, enforcement is questionable (John et al., 2018). There have also been instances of horseshoe crabs being importuned to North America to be used as bait, but this practice has been prohibited by the IUCN in 2013 (D. R. Smith et al., 2017).

Even with more management focus on *L. polyphemus,* there is still active advocacy for all species. For instance, the Ecological Research & Development Group (ERDG) and IUCN's horseshoe crabs Species Survival Commission (IUCN SSC) have an active role in all 4 species which could lead to favorable global regulation (John et al., 2018).

1.4 Why Focus on Horseshoe Crab Culturing

With all the attention on horseshoe crabs' dwindling numbers, solutions have been proposed to attempt to mitigate their decrease in the wild. Earlier horseshoe crab scientific studies focused on physiology, medical value, and ecological conservation, but a growing interest in aquaculture has created the modern-day focus on culture conditions for stock reestablishment (Luo et al., 2020; Sheikh et al., 2019; Tinker-

Kulberg, Dellinger, Brady, Robertson, Levy, et al., 2020).

While some believe that the rearing of horseshoe crabs is impractical due to their slow growth and lengthy onset of puberty, successful juvenile rearing programs have occurred in Japan, China, and Taiwan. Reintroduction programs take eggs from nests and raise them in more controlled environments for six to twelve months before releasing them into the wild. The added benefit of reintroduction programs is that the shorter time for animal growth allows educational programs to coincide with these experiences. These reintroduction programs allow for higher chances of survival for the horseshoe crabs while also offering curriculum possibilities.

However, culturing techniques for juvenile horseshoe crabs vary from place to place. Previous aquaculture studies focus on the optimal conditions for rearing, but differences arise. For instance, M.L. Botton and Itow (2009) found the optimal conditions for *L. polyphemus* to be 25-33 °C and 20-40ppt, while Smith and Berkson (2005) found that 15-21 °C and 27 ppt were optimal. A summary of defined sets of culturing techniques for all species of horseshoe crabs needs to be defined and used to optimize culturing efforts for the future.

1.5 Literature Review of Culture Conditions for the Past 40 Years

This section focuses on compiling themes from previous research on the culture conditions of the four horseshoe crab species.

Google Scholar and Web of Science were used to find journal articles, books,

book chapters, and early access published papers in English. No time limits were set during the search, and articles until January 2024 were considered. The keywords of "horseshoe crab," "aquaculture," "culture," "*Limulus polyphemus*," "*Tachypleus gigas*," "*Tachypleus tridentatus*", and "*Carcinoscorpius rotundicauda*" were used. Further articles in the focus area were found through literature references. Full articles that were accessible through the university (UMBC) or were open access were considered with a focus on compiling data that included parameters related to culture conditions for horseshoe crabs.

Overall, 40 studies were found including culture conditions for all species, with *L. polyphemus* and *T. tridentatus* being the most represented species. The most common studies focused on diet, culture setup, temperature, and salinity, with the juvenile life stage being the most observed.

Table 1. ***Articles by Species and Life Stage.*** *It is important to note that one study could contain information on more than one life stage.*

	Number of Articles	**Egg**	**Juvenile**	**Adult**
L. polyphemus	15	6	6	7
T. gigas	5	1	3	2
T. tridentatus	14	3	9	4
C. rotundicauda	6	1	3	2

The forty published articles describing culture conditions for the four species of horseshoe crabs are summarized (Table 1). There is more of a focus on *L.*

polyphemus (n=15) and *T. tridentatus* (n=14) compared to *C. rotundicauda* (n=6) and *T. gigas* (n=5). Of these studies, the species' distribution of life stages was relatively even except for *T. tridentatus*, which mainly focused on juveniles. Altogether, juveniles (n=21) were the most studied, followed by adults (n=15) and then eggs (n=11).

Table 2. ***Focus of Literature Studies.*** *The study focus was analyzed for all papers. Culture setup describes studies on aquaculture and husbandry conditions. The 'Other' category included immunology, spawning behavior, molting, and tidal cycle impacts on juveniles. One study could include multiple factors studied, so the number does not equal the total number of papers but the number of subjects mentioned for all studies.*

Study Focus	***L. polyphemus***	***T. gigas***	***T. tridentatus***	***C. rotundicauda***
Culture Setup	4	1	3	1
Diet	3	1	4	2
Temperature	3	1		1
Salinity	2	2		1
pH		1	1	1
pollutants/contaminants	1		3	
osmotic pressure	2			
bleeding	1	1		
morphology	2		1	
other			2	3

Experimental types for all species were examined (Table 2). The most common studies included diet (n=10), culture setup (n=9), temperature (n=5), salinity

(n=5), and other (n=5). The three most common conditions studied in *L. polyphemus* were culture setup, diet, and temperature. *T. gigas* had salinity as the most common, with everything else tied for 2nd. For *T. tridentatus*, it was diet, culture setup, and pollutants/contaminants. *C. rotundicauda* had other, diets, and everything else tied as their most common study focuses.

Table 3. ***Aquaculture Setup for L. polyphemus.*** *Studies detailing aquaculture setup for L. polyphemus. – indicates no data and * means no further information.*

Source	Culture Duration	Life Stage Focus	Enclosure Dimensions
(Schreibman & Zarnoch, 2009)	1.5 years	Egg and Juvenile	Egg: 300-500 eggs in 6-liter McDonald jar with flow 40 liter/min, Juvenile: 320L downweller with 1.05-2L/min, then moved to RAS tank with sand
(Laughlin, 1983)	-	Egg	Bowl*
(Ehlinger & Tankersley, 2004)	75 days	Egg, Juvenile, Adult	1.5cm diameter x 1.5cm depth (n=30), Adult: Fiberglass tank (2.7x1.7x1m)
(M. L. Botton et al., 2006)	-	Egg	Petri dish with 15mL water
(Greene et al., 2011)	-	Egg	Sand free glass finger bowls*
(Sekiguchi et al., 1988)	7 years	Juvenile	Egg: Glass bowl (6cm Diameterx3cm) (n=20) with sand Juvenile: largest container 36x25.5x10 cm for 14th instar
(Carmichael et al., 2009)	128 days	Juvenile	Reared 15x24 cm and 20x229 cm (n=13)
(Coates et al., 2012)	56 days	Adult (n=48)	closed circulation, 200L (n=3 per tank)
(Tinker-Kulberg, Dellinger, Brady, Robertson, Levy, et al., 2020)	12 months	Adult(n=24)	Pair of Ras, tanks (4 ft × 6 ft × 1 ft)

(Shinn et al., 2015)	2 months	Egg to 3rd Instar	Eggs: 6 L plastic tubs on a flow-through system, 150 mL min1 flow rate Juvenile: downweller (d=315 mm h= 150 mm), with 50 lm-400 lm mesh with 7 units per 200 L trough. Flow rate of 1 L min1.
(Tzafrir-Prag et al., 2010)	2 months	6–12-month-old	Eggs: McDonald jars for about 1 month Juveniles: RAS with downweller, then moved to Ras (227L) with sand substrate.
(Tuxbury et al., 2014)	3 years	Adult	3 different setups: Quarantine tank, Holding tank for educational program use, and Touch tank exhibit with gravel/crushed coral substrate. Rotation of 1 wk on-exhibit, 2 wk off-exhibit in holding tanks.
(Tinker-Kulberg, Dellinger, Gentit, et al., 2020)	6 months	Adult Horseshoe crabs (n = 64)	Indoor: Pair of RAS Outdoor: Tidelands pond pen culture system in a semi-tidal saltwater pond, including four outdoor PCS enclosures (15 ft long × 5 ft high × 5 ft wide) separated by 4 ft.
(Tinker-Kulberg, Dellinger, Brady, Robertson, Goddard, et al., 2020)	8 week feeding trials	Adult (n=24)	Recirculating aquaculture system with four holding tanks (40 × 6 0 × 1 0); a biofiltration tank, stocking density of 1.0-lb per square foot
(Leschen & Correia, 2010)	17 days	Adult (n=310)	Six flow-through seawater tanks (differing in volume) with 5 cm sediment

Table 4. ***Aquaculture Setup for C. rotundicauda.*** *Studies detailing aquaculture setup for C. rotundicauda. – indicates no data.*

Source	Culture Duration	Life Stage Focus	Enclosure Dimensions
(Wu et al., 2019)	2-week acclimation period	**Adult (n=12)**	-
(Lim et al., 2022)	18 months	1st-7th Instar	RAS system with sand/mud substrate.
(Hu et al., 2010)	7 weeks	Adult	RAS system with 12 tanks (50 cm x 40cm x 30cm, water volume: 20 L)

(Kuang et al., 2022)	8 weeks	Egg (n=150)	5 replicates of experimental setup: Acrylic tank (130cm ×30cm×30cm) with air and water pump and rectangular acrylic "experimental" box (15cm×15cm× 15cm) containing 9 cm sand substrate. Water pump connected to the reservoir to simulate the tidal cycles.
(Srijaya et al., 2014)	-	2nd instar (n=990)	Preparation tank of 50 L for acclimation and experiment
(Hu et al., 2018)	84 days	Juveniles with prosomal width of 14–17 mm, and sixth instar for weight	27 plastic tanks (25 cm x 15 cm x 10 cm)

Table 5. ***Aquaculture Setup for T. gigas.*** *Studies detailing aquaculture setup for T. gigas. – indicates no data. * means no further information.*

Source	**Culture Duration**	**Life Stage Focus**	**Enclosure Dimensions**
(Zaleha et al., 2011)	-	Egg and Juvenile	Triplicate 500 ml glass beaker container covered with parafilm.
(Sheikh et al., 2021)	5 months	Adult (n=18)	Aquarium 92" × 48" × 30" (L × W × D) with stocking density two pairs/square meters.
(Faizul et al., 2011)	6 months	6-11 month old (n=300)	Conventional: 3 rearing trays (n=50 each) Unconventional: 3 rearing trays connected to RAS system (n=50 each) with mechanical and biological filter and polyethylene tray.
(Razak & Kassim, 2018)	-	Adult (n=60)	Acclimation period: 6 round black tanks (n=5 each) with no substrate. Test enclosure: transparent aquarium (130×130×30 cm)
(Suniza et al., 2011)	About 80 days	2nd Instar	Five 5L acclimatization tanks*

Table 6. ***Aquaculture Setup for T. tridentatus studies.*** *Studies detailing aquaculture setup for T.*

*tridentatus. – indicates no data and * means no further information.*

Source	Culture Duration	Life Stage Focus	Enclosure Dimensions
(Y. Chen et al., 2010)	15.5 months	Egg to 9th Instar	Fiberglass tank (200x100x25 cm) with running water and coral sand
(Ying et al., 2022)	4 months	Adult	Two indoor cement ponds (4mX4mX0.8m) with sand substrate (n=10)
(Arif et al., 2022)	About 30 days	Juvenile with body length 0.622 cm ± 0.131 cm.	Glass Tanks*
(Liu et al., 2022)	1-2 months	1st Instar (n=720)	six glass tanks (30 cm × 20 cm × 15 cm), with 2 cm-thick fine sand.
(Kwan et al., 2015)	12 weeks	7th Instar (n=120)	15 indoor aquarium tanks (35 × 22 × 20 cm) with 4 cm sand layer
(C.-P. Chen et al., 2016)	150 days	1st and 2nd Instars	Two outdoor cement ponds with sand substrate, 2,365 m^2 and 2,150 m^2, 1.5 m height.
(Kwan et al., 2014)	12 weeks	7th Instar (n=96)	Twelve indoor aquarium tanks (35 × 22 × 20 cm), connected to a filter tank and water circulating system (flow rate: 1.5 l min−1)
(Wu et al., 2019)	2-week acclimation	Adult (n=12)	-
(Yamamichi & Sekiguchi, 1974)	4 months	Egg	hanging-drop, lens-paper and petri-dish methods for embryo culture.
(Hu et al., 2010)	7 weeks	Adult	RAS system containing 12 tanks (66 cm x 46 cm x 50 cm, and water volume: 60 L)
(Xiong et al., 2023)	60 days	1st Instar (n=600)	Glass tank (1.5 m × 0.7 m × 0.3 m) with a circulating aquaculture system containing six baskets (0.35 m × 0.2 m × 0.1 m) (n=100) with 2cm sand substrate.

(Z. Xu et al., 2020)	28 days	Adult (n=27)	3 replicates of nine circular circulating aquaculture tanks (diameter, 2 m; water depth, 0.9 m).
(Hu et al., 2018)	84 days	Juveniles with prosomal width of 14–17 mm, and fifth instar for weight	27 plastic tanks (25 cm x 15 cm x 10 cm)
(Sekiguchi et al., 1988)	7 years	Egg to 10^{th} Instar	Cultured in bowls (8.5 cm diameter and 4.5 cm depth) (n=20) then reared individually after 1st post hatch in containers with sand substrate varying in size with smallest (34x34x30 mm deep).

The culture conditions for all horseshoe crab species were detailed (Table 3, Table 4, Table 5, Table 6). The study durations varied from the shortest of 2 weeks to the longest of 7 years, with an overall average for all species around 250.27 ± 419.71 days, with the outlier of 7 years influencing this value. For each species, the average duration of study/ study length was *L. polyphemus* (n=12) 433.25 ± 737.12, *T. gigas* (n=3) 138.17 ± 53.63 days, *T. tridentatus* (n=14) 279.55 ± 664.55, and *C. rotundicauda* (n=5) 150.1 ± 223.55 days.

For enclosure design, Tables three through six show how each system was unique in animal density and design. Shared similarities included substrate presence and RAS/flow-through systems. Out of all the species, sand/gravel substrate was specifically mentioned in 14 out of the 40 articles with *T. tridentatus* (n=7), *L. polyphemus* (n=5), and *C. rotundicauda* (n=2). RAS or flow-through systems were mentioned in 16 out of the 40 articles, with *T. tridentatus* (n=5), *L. polyphemus* (n=8),

T. gigas (n=1), and *C. rotundicauda* (n=2).

Table 7. ***Water Maintenance for All Species.*** *Water Maintenance intervals for all species and the number of articles that report this setting.*

Water Change Frequency	***L. polyphemus***	***T. gigas***	***T. tridentatus***	***C. rotundicauda***
Daily	1	2	4	1
Every other day	2	1	1	
every 3 days			1	
3x per week	1	1		
5x per week	2			
every week	5		2	
every 2 weeks	1			
2x monthly			1	
RAS/Flowthrough	10	1	5	2

The water maintenance was reported across all species, with the most common water changes/setups being RAS/flow-through systems (n=18), daily water changes (n=8), every week water changes (n=7), and every other day water changes (n=4) (Table 7). The most common for *L. polyphemus* was RAS/flow-through, weekly water changes, every other day, and 5x per week. For *T. gigas*, daily was most common. For *T. tridentatus*, RAS/flowthrough was the most common, followed by daily. For *C. rotundicauda*, the most common water maintenance was RAS, followed by daily water changes.

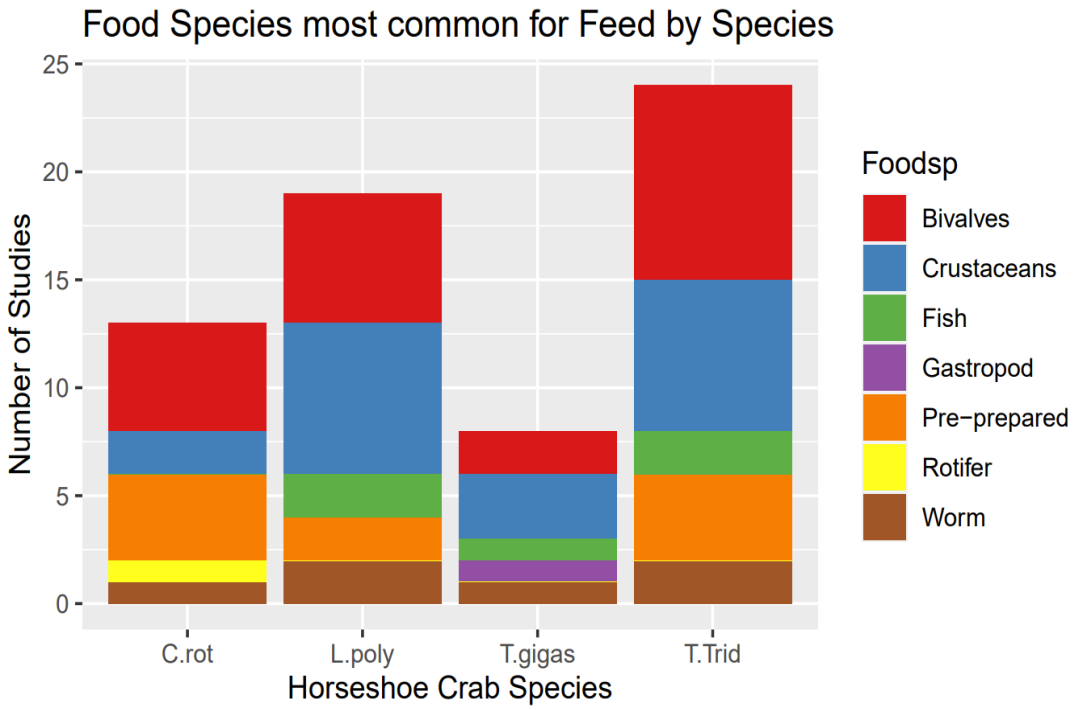

Figure 2. ***Food Types Most Commonly Used By Species.*** Pre-prepared meals include fish meal, blood meal, meat and bone meal, poultry by-products, and a gelatin mixture (Tinker-Kulberg, Dellinger, Brady, Robertson, Levy, et al., 2020). The bivalves included clams, mussels, and oysters. The crustaceans included artemia, shrimp, prawn, krill, crab, and copepods.

The most common food types for horseshoe crab culture were bivalves (n=22), crustaceans (n=19), and pre-prepared meals (n=10) (Figure 2). Overall, 18 different types of food were grouped into seven categories. The bivalves included clams (n=16), mussels (n=4), and oysters (n=1). For crustaceans, the species included artemia (n=10), shrimp (n=5), prawn (n=1), copepod (n=1), krill (n=1), and crab (n=1). The pre-prepared meals included standard meals formulated with specific concentrations before feeding the horseshoe crabs. These included fish meal (n=2), blood meal (n=2), meat and bone meal (n=2), poultry by-product (n=2), and a gelatin

mixture.

Of all food types, clams, artemia, and worms were the most used across all horseshoe crab species. Of the 18 food types, *C. rotundicauda* and *T. Tridentatus* included 11, *L. polyphemus* included 9 and *T. gigas* included 7. For *L. polyphemus*, the top 3 most common food types were artemia, clams, and mussels. For *T. gigas*, clams were the most common. For *T. tridentatus*, clams and artemia were the most common. For *C. rotundicauda*, clams were the most common.

For the temperature and salinity, the combined average temperature range for all species was 23.80 ± 4.44 °C to 28.031 ± 4.22 °C. The combined average salinity range was 25.07 ± 7.10 ppt to 31.63 ± 4.78 ppt. However, this data includes the five studies testing temperature and salinity, which skews the data. Temperature studies included *L. polyphemus* (n=3), *T. gigas* (n=1), and *C. rotundicauda* (n=1). Salinity studies included *L. polyphemus* (n=2), *T. gigas* (n=2), and *C. rotundicauda* (n=1). Without the studies testing these factors, the new temperature range was 24.6 to 26.6 °C, and the salinity range was 28.2 to 30.3 ppt. *L. polyphemus* has an average temperature range of 20.1 to 21.6 °C and salinity of 25.2 to 27.6 ppt, *C. rotundicauda* had a temperature range of 26.4 to 28.8 °C and salinity of 29 to 30.2 ppt, *T. gigas* 26 to 28 °C and 28.5 to 31.5 ppt, and *T. tridentatus* 26 to 28 °C and 30 to 32 ppt.

The ideal range of temperature and salinity across all species was 24.6-26.6 °C and 28.2-30.3 ppt, reflecting warmer temperatures and salinities slightly lower than seawater. Enclosures were not standardized but had commonalities like filtration

and substrate. Feed mimicked reality, with bivalves and crustaceans being the most popular food categories, specifically artemia and clams.

1.6 Summary of Literature Review

1.6a Study Focuses

In the papers analyzed, it was found that there was an almost even distribution of interest across all life stages for all species. Juveniles (n=21) had the highest focus across all species. This interest in juveniles could be due to the importance of this life stage and the numerous factors that affect them. *T. tridentatus* (n=9) had the most representation from the juvenile-focused papers. This could be due to the reintroduction programs currently in progress for this species (M. L. Botton et al., 2022). The egg stage (n=11) had the least studies, potentially due to fewer variables affecting them than other life stages. In the adult stage (n=15), half the papers focused on *L. polyphemus* alone.

The most common study focuses were on diet, culture setup conditions, temperature, and salinity. These focuses all play a role in the culturing. Other than *C. rotundicauda*, the other species followed this pattern of diet, culture setup, temperature, and salinity being the most frequently studied factors. This is expected as they all relate to culturing techniques.

1.6b Culture Duration

The overall mean study duration for all species was 250 days (approximately 8 months) with a minimum of 14 days and a maximum of 2555 days (7 years). This mean exacerbated the length, when in reality, approximately 79% of all culture durations were under 6 months, and approximately 59% of the study culture durations were under 3 months. The median of 98 days better represents culture duration across all species. These three to six months of study focus coincide with the potential improvement of culture conditions for juvenile horseshoe crab re-introduction programs.

L. polyphemus and *T. tridentatus* had the highest culture durations due to three studies over 1 year for *L. polyphemus* and two studies over 1 year for *T. tridentatus*. Both species had more articles represented, which could indicate a correlation between the number of studies and funding for long-term studies. Of the studies longer than 6 months, the breakdown of life stages were eggs (n=1), juveniles (n=6), and adults (n=3). The longer studies focused on culture conditions, with seven out of eight focusing on aquaculture improvement.

1.6c Temperature and Salinity Conditions

For the temperature and salinity, there was an overall range of 24.6 to 26.6 °C and 28.2 to 30.3 ppt for all species. There was a marked difference between *L. polyphemus* and the Asian Horseshoe crabs, with *L. polyphemus* having average ranges of 20.1 to 21.6 °C and 25.2 to 27.6 ppt compared to the average of the Asian

horseshoe crab species of 26.1 to 28.3 °C and 29.2 to 31.2 ppt.

Temperature is a barrier in the global horseshoe crab distribution (Shuster & Sekiguchi, 2009) which could reflect the difference in temperature and salinity conditions by species. Meilana et al. (2021) stated that the temperature for the juvenile Asian horseshoe crab had a range of 28.1-34.7 °C and 30-40 ppt, which corresponds to the higher temperature range found for this species compared to *L. polyphemus*.

1.6d Enclosure and Water Maintenance

Enclosure and water maintenance go hand in hand with large systems requiring less frequent water changes and smaller, short-term experiments requiring daily water changes. Aeration or flowing movement is essential. P. Xu et al. (2021) detail how continuous air pumping, especially for eggs, within the culturing containment provides more DO and agitates the eggs to avoid mass infections. While only a few studies reported the use of aeration, there is merit in this practice when there is no flowthrough system in place.

Substrate is another important factor for enclosures as adult horseshoe crabs are benthic. 35% of the studies mentioned a substrate in some life stage. While substrate mimics the natural environment more thoroughly, water changes, and maintaining a clean environment is more difficult with substrate. It is recommended that substrate be included for experiments with later-stage juveniles and adults who are more stable. For short-term juvenile studies, it is recommended that substrate be

omitted as it could create problems with water changes.

The most common methods for water exchanges were using a RAS/Flow-through system, daily water changes, and one water change every week. More extensive systems that house many horseshoe crabs are more likely to need less frequent changes, while the shorter-term studies with no aeration need to be changed daily. It is recommended that 25-30% of the water should be changed every 3-4 weeks to reduce the amount of accumulated nitrates (S. A. Smith & Berkson, 2005). Tinker-Kulberg, Dellinger, Gentit, et al. (2020) found that indoor RAS systems allowed adults to have higher rebound kinetics with respect to LAL reactivity and 100% survival, while outdoor systems had a 15% mortality rate. While daily water exchanges are common for shorter experiments, a flowthrough system could reduce labor and create a more stable environment to support further culturing projects.

1.6e Feed

Concerning feed, wild horseshoe crabs have many food sources of dead fish, algae, mollusks, worms, bivalves, polychaetes, crustaceans, etc., which cannot be replicated fully in captive populations with the limit of food sources available (M. L. Botton & Haskin, H., 1984; M. L. Botton & Ropes, 1989; Friel et al., 2020). Bivalves are argued to be the primary food source, especially for adults, whereas the juveniles eat smaller benthic polychaetes, amphipods, and isopods (M. L. Botton & Haskin, H., 1984; M. L. Botton & Ropes, 1989; Gaines et al., 2002; Lee et al., 2021).

The diet distribution found in this study reflects real-world food sources, with

bivalves and crustaceans being the top two feed categories. However, the third most common diet was the pre-prepared options. While pre-prepared diets offer a more controlled ratio of food, frozen individual species were preferred. Clams, artemia, and worms were the most common feed for horseshoe crab species.

While crustaceans are mentioned in the natural wild diet, artemia are not. However, artemia is a food source well established as fish feed and is easily accessible for facilities, which could account for its popularity for horseshoe crabs (Madkour et al., 2023). Artemia can be used for early life stages as they grow and switch to a combination of crustaceans and clams for a more realistic diet. Pre-prepared meals could be utilized to make the perfect combination of these species, but more research is needed.

1.6f Summary of Literature Review

Horseshoe crab studies in the past 30 years have centered around physiological characteristics, medical value, and ecological conservation (Luo et al., 2020). The most common application of horseshoe crab aquaculture has been framed as stock enhancement by releasing juveniles into the wild (Schreibman & Zarnoch, 2009). Successful rearing programs have been established in Japan and China (M. L. Botton et al., 2022), with studies finding that size could be a leading determination of release success, with hatchery-bred juveniles at their 6^{th} instar stage more likely to survive (P. Xu et al., 2021). More standardization in procedures is needed to implement these types of programs for *L. polyphemus*.

1.7 Research Objectives

Further research needs to be conducted on standardizing enclosure/environment types with conditions optimized for aquaculture in captivity. In the past, most researchers have used their facility's equipment rather than trying to create a standardized, long-lasting setup. Good examples of long-lasting culturing setups were from the multiyear studies described in Sekiguchi et al. (1988) and Schreibman & Zarnoch (2009). It is helpful to have a structured outline for each life stage, as there are different requirements for each life stage. More multi-stage projects should be conducted to standardize optimal growth conditions.

Aquaculture technology, especially optimizing culture conditions and spawning in captivity, has been employed to produce seafood for many economically valuable species and conserve endangered species. With a recent decline in the horseshoe crab population, this research aims to optimize growth conditions with a focus on temperature and its effect on growth and survivorship for *L. polyphemus* in culture conditions.

Furthermore, this research aims to expand on the temperature experiments by working with outside organizations to create horseshoe crabs-related educational curricula that coincide with reintroduction programs. Educational programs like the ones described later in the thesis bring interest to the species while allowing students to better understand scientific practices related to animal husbandry.

Therefore, the main research questions for this thesis include:

1. Does temperature accelerate the growth/molting interval of *L. polyphemus*?
2. Does temperature influence the survival rate of *L. polyphemus*?
3. Can experimental setup and practices for horseshoe crabs be translated into an educational setting?
4. How do environmental education programs like this impact the students and teachers in the program?

Chapter two will include findings from three temperature experiments on the effect of *L. polyphemus* growth and survivorship. Chapter three will be centered around the impacts of applied research programs and their success in supporting educational programs, specifically the program "Horseshoe Crabs in the Classroom." Finally, the last chapter will summarize the scholarship that motivated this research while also exploring future directions and key takeaways from this thesis.

Chapter 2: Horseshoe Crab Temperature Experiments

2.1 Background

2.1a Chapter Overview

Four species of horseshoe crabs (Chelicerata: Limulidae) are present globally: *Limulus polyphemus, Carinoscorpius rotundicauda, Tachypleus gigas,* and *Tachypleus tridentatus*. The Atlantic horseshoe crab, *L. polyphemus*, inhabits the coast of North America from Maine to the Yucatan peninsula (S. A. Smith & Berkson, 2005). The latter three species are found in Southeast Asia from India to Japan, including the East Indies and the Philippines (John et al., 2018). Molts and instar stages classify all horseshoe crabs' growth. Specifically, *L. polyphemus* reaches sexual maturity in about 10 years with 16-17 instar stages (Gaines et al., 2002; Sekiguchi et al., 1988; S. A. Smith & Berkson, 2005) Horseshoe crabs have an approximately 18-22 years lifespan with reported tolerance ranges of -5-35 °C (temperature) and 5-30 ppt (salinity) (S. A. Smith & Berkson, 2005).

Horseshoe crabs have three main life stages of eggs, juveniles, and adults. In ideal conditions, eggs take roughly one month to develop, juveniles take approximately 10 years, and adults can live greater than 10 years before death. The beginning months of life are crucial to development with horseshoe crabs undergoing numerous molts compared to the older juveniles that only molt once per year. This initial period of growth and molting activity makes the initial stages of eggs and

younger juveniles more appealing to study by researchers. Furthermore, the time to reach trilobite stage, also known as the first instar, is influenced by environmental factors like temperature, salinity, dissolved oxygen (DO), and the presence of pollutants (M. L. Botton et al., 2010). The factors experienced in the eggs stage and early juvenile can potentially be the most impactful in a horseshoe crab's development out of any life stage.

This chapter describes three temperature experiments run on *L. polyphemus* starting at different life stages to test the development and survivorship affected by these conditions.

2.1b Growth and Egg Development

L. polyphemus lay their eggs on sandy beaches. The sediment in which a horseshoe crab is born impacts their growth. Sediment temperature, amount of oxygen, contaminants, etc., all affect the time to hatch and survivorship (Vasquez et al., 2015). For instance, one study found that the sediment temperatures in different regions of the beach (high tide vs low tide areas) affected survivorship with high tidal areas exposed to 35 °C and more oxygen resulting in an 80-90% developmental success compared to the lower tidal zones with lower temperatures and less oxygen, resulting in a developmental success of 25% less than the control condition eggs (Vasquez et al., 2015).

Eggs are expected to hatch within two to four weeks after fertilization, with two months being the standard cutoff for when they will no longer hatch (Kendrick et

al., 2021).

Figure 3. ***Freshly Retrieved and Washed Day-Old Horseshoe Crab Eggs****. These eggs were retrieved from Ocean City, MD from actively spawning horseshoe crabs. They were separated and rinsed with filtered water before being counted and divided into experiments.*

The most recent classification of horseshoe crab egg development divides the egg's growth into five stages, ranging from A-E, followed by the 1st instar stage after they have hatched. Horseshoe crab eggs start as unfertilized eggs that are greenish blue/grey, with a select few being pink/orange (**Error! Reference source not f ound.**). These eggs are covered in an adhesive material that allows them to stick to the eggs surrounding them and to sand grains.

After fertilization, eggs move into stages A and B. These stages are relatively hard to distinguish from each other due to their similar appearance. Stage A is the first stage after fertilization where the egg still exhibits mostly of yolk with minimal free space surrounding the embryo. Stage B exhibits a small clear space surrounding the yolk due to the germ disk expansion. Limb buds begin to form from the continued

differentiation of the germ disks, causing slight rises in the main embryo mass. Stage C is characterized by the rudimentary prosomatic appendages elongating and becoming more recognizable while more space surrounds the yolk (M. L. Botton et al., 2010). Stage D is classified by the rupture of the chorion, which causes two semicircles of egg material at the top of the embryo. Stage D is the time when the eggs are first able to be classified as embryos (M. L. Botton et al., 2010). Stage D is the transformative stage where the legs have unfolded, the shell buds from the central egg, and the full body of the embryo is visible. The final stage of development is stage E, where the horseshoe crab's body is visible, the egg is much larger than before, and the horseshoe crabs can move around in the clear inner egg membrane. The legs are fully segmented, and the book gills are developing rapidly. Stage E embryos are nearly double in diameter compared to unfertilized eggs (M. L. Botton et al., 2010). Their compound eyes are visible, and the horseshoe crabs resemble their adult counterparts.

Horseshoe crab eggs hatch after Stage E, resulting in what some refer to as instar one, or the trilobite larval stage. These trilobite larvae emerge from beach sediments, where they have a brief planktonic period before settling to the benthos and molting to instar two. Instar two is when the telson forms and they become sedimentary for the rest of their lives (M. L. Botton & Loveland, 2003). The fourth instar is usually reached by the end of their first summer when they move to deeper waters away from the beach they hatched in (M. L. Botton & Loveland, 2003).

A couple of size indicators and guides are used to determine the instar stages of horseshoe crabs. One such example is provided by (Sekiguchi et al., 1988) where they outline the sizes of multiple body parts of the horseshoe crabs in their experiments. However, even with guidelines provided, these are not as reliable with variations in sizing, making classification based on instar size difficult. It is also challenging to correctly size the live smaller horseshoe crabs to the mm of accuracy that is needed for correct assessment. To accurately track a base line for the horseshoe crabs requires sizing them using previous size guides and tracking the next egg/molting size to ensure that the previous estimate was correct.

2.1c Why Focus on Temperature

This chapter is centered around temperature's effects on the growth and survivorship of egg/juvenile horseshoe crabs. Temperature was chosen because it was one of the more studied factors involving *L. polyphemus, as* detailed in the previous chapter's literature review. Furthermore, in the study that found that eggs developed at a more successful rate in high tide vs lower tide, they concluded that the site success was heavily influenced by avoiding areas of hypoxia and hydrogen sulfide, but there was also success in areas with higher temperatures (Vasquez et al., 2015). They found survival rates at temperatures as high as 35 °C led to success, which is on the edge of the reported temperature range for *L. polyphemus* of -5-35 °C (S. A. Smith & Berkson, 2005). Temperature could play an integral role as a key factor in speeding up growth and affecting survivorship. In the wild, it has been seen that eggs

laid in a marsh, which has lower temperatures and higher levels of anoxic conditions, influenced the hatching rate by potentially slowing egg growth and causing an increase in discolored eggs (Kendrick et al., 2021).

The influence of temperature on growth and survival motivated the idea of using temperature to produce larger juveniles in a shorter time. If successful, the time necessary to raise juveniles to adulthood can be greatly reduced while also balancing a steady survivorship.

2.1d Previous Juvenile Aquaculture

The most common application of horseshoe crab aquaculture has been framed as stock enhancement by releasing juveniles into the wild (Schreibman & Zarnoch, 2009). Successful rearing programs have been established in Japan and China (M. L. Botton et al., 2022), with studies finding that size could be a leading determination of release success, with hatchery-bred juveniles at their 6^{th} instar stage more likely to survive (P. Xu et al., 2021).

Chapter one's literature review found that aquaculture studies of *L. polyphemus* in the last 40 years have used a range of conditions with a focus on having average temperature ranges of 20.1-21.6 °C and 25.2-27.6 ppt. Aeration or flowing movement within the culturing containment is essential, with P. Xu et al. (2021) detailing how continuous air pumping , especially for eggs, provides more DO and agitates the eggs to avoid mass infections. While these substrates mimic the natural environment more thoroughly, water changes, and maintaining a clean

environment is more difficult with substrate. It is recommended that substrate be included for experiments with later-stage juveniles and adults who are more stable. For short-term juvenile studies, it is recommended that substrate be omitted as it could create problems with water changes. It is recommended that 25-30% of the water should be changed every three to four weeks to reduce the amount of accumulated nitrates (S. A. Smith & Berkson, 2005)

In relation to feed, wild horseshoe crabs have many food sources (*e.g.* dead fish, algae, mollusks, worms, bivalves, polychaetas, and crustaceans), which cannot be replicated fully in captive populations with the limit of food sources available (M. L. Botton & Haskin, H., 1984; M. L. Botton & Ropes, 1989; Friel et al., 2020). Chapter one's literature review found that clams, artemia, and worms were the most common feed across all horseshoe crab species.

Using the findings from previous experimental studies describes in chapter one's literature review, the ideal experimental setups for less than a year include tanks with aeration, no substrate on the bottom, frequent water changes, a salinity around 30 ppt, and a feed of artemia. Since temperature was the experiment's focus, three temperature groups were chosen: Room Temperature (RT) from 20-22 °C was the control, Medium Temperature (MT) from 23-25 °C, and High Temperature (HT) from 28-30 °C.

2.2 Experimental Design

2.2a General Timeline of All Research Projects

Three research experiments were conducted from the Summer of 2023 to the Winter of 2025. Two six-month long-term experiments occurred from summer to winter in 2023-2024 and 2024-2025. A shorter two-month experiment focusing on eggs occurred in the summer of 2024. These experiments tested the effect of three set temperatures on growth and survivorship. The experimental temperatures were Room Temperature (RT) from 20-22 °C, Medium Temperature (MT) from 23-25 °C, and High Temperature (HT) from 28-30 °C. The longer experiments included exposure to higher temperatures from the starting stages of eggs and instar 2. The shorter experiment focused on egg growth and the progression of egg stages over 6 weeks. For both years, eggs were received in June from Ocean City, MD, with help from the Maryland Department of Natural Resources.

2.2b Egg Experiment

This study lasted 6 weeks from receiving the eggs. This study was designed to test how temperature might affect early-stage eggs. Eggs were obtained from Ocean City, MD, on Thursday, June 20th, 2024. The eggs were cleaned twice in filtered 30 ppt water and sorted from external debris in the following days. The experiment started on Monday, June 24th after eggs were sorted into stages A and B, the earliest stages of egg development. Official eggs for the experiment were determined by

Wed, June 26th.

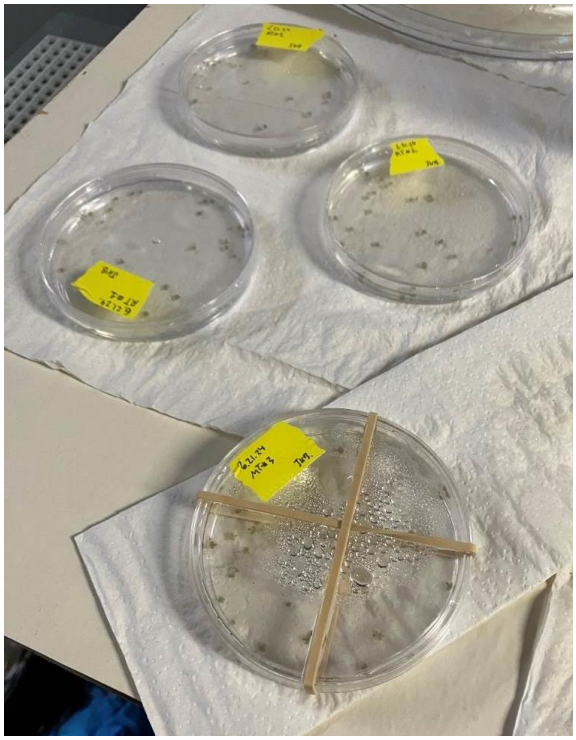

Figure 4. ***Setup for Egg Experiment****. Three room-temperature egg setups are shown. One medium-temperature petri dish is shown wrapped in rubber bands.*

Three Petri dishes (9 cm diameter) were used for each of the three temperature treatments. Each dish contained 30 eggs starting in the earliest egg stages of A and B totaling 90 horseshoe crabs in each temperature treatment. The Petri dishes contained about 1 cm depth of 30 ppt filtered water.

The Petri dishes had a diameter of 9 cm, resulting in a surface area of 63.6 cm^2 and a volume of 63.6 cm^3 when filled up 1 cm. This gave about 2.1 cm^2 of space on the bottom for each horseshoe crab and 2.1 cm^3 of water. The Petri dishes were placed in a heated water bath to achieve the desired temperatures for the MT and HT

treatments (Figure 4). The petri dishes were secured by rubber bands and floated in a heated water bath.

For the egg-to-two-month experiment, since they were mostly eggs, they did not receive food until instar 2 was reached toward the end of the experiment. Instar 1 lives off of the nutrients from their yolk and do not require feeding. These eggs were stored in Petri dishes and only required water changes when a buildup of external particles like leftover sand or shed egg reached a higher amount. The instar 2 horseshoe crabs were fed a limited amount of diluted solution of baby brine shrimp from a pipette. This amount would then be cleaned out on a Monday, Wednesday, Friday (MWF) basis when routine water changes were conducted. Upkeep included routine cleaning and counting every MWF. Cleaning included removing debris like discarded eggshells and sand/dirt from the transfer. Even though no aeration was provided for the Petri dishes, the eggs and beginning instars were moved around every MWF when stage sizes were assessed. This allowed enough movement for almost no mold to grow on the experimental eggs.

2.2c Six-Month Temperature Experiment from Eggs (2024-2025)

Eggs were obtained from Ocean City, MD, on Thursday, June 20th, 2024. The eggs were cleaned twice in filtered 30 ppt water and sorted from external debris in the following days. Eggs were sorted on Monday, June 24th, to choose eggs in stages A and B for the experiment. Three replicate procedures were followed for the temperature of Room Temperature (RT) from 20-22 °C, Medium Temperature (MT)

from 23-25 °C, and High Temperature (HT) from 28-30 °C. As the horseshoe crabs grew, their containment equipment also adapted with incremental size increases, and aeration and filtration equipment improved with each stage (Figure 5).

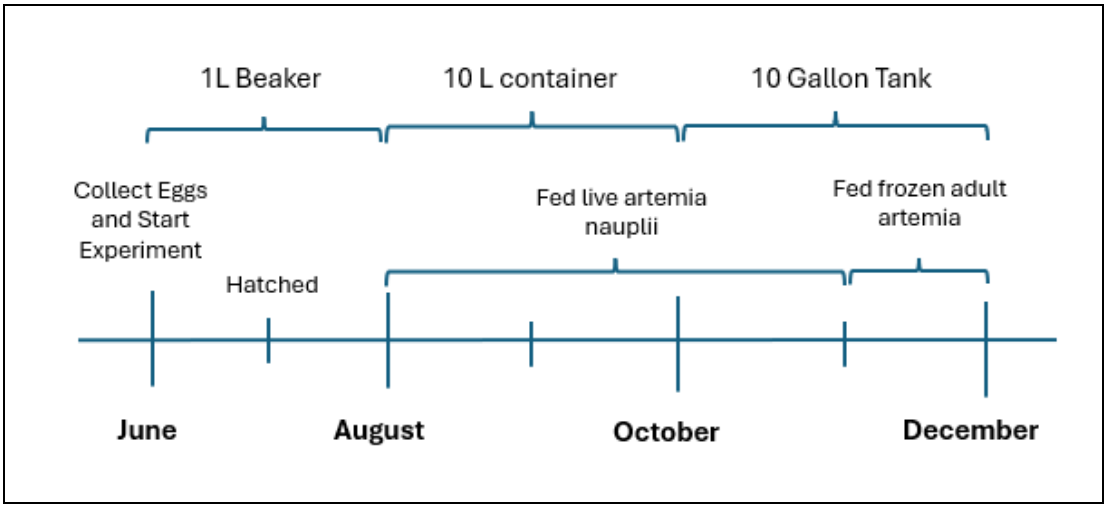

Figure 5. ***Timeline of Temperature Experiment from Egg.*** *Detailed is the experimental progress from collecting eggs in June 2024 to housing them in 1 L containers, then 10 L, and then 10-gallon tanks.*

Roughly 100 eggs were chosen for each treatment in the A and B egg stage. RT included 101 eggs, MT included 102, and HT included 100 eggs. The eggs were placed in 1 L cylindrical beakers with 30 ppt water from June 2024 to August 2024 (Figure 6). When eggs started to hatch, aeration was added to the 1 L containers. The 1 L beaker had a diameter of 10 cm and a height of 10 cm. This gave a total surface area of 78.5 cm^2 and a total volume of 785.4 cm^3. For each horseshoe crab, they had approximately 0.79 cm^2 of space on the bottom and approximately 7.9 cm^3 of water. This container was for eggs and early-stage juveniles < 2 cm long. This container included instar 1, the trilobite stage, where they are more free swimming than the

later bottom-dwelling stages.

After most eggs had hatched, all eggs/juveniles were moved to cylindrical 10 L containers in August 2024. The 10 L containers had aeration and no substrate. The 10 L containers were placed in a heated water bath for MT and HT to maintain the appropriate temperatures. The 10 L cylindrical containers had a diameter of 26 cm and a height of 15 cm. This resulted in these 10 L containers having a total surface area of 530.9 cm^2 and a volume of 7963.9 cm^3. The initial amounts of horseshoe crabs added to these containers were RT = 94, MT = 93, and HT = 94. For each horseshoe crab, there was about 5.6 cm^2 of space on the bottom and 84.7 cm^3 of volume above. This space quickly became limiting for the faster-growing HT.

After October 2024, the juveniles were moved from the 10 L containers to 10-gallon tanks. These tanks included bubble air filters for all tanks and heaters for MT and HT treatments. The 10-gallon tanks had a length of 51.435 cm, width of 26.67 cm, and height of 24.05 cm. The total surface area for one tank equals 1371.8 cm^2 and a total volume of 32,991.1 cm^3. The initial amount of horseshoe crabs added to this setup from each temperature was RT = 76, MT = 72, and HT = 44. At the start of the 10-gallon tanks, the space available for one horseshoe crab for RT = 18 cm^2, MT = 19.1 cm^2, and HT = 31.2 cm^2. The volume available for each horseshoe crab at the start of the 10-gallon tanks was RT = 434.1 cm^3, MT = 458.2 cm^3, and HT = 749.8 cm^3.

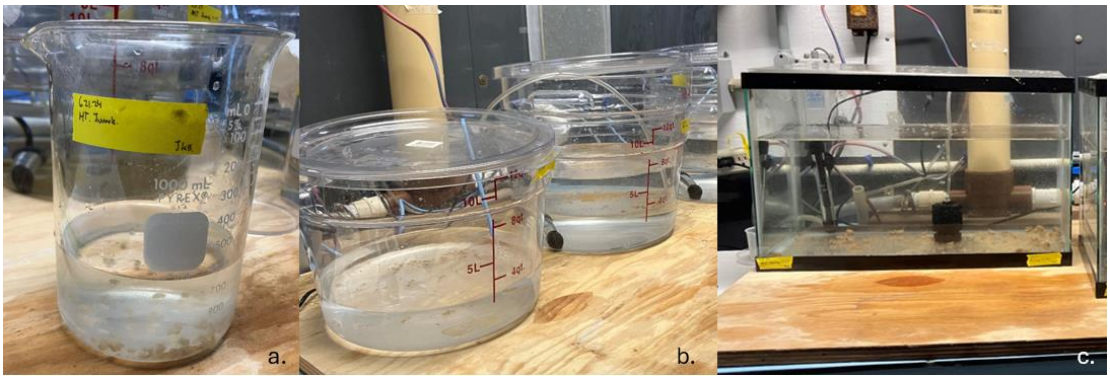

Figure 6. ***Setup for Egg to Six Month Temperature Experiment.*** *(A) 1 L container for eggs to young juveniles. (B) 10 L containers with MT being in a water bath. (C) 10-gallon tank setup for MT treatment.*

Throughout the experiment, feeding was done on MWF with differing amounts of live baby brine shrimp that steadily increased as the horseshoe crabs grew. Once the horseshoe crabs reached approximately instar 6, frozen adult brine shrimp cubes were added to the live artemia diet.

Water changing frequency varied from each containment type. In the beginning stages of the 1 L container, there were minimal water changes as there was less of an excess of food left over, and most of the horseshoe crabs were still in their egg stages. The 1 L containers had aeration, which allowed a more uniform distribution of movement and prevented mold from forming. When the horseshoe crabs moved to the 10 L containers, feedings were limited to MWF, but more frequent water changes were required. The 10-gallon tanks were able to return to the MWF feeding arrangement and water changes. The water changes were decreased in frequency to about once per week due to the addition of filtration from bubble filters in the tanks. This allowed little water changes and filter cleanings throughout the

week, and only the larger water changes occurred on Fridays when the horseshoe crabs were measured and counted. Water changes were completed using a siphon, and 30 ppt of replacement water was added.

Molts were removed from their enclosures every MWF. Molts were allowed to dry out before being photographed and measured for prosomal width and length from the imaged carapace. The molts were measured and cross-referenced from previous instar stages before being pictured and measured in ImageJ software for accurate molt size comparisons. After the horseshoe crabs were transferred to the 10-gallon tanks in October 2024, all live experimental animals were measured by hand using a ruler on Fridays. The prosomal width and length of the horseshoe crabs were recorded for all measurements. These measurements were used to determine the mix of instar stages in all experimental tanks. If dead horseshoe crabs were found, they were measured, and the dead body's data and potential cause of death were noted. The temperature experiment ended on December 20th, 2024, when all horseshoe crabs were measured by hand before their instar stages were determined.

2.2d Six-Month Temperature Experiment from Instar 2 (2023-2024)

The eggs for the temperature experiment were collected on June 19th, 2023, from Ocean City, Maryland, with the help of the Maryland Department of Natural Resources. The eggs were filtered and cultured in 12-well plates until they hatched in July. After they hatched, each individual horseshoe crab was moved to a six-ounce (100 ml) container full of 30 ppt water. The juveniles that reached instar 2 by August

8th were used in the temperature experiment (Figure 7).

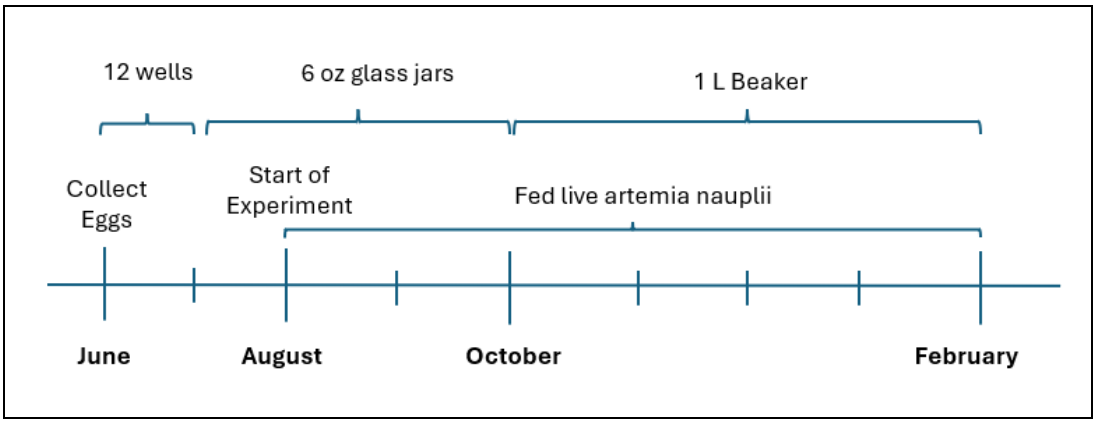

Figure 7. ***Instar Two to Six Month Timeline.*** *The eggs were collected in June and grown in normal lab conditions until August when the temperature experiment started. In the experiment, they were fed live artemia and moved through two different enclosure types.*

The experiment started using instar 2 horseshoe crabs grown at RT. The experiment started on August 11th, 2023, with a total of 44 horseshoe crabs in RT treatment, 45 in MT treatment, and 44 in HT treatment. Five juveniles were added to six-ounce jars, with six to seven jars per temperature. Replacements for dead horseshoe crabs were added during the first 10 weeks of the experiment due to the high initial mortality. The temperature treatments included RT: 20-22 °C, MT: 23-25 °C, HT: 28-30 °C. Aeration for the six-ounce jars was added six days into the experiment and kept for the remainder of the experiment. The six-ounce container had a diameter of 6.5 cm and a height of water of 5 cm. The surface area for one six oz jar was approximately 33.2 cm^2, and the volume was 165.9 cm^3. On average, one horseshoe crab was allowed 6.6 cm^2 of space on the bottom and 33.2 cm^3 of the water volume to themselves.

The horseshoe crabs were moved to 1 L containers in September of 2023. The 1 L containers held approximately 10 horseshoe crabs with three containers in every temperature treatment. The 1 L beaker had a diameter of 10 cm and a height of 10 cm. This resulted in one of the 1 L containers having a total surface area of 78.5 cm^2 and a total volume of 785.4 cm^3. This means that, on average, one horseshoe crab would have 7.9 cm^2 of space on the bottom and 78.5 cm^3 volume of water in the container. These 1 L containers were used until the six-month experiment ended in February 2024 (Figure 8).

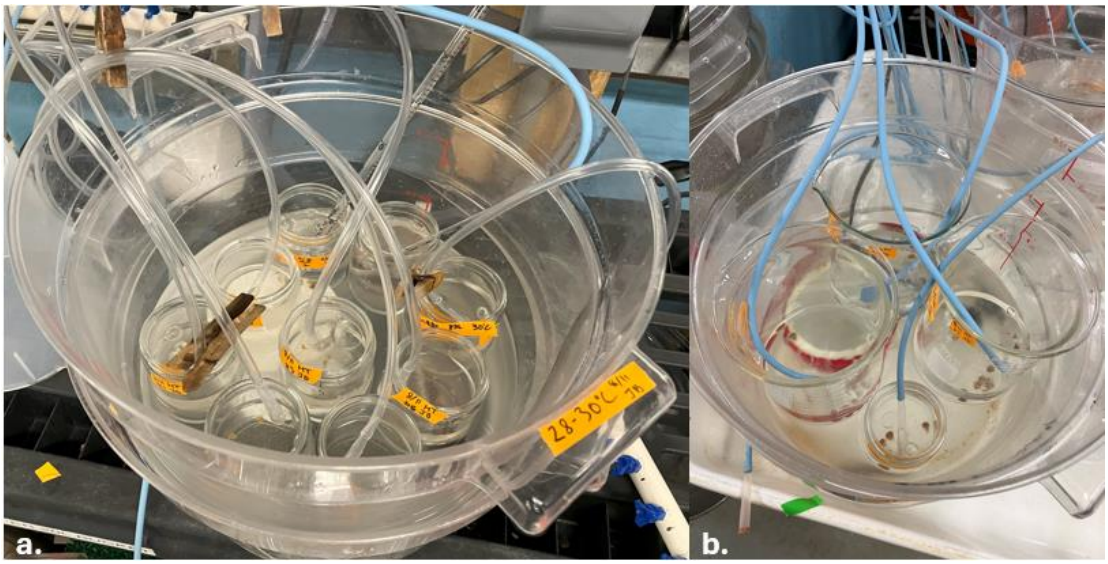

Figure 8. ***Temperature Study Setup for Six Month Experiment from Instar 2.*** *(A) The six-ounce jars containing five horseshoe crabs in each container. Air hoses with pipette tips were used to provide aeration to each container. (B) 1 L containers in a water bath containing about 10 horseshoe crabs in each container.*

Throughout the experiment, juveniles were fed baby brine shrimp daily, with water changes occurring daily in the smaller 6 oz containers. Since these were smaller containers, they quickly became dirty and needed to be changed to prevent bacterial

growth. They needed to be fully cleaned one a week to scrub off excess artemia. During the weekly jar cleanings, the horseshoe crabs would be transferred to another holding facility where they would be sized. Aeration was provided by air tubes with pipette tips attached at the ends. These pipette tips were changed every month.

Molts were collected daily and compared to previous data for size determination. The molts were dried, pictured, and measured using ImageJ software. The live juveniles were analyzed every Wednesday using size comparisons to determine a more accurate weekly prediction of instar stages for each temperature. If dead horseshoe crabs were found, they were measured, and the dead body's data and potential cause of death were noted. The amount of artemia eaten by all horseshoe crabs in the experiment was attempted to be counted. However, these numbers proved to be unreliable and could not be used in the final data.

After the experiment concluded, the remaining juvenile horseshoe crabs were transferred to three 10-gallon tanks to continue the temperature experiment. These tanks contained a sand substrate, bubble filter, and heater. They were switched to a diet containing artemia and frozen brine shrimp cubes. Maintenance switched to a MWF schedule, with feed, water maintenance, and molt collecting occurring on those days. They were kept in the 10-gallon tanks from February 2024 to June 2024. All horseshoe crabs were released on June 3rd at Sandy Point State Park with the Maryland Department of Natural Resources where the water temperature was 25 °C in the wild.

2.3 Results

2.3a Egg Experiment

Out of the 90 horseshoe crabs per temperature, most survived to the end of the treatment (Table 8)

Table 8. ***Final Data from Egg Experiment.*** *Each treatment contained n=90 horseshoe crabs. The progression of eggs was tracked with a focus on hatch time and survival.*

	Room Temp (20-22 °C)	Medium Temp (23-25 °C)	High Temp (28-30 °C)
Did not hatch	5.6%	4.4%	10%
Died in experiment	3.3%	4.4%	23.3%
Alive at end	91.1%	91.1%	66.7%
Time of 1st Instar 1	Week 3	Week 3	Week 2
Time of 1st Instar 2	Week 6	Week 5	Week 4
Time of 1st Instar 3	n/a	n/a	Week 6

The animals in HT had the highest mortality and percentage of eggs that did not hatch. Animals in RT and MT treatments had similar amounts of eggs that did not hatch and mortality by the end of the experiment. The progression of egg stages occurred swiftly, with the first instar appearing in HT treatment by week 2. In contrast, animals in RT and MT treatments reached the first instar in week three. These data are consistent with the 2-4 weeks it typically takes fertilized eggs to develop in the wild. The development to instar 2 followed the similar trend of HT treatment occurring faster, followed by MT treatment, and finally, RT treatment.

Horseshoe crabs in HT treatment were the only ones to reach instar 3 by week 6. Even in the small scale of a six-week study, the trend of higher temperatures accelerating growth while lowering the survivorship of the horseshoe crabs was initially shown.

2.3b Six-Month Temperature Experiment from Eggs (2024-2025)

The six-month temperature experiment from eggs started in June 2024 when eggs were collected from Ocean City, MD. The horseshoe crabs were raised in 1 L beakers until August 2024, when they were transferred to the 10 L containers until the experiment ended in October 2024. After October, they were transferred to 10 gallon tanks.

After six months of culturing, weekly estimates of instar stages for the whole tanks were taken by using measured molt sizes and measurements of live horseshoe crabs in the experiment compared to previous size guides on instar size. Since the horseshoe crabs were kept in one containment, individual horseshoe crabs could not be distinguished, and a bulk estimation was determined.

Throughout the study, the increased temperature generally resulted in a faster molting rate. Horseshoe crabs in the HT treatment reached every instar stage faster than any other temperature except for instars 1 and 3 (Figure 9). Horseshoe crabs in the RT treatment took the longest to reach every stage. Horseshoe crabs in MT and HT treatments had similar trends of time to reach the next instar stage, but started to have larger and larger time gaps between each instar stage, resulting in a higher instar

stage reached for the HT treatment.

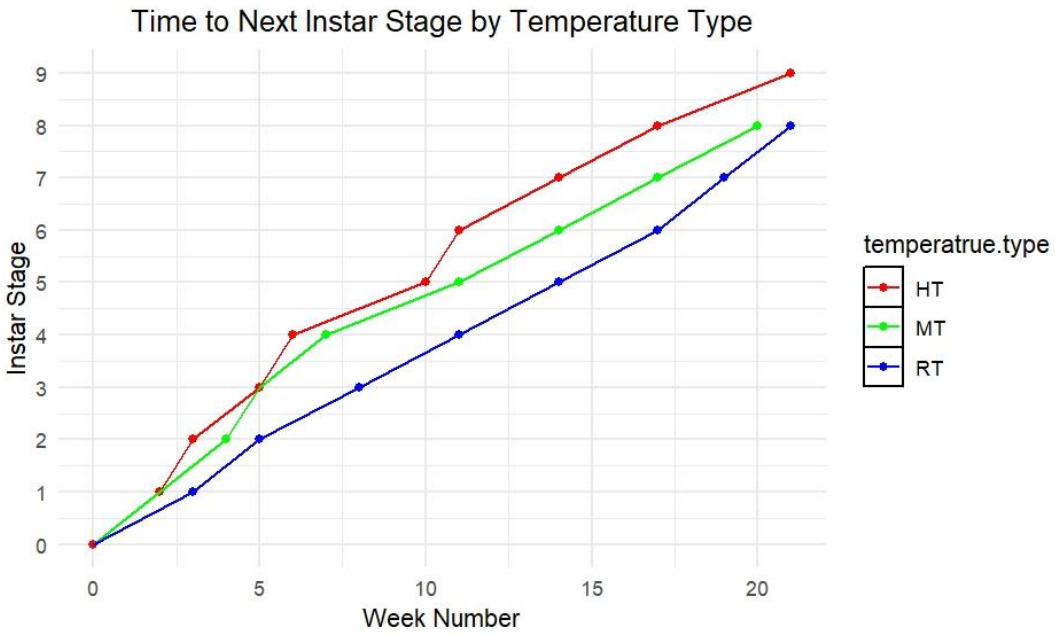

Figure 9. ***Time to Reach Instar Stage by Temperature.*** *High temperature treatments are represented in red, medium temperature treatments in green, and room temperature treatments in blue.*

On the final day of the six-month temperature experiment, all live horseshoe crabs were measured by hand with a ruler (Figure 10). While there are a number of size distributions by the horseshoe crabs in each individual temperature, general trends of larger horseshoe crabs can be seen in the higher temperatures. The RT treatment horseshoe crabs (n= 72) have an average size of 14.8 ± 4.8 mm for prosomal width and 23.5 ± 8.7 mm for length which correlated to the size of a typical instar 6. The MT treatment horseshoe crabs (n= 70) have an average size of 21.3 ± 6.1 mm for prosomal width and 35.3 ± 11.8 mm for length, which correlates to a typical instar 7. The HT horseshoe crabs (n= 39) have an average size of 30.7 ± 8 mm for prosomal width and 53.6 ± 16.4 mm for length, which correlates to a typical

instar 9 size.

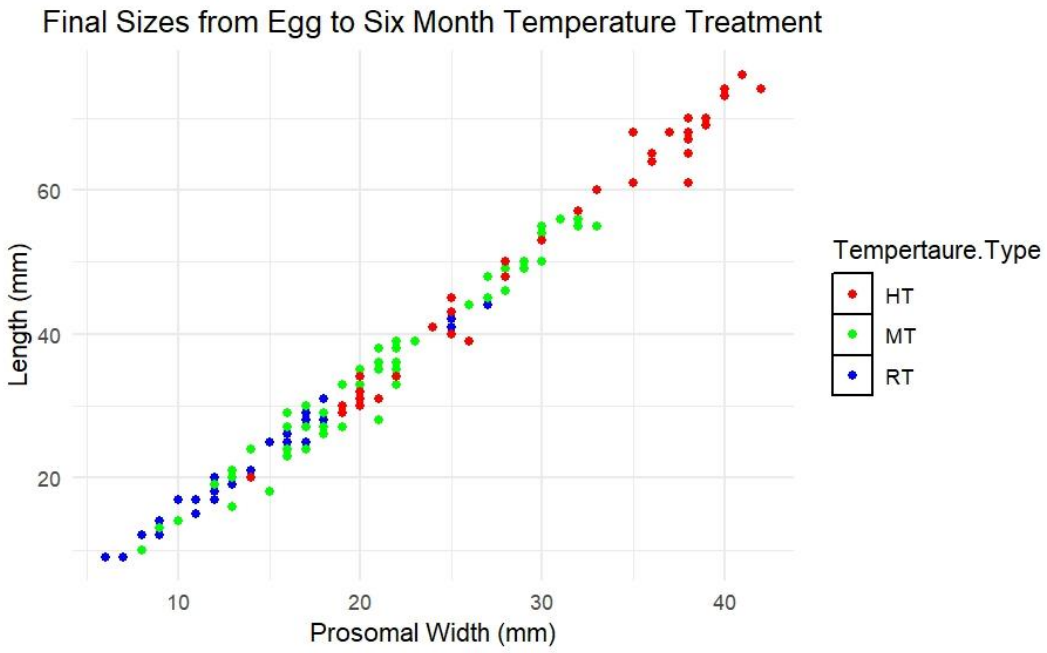

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Figure 10. ***Final Sizes of Horseshoe Crabs after Six Month Temperature Experiment from Eggs.*** *Horseshoe crab prosomal width and length were measured by hand in mm. High temperature treatment is represented in red, medium temperature treatment in green, and room temperature treatment in blue.*

*To test the significance of the differences in sizes between the temperature treatment types, an ANOVA: Single Factor test was ran comparing the prosomal width and lengths of all treatment live animals ($p < 0.05$; Table 9****Error! Reference source not found.****). To compare the size differences between each temperature treatment against each other, a two sample T-test assuming unequal variances was run for RT vs. MT, RT vs. HT, and MT vs. HT treatments for prosomal width and length ($p < 0.05$;* Table 10). Both of these statistical tests were ran using the Data Analysis tool in Microsoft Excel.

Table 9. ***ANOVA: Single Factor for Final Sizes of Live Animals.*** *An ANOVA single factor test was ran to compare the live animals in RT, MT, and HT treatments for prosomal width (PW) and length (L). Bolded are the significant p-values lower than 0.05.*

	source	*ss*	*df*	*MS*	*F*	*P*	*F crit*
Prosomal Width	Between Groups	6487.652	2	3243.826	85.74857	**8.33E-27**	3.046721
	Within Groups	6733.652	178	37.8295			

Length	Between Groups	22965.63	2	11482.81	79.70873	**1.91E-25**	3.046721
	Within Groups	25642.62	178	144.0597			

Table 10. ***T-Test: Two-Sample Assuming Unequal Variances for Final Sizes of Live Animals.*** *The lower temperature was variable one and the higher temperature was variable 2, resulting in a one-tailed test. The prosomal width and length were compared between each temperature treatment. Bolded are the significant p-values lower than 0.05.*

	test	*df*	*t Stat*	*P(T<=t) one-tail*	*t Critical one-tail*
Prosomal Width	RT vs MT	131	-7.08648	**3.81E-11**	1.656569
	RT vs HT	53	-11.2691	**5.47E-16**	1.674116
	MT vs HT	63	-6.30742	**1.58E-08**	1.669402
Length	RT vs MT	126	-6.73057	**2.67E-10**	1.657037
	RT vs HT	50	-10.5471	**1.3E-14**	1.675905
	MT vs HT	60	-6.06119	**4.85E-08**	1.670649

The temperature treatments had a significant effect on the prosomal width and length of the horseshoe crabs in all treatments ($p < 0.05$; Table 9). Furthermore, when comparing individual temperature treatments against each other, all treatments were significantly different ($p < 0.05$;

Table 10).

Regarding survivorship, higher temperatures experienced higher mortality rates compared to other temperatures. Horseshoe crabs in the HT treatment had the

highest mortality rate, with only 39% of the population living until six months (Figure 11). Horseshoe crabs in the RT and MT treatments had similar amounts of survivorship, with 71.3% survival for RT treatment and 68.6% for MT treatment.

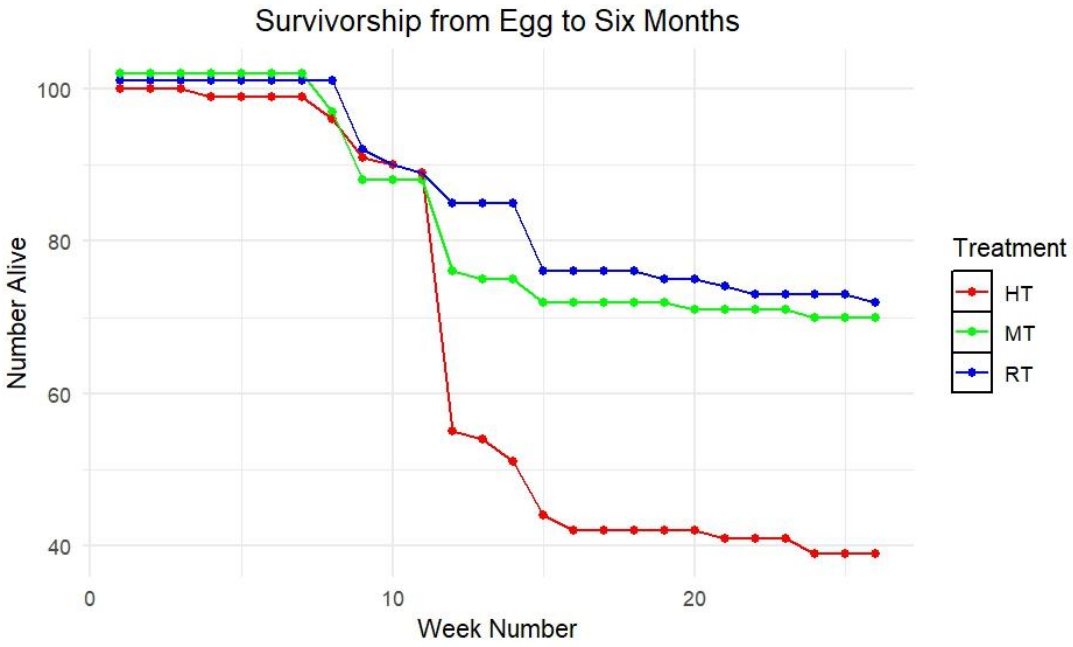

Figure 11. ***Survivorship of Horseshoe Crabs in Temperature Experiment from Egg to Six Months***. *The red corresponds with HT treatment, green for MT treatment, and blue for RT treatment. The number of horseshoe crabs started at about 100 horseshoe crabs for each experimental treatment.*

The beginning of the experiment had a steady survivorship until week 9 when the unhatched eggs were discarded. The unhatched eggs included 7 from RT treatment, 9 from MT treatment, and 5 from HT treatment. In all temperatures, there was an increase in mortality around weeks eleven and twelve, occurring at the beginning of September 2024. This spike in mortality occurred around approximately three months, or halfway, into the experiment. After this mortality spike was discovered, the horseshoe crabs were moved to the 10-gallon tank setup. After week

15, the survivorship stabilized to a relatively steady decrease until the end of the experiment.

Molts from each temperature were collected throughout the experiment. These molts were allowed to dry out, then analyzed with ImageJ measured for prosomal width and length. These molts were categorized by their instar stages (Table 11). This experiment was conducted as a batch experiment with individual horseshoe crabs unable to be individually tracked through their molt sizes. Previous size data collected by the lab was used as a basis for determining instar stages of these molts. Lab size data was used instead of previous research guides due to the labs data being produced from horseshoe crabs gathered from the same area and time as this experiments horseshoe crabs. The average sizes of the molts were measured as they provide a more accurate measurement of horseshoe crab size than live animals measured by hand.

Table 11. ***Average size of molts by instar stage across the different temperature treatment****. Molts were collected, dried out, and measured using ImageJ. The average value is given for each temperature and instar stage. ANOVA: single factor tests were run on instars one through six. (*) indicates a column where the null hypothesis of all categories being equal, was rejected. N represents the number of molts measured, PW equals the prosomal width in mm, and L is the length measured in mm.*

	Instar 1			Instar 2			Instar 3			Instar 4			Instar 5			Instar 6			Instar 7			Instar 8		
	n	PW	L	n	PW	L	n	PW *	L *	n	PW	L	n	PW	L *	n	PW*	L *	n	PW	L	n	PW	L
RT	83	2.87	3.34	79	4.78	6.06	67	6.99	9.56	48	9.03	13.38	31	12.43	19.55	9	17.06	28.17	2	18.89	30.64	-	-	-
MT	93	2.88	3.39	66	4.81	5.81	74	6.63	8.74	74	9.14	13.28	56	12.17	18.83	45	15.95	26.21	21	21.66	36.11	-	-	-
HT	90	2.87	3.38	81	4.79	5.91	61	6.39	8.49	60	9.21	12.84	39	12.37	18.09	38	16.33	25.60	27	21.43	34.94	20	28.64	47.95

To test the significance of differences in molt sizes between the temperature treatments, an ANOVA: Single Factor test was ran comparing the prosomal width

and lengths of each instar stage molts ($p < 0.05$; Table 12). This statistic test was run using the Data Analysis tool in Microsoft Excel. While there is data for instar molts from instar 1 through 8, an ANOVA: Single Factor test was run on only instar molts from instar 1 through 6 due to sample size. Temperature treatments proved to be significant on molt size for instar 3 PW and L, instar 5 L, and instar 6 PW and L. In previous experiments, molts have been relatively equal in size, however, external lab factors might have played in the mix, resulting in the differences in sizes. Some potential reasons for the differences in sizes could be contributed to challenges encountered in the experiment, namely, cannibalism.

Table 12. ***ANOVA: Single Factor Test for Molt size by Instar Stage in Temperature Experiment Egg to Six Month.*** *An ANOVA single factor test was ran to compare the RT, MT, and HT treatment final molt sizes for prosomal width (PW) and length (L) of the molts dried out and measured with ImageJ software. The p-values that are significant are bolded.*

	source	*SS*	*df*	*MS*	*F*	*P*	*F crit*
Instar 1: PW	Between Groups	0.002378	2	0.001189	0.028317	0.972084	3.030116
	Within Groups	11.0452	263	0.041997			
Instar 1: L	Between Groups	0.098363	2	0.049182	0.391097	0.676707	3.030116
	Within Groups	33.07303	263	0.125753			
Instar 2: PW	Between Groups	0.030107	2	0.015054	0.146949	0.863421	3.036339
	Within Groups	22.84434	223	0.102441			
Instar 2: L	Between Groups	2.268647	2	1.134324	2.816574	0.06194	3.036339

	Within Groups	89.80918	223	0.402732			
Instar 3: PW	Between Groups	11.59682	2	5.79841	24.80837	**2.4E-10**	3.041286
	Within Groups	46.51187	199	0.233728			
Instar 3: L	Between Groups	41.40563	2	20.70282	14.48957	**1.33E-06**	3.041286
	Within Groups	284.3329	199	1.428808			
Instar 4: PW	Between Groups	0.905882	2	0.452941	0.815378	0.444109	3.046433
	Within Groups	99.43419	179	0.555498			
Instar 4: L	Between Groups	9.823487	2	4.911744	2.009342	0.13709	3.046433
	Within Groups	437.5573	179	2.444454			
Instar 5: PW	Between Groups	1.593411	2	0.796706	1.139043	0.323478	3.069894
	Within Groups	86.03255	123	0.699452			
Instar 5: L	Between Groups	37.01843	2	18.50922	4.147634	**0.018065**	3.069894
	Within Groups	548.8993	123	4.462596			
Instar 6: PW	Between Groups	10.23184	2	5.115919	4.829068	**0.010208**	3.09887
	Within Groups	94.28669	89	1.059401			
Instar 6: L	Between Groups	48.40287	2	24.20144	3.658633	**0.029718**	3.09887
	Within Groups	588.7247	89	6.614884			

Overall, this experiment observed a trend where higher temperatures resulted in larger instars but higher mortality. The times of highest mortality occurred when the eggs were discarded and halfway through the experiment. Other than these times of higher mortality, the survivorship remained relatively constant with a steady decline across all temperatures.

2.3c Six-Month Temperature Experiment from Instar 2 (2023-2024)

The experiment started in August 2023 when previously grown instar 2's were moved into the temperature treatments. The instar 2 horseshoe crabs chosen for this experiment were grown at room temperature until experiment time. They were kept in the six-ounce containers for two months and then moved to 1 L beakers for the remaining four months, finishing the experiment in February 2024. This six-month experiment was comprised of smaller containers focused on identifying individual instar stages with smaller numbers of animals being analyzed per temperature treatment.

Overall, similar trends to the previous two temperatures were exhibited in this study, with higher temperatures resulting in a faster molting rate but lower survivorship. Horseshoe crabs in the HT treatments had a faster molting rate reaching instar 10 compared to MT horseshoe crabs reaching instar 8 and RT horseshoe crabs reaching instar 6 (Figure 12). Interestingly, animals in the MT and HT treatments had relatively similar molting rates from instar 2 until instar 4 until they started diverging

around instar 5. Animals in the RT treatment had a slower molting rate throughout the whole experiment.

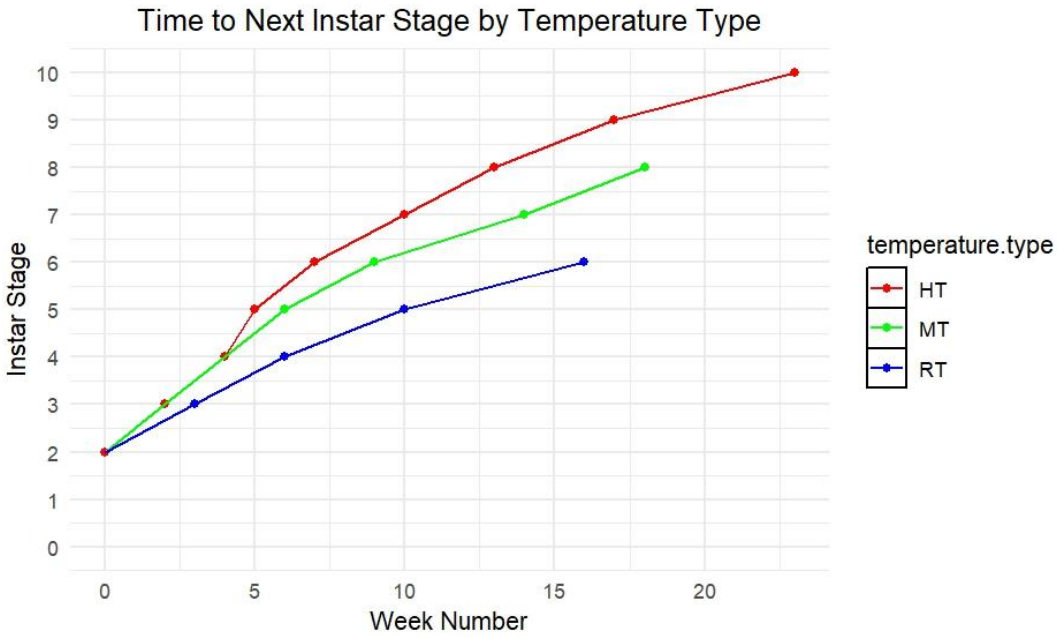

Figure 12. ***Time to Reach the Next Instar Stage by Temperature.*** *HT animals reached the largest instar stage of 10 compared to MT animals reaching instar 8 and RT animals reaching instar 6.*

Each temperature type had an incremental growth difference, resulting in a 2-step instar stage increase by each temperature treatment by the end of the experiment (Figure 13).

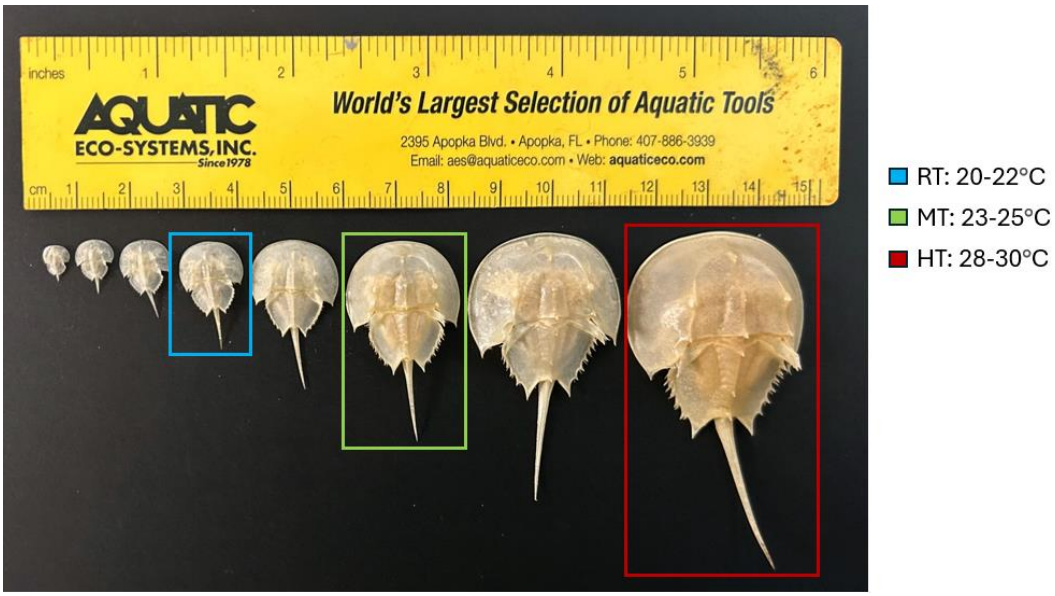

Figure 13. ***Final Molt Size Comparison of Temperature experiment from Instar 2****. Boxed in blue is RT with the instar 5 molt, boxed in green is the MT final molt size of instar 7, and boxed in red is the final molt size of 9.*

While the horseshoe crabs had higher growth rates in higher temperatures, mortality was also increased. By the end of the six-month experiment, HT treatment only had 7 horseshoe crabs remaining, creating a 16% survival rate. MT treatment had 19 animals remaining for a 42% survival rate. RT treatment had 25 animals remaining for a 57% survival rate. Overall, there was a steady decrease in survivorship throughout the study, with no one time resulting in a spike in mortality (Figure 14). However, it is important to note that these survival rates are much greater than what is typically seen in the wild, with studies reporting only approximately 0.001% survival for *L. polyphemus* through the first summer (M. Botton et al., 2003; S. A. Smith & Berkson, 2005).

Figure 14. ***Survivorship for Temperature Experiment from Instar 2 to Six Months****. RT treatment is in blue, MT treatment is in green, and HT treatment is in red. All groups started with approximately 45 horseshoe crabs.*

Finally, molts were removed daily and sorted into their respective instar stages. These molts were dried out and analyzed with ImageJ. The average PW and L were computed for each temperature and instar stage (Table 13). Since the enclosures were smaller and more divided, individual horseshoe crabs were able to have their instar stages confirmed, resulting in a base data set that was used as a reference for future experiments.

Table 13. ***Sizes of Instar Stages by temperature.*** *Molts were collected, dried out, and measured using Image J. The average value is given for each temperature and instar stage. ANOVA: single factor tests were run on instars two through 5 and a two variable t test with unequal variance was used for instar six. All the other instars did not have enough values or were only one variable. N represents the number of molts measured, PW equals the prosomal width in mm, and L is the length measured in mm. * indicates a column where the null hypothesis of all categories was equal, was rejected.*

	Instar 2			Instar 3			Instar 4			Instar 5			Instar 6			Instar 7			Instar 8			Instar 9		
	n	PW	L	n	PW	L *	n	PW	L	n	PW	L	n	PW	L	n	PW	L	n	PW	L	n	PW	L
RT	29	4.71	6.55	23	6.77	10.08	14	9.29	14.01	5	12.70	20.29	-	-	-	-	-	-	-	-	-	-	-	-
MT	37	4.70	6.40	36	6.81	9.90	26	9.49	14.58	15	12.58	20.61	11	16.37	27.39	1	23.12	40.04	-	-	-	-	-	-
HT	34	4.77	6.46	31	6.73	9.68	23	9.26	14.19	12	12.88	20.74	8	16.43	27.24	5	21.33	36.67	4	27.76	50.48	2	37.68	70.95

To test the significance of the differences in molt sizes between the temperature treatments, an ANOVA: Single Factor test was ran comparing the prosomal width and lengths of each instar stage molts ($p < 0.05$; Table 14). This statistic test was run using the Data Analysis tool in Microsoft Excel. While there is data for instar molts from instars 2 through 9, an ANOVA: Single Factor test was run on only instar molts from instar 2 through 5 due to sample size. Temperature treatments proved to be significant on molt size for instar 3 L. The statistically significant result from this dataset is surprising as there were no signs of cannibalism that could have contributed to the earlier differences in length in molt sizes.

Table 14. ***ANOVA: Single Factor Test for Temperature Experiment Instar 2 to Six Month.*** *An ANOVA single factor test was ran to compare the RT, MT, and HT treatment final molt sizes for prosomal width (PW) and length (L) of the molts dried out and measured with ImageJ software. The p-values that are significant are bolded.*

	source	*SS*	*df*	*MS*	*F*	*P*	*F crit*
Instar 2: PW	Between Groups	0.110456	2	0.055228	0.38158	0.683802	3.090187
	Within Groups	14.0393	97	0.144735			
Instar 2: L	Between Groups	0.360922	2	0.180461	1.416173	0.247612	3.090187
	Within Groups	12.36059	97	0.127429			
Instar 3:	Between Groups	0.097249	2	0.048625	0.308828	0.735108	3.101296

PW	Within Groups	13.69805	87	0.157449			
Instar 3: L	Between Groups	2.161683	2	1.080842	3.652289	**0.029986**	3.101296
	Within Groups	25.74638	87	0.295935			
Instar 4: PW	Between Groups	0.752089	2	0.376045	1.231509	0.299122	3.150411
	Within Groups	18.32115	60	0.305353			
Instar 4: L	Between Groups	3.430615	2	1.715308	1.275544	0.286742	3.150411
	Within Groups	80.68596	60	1.344766			
Instar 5: PW	Between Groups	0.637362	2	0.318681	0.935125	0.404054	3.327654
	Within Groups	9.882898	29	0.34079			
Instar 5: L	Between Groups	0.710742	2	0.355371	0.340329	0.71434	3.327654
	Within Groups	30.28173	29	1.044198			

Overall, this experiment was a smaller scale focus on temperature effects after exposure to normal conditions for two months. The higher temperatures resulted in faster growth but higher mortality.

2.3d Comparison of Three Temperature Experiments (2023-2025)

Final Instar Stages from All Experiments

The largest instar stages were reached in the HT treatments of both six-month studies (Table 15**Error! Reference source not found.**). It is important to emphasize that horseshoe crabs in one experiment started from eggs compared to the other experiment that started from instar 2. From this information, it would be predicted that the animals starting from the larger stage would have a higher instar stage. However, while the HT treatment was able to reach the largest instar compared to the other experiment, none of the other treatments exceeded the animals grown from eggs.

Table 15. ***Largest Instar Stage after Long Experiments.*** *Two longer-term experiment results are shown, the egg-six-month and the instar 2-six-month experiments.*

	Egg- Six Month	**Instar 2- Six Months**
Room Temperature	Instar 8	Instar 6
Medium Temperature	Instar 8	Instar 8
High Temperature	Instar 9	Instar 10

Mortality

Overall, the egg to two-month experiment had the highest survivorship across all temperatures, followed by the egg to six months, and then instar 2 to six months (Table 16). Across all experiments, RT had the highest survivorship compared to the other temperature treatment. RT and MT treatment survivorships were more closely

related than the HT treatment survivorship which was substantially lower across all studies. This reiterates the trend of HT treatments having the lowest survivorship followed by MT and RT treatments.

Table 16. ***Survival percentages at the End of Experiments.*** *This includes the final survival percentages for the shorter-term two-month experiment and the two longer six-month experiments.*

	Egg- Two Month	**Egg- Six Months**	**Instar 2- Six Months**
Room Temperature	91.1%	71.3%	57%
Medium Temperature	91.1%	68.6%	42%
High Temperature	66.7%	39%	16%

This variation in survivorship could be due to a number of different factors outside of strictly temperature, including length of study, horseshoe crab density, and general feeding/water change procedures.

The egg experiment lasted the shortest amount of time, only 6 weeks in total, which could create inconsistencies when compared to the longer studies. When comparing the survivorship by the other two experiments at six weeks, the egg-to-six-month experiment has RT = 93.1%, MT = 91.2%, and HT = 94%, and the instar 2-to-six-month experiment has RT = 72.7%, MT = 82%, and HT = 72.7%. This comparable marking of six weeks across all experiments has different results than the overall trends initially seen in the one short term study.

Furthermore, in the egg to six-month experiment, the six-week mark accounted for 24% of the total mortality in RT, 28% of the total mortality for MT,

and 9.8% of the total mortality in HT. This initial six weeks accounted for the most mortality in MT treatment, then RT treatments, and finally HT treatments. In the instar 2-to-six-month temperature experiment, the six-week mark accounted for 63% of the total mortality in RT, 31% of the total mortality for MT, and 32% of the total mortality in HT. This initial six weeks accounted for the most mortality in the RT treatment, then HT treatment, and finally MT treatment.

2.3e Challenges in Experiments

These experiments came with many unforeseen challenges. While some were physical ailments to the horseshoe crabs, others occurred from the troubles of procedural management. When observing the horseshoe crabs, certain concerning trends appeared over the course of multiple studies. Several horseshoe crabs had twisted legs, artemia growth on their shells, disease, and died mid-molt. Furthermore, the rise of cannibalism occurred in the egg to six-month experiment when animals were contained in the 10 L containers. However, the most overarching struggle with these experiments was due to the uncertainty in exact sizes for instar stages, leading to estimations of instar stages as time progressed.

Body Malformations

Some body malformations occurred throughout both experiments. Some horseshoe crabs developed twisted legs (Figure 15). While live horseshoe crabs were found to have these legs, these legs could become a hazard when molting to the next stage. Another common body malformation that proved to be a common cause of

death during both years of experiments was the horseshoe crab getting stuck mid-molt (Figure 16).

Figure 15. ***Twisted Legs on Live Horseshoe Crabs.*** *(A) Horseshoe crab from instar two to six-month experiment. (B) Horseshoe crab from egg to six-month experiment. Both animals were still alive and active at time of picture.*

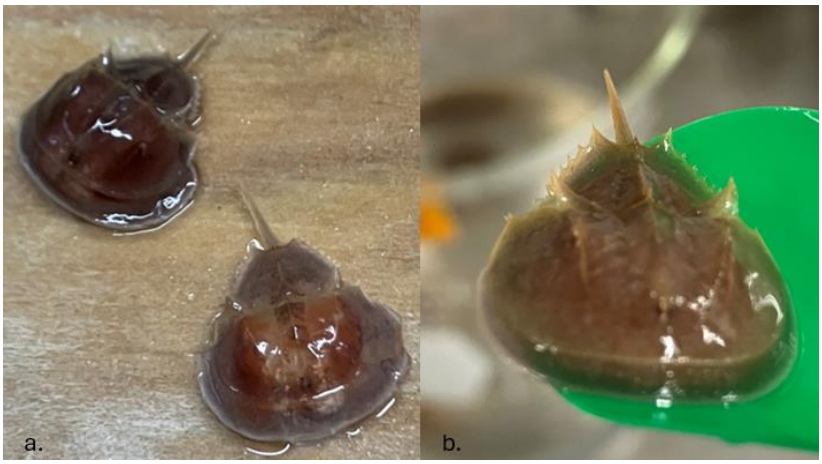

Figure 16. ***Horseshoe Crabs Stuck Mid-Molt.*** *(A) Horseshoe crabs from egg to six-month experiment. (B) Horseshoe crab from instar two to six-month experiment. The molts are the casing covering the emerging horseshoe crab.*

Disease/growths

Two cases of white spots appeared on horseshoe crabs in the egg six-month experiment (Figure 17). These white spots had a central darker orange spot with white

discolored flesh emulated from it. These spots were only seen in the HT treatment tank but were noticed in other horseshoe crabs grown in the lab.

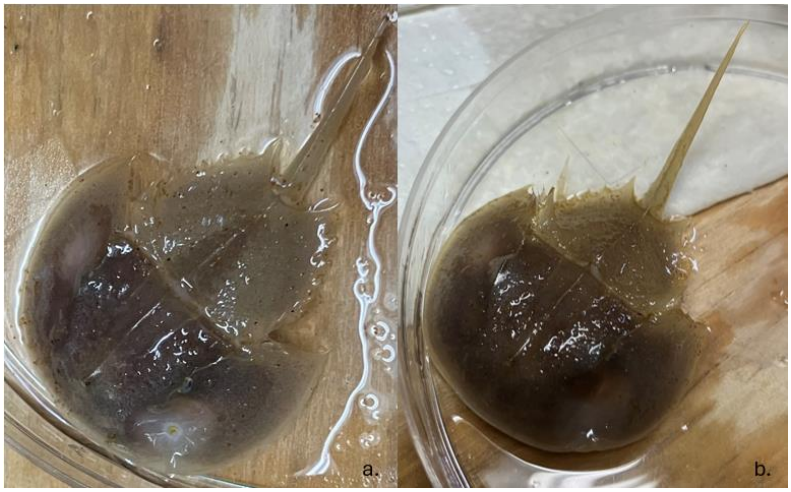

Figure 17. ***White spots on Horseshoe Crabs****. (A) A horseshoe crab with two white spots pictured on November 1st, 2024. (B) The same horseshoe crab pictured on December 13th, 2024, with visibly reduced infection in both spots.*

Artemia growth was observed in horseshoe crabs from the instar 2 to six-month experiment (Figure 18). This growth did not appear to harm the horseshoe crabs, but this buildup could indicate weaker animals that cannot clean their shells.

Figure 18. ***Excess Artemia Growth on Horseshoe Crab****. Horseshoe crab from instar 2 to six-month temperature experiment. The excess growth could be removed with repeated water rinses.*

Cannibalism

In week 11 in the temperature experiment from egg to six months, it was discovered that horseshoe crabs were missing from all tanks. Four horseshoe crabs from RT treatment, 13 from MT treatment, and 34 from HT treatment. Water changes were done in a way that ensured no escaped animals, but nonetheless, animals were missing. All horseshoe crabs were recounted, measured by hand, and transferred to a bigger tank. Not only were horseshoe crabs missing, but of the living horseshoe crabs, there were signs of shorter tensions and chunks of the carapace missing (Figure 19).

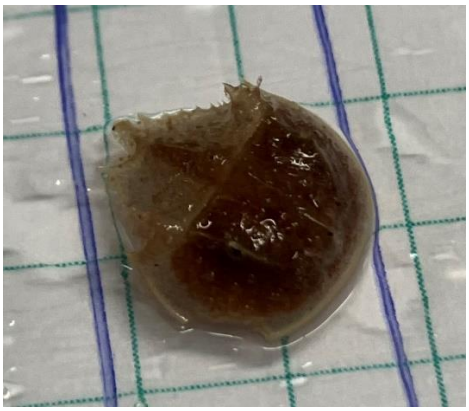

Figure 19. ***Live Horseshoe Crab with Signs of Cannibalism.*** *This horseshoe crab has a shortened telson compared to others of similar size and a portion of the prosoma to missing. These shortened telsons make adjustments to surroundings harder.*

Once all of the horseshoe crabs were remeasured, the missing instars' stages were determined. For RT treatment, 4 were missing from instar 3. The majority of the living horseshoe crabs in RT were in instar 3 (83%), instar 4 (15%), and in instar 2 (2%). For MT, of the 13 that disappeared, they were in instar 3 (n=7), instar 4 (n=5), and instar 2 (n=1). At this time, MT treatment had a majority of live animals in instar

4 (64%), instar 5 (20%), and instar 3 (16%). In HT, of the 34 animals missing, they were in instar 3 (n=18), instar 4 (n=10), instar 2 (n=3), instar 6 (n=2), and instar 5 (n=1). For the live horseshoe crabs remaining in HT most were in instar 5 (76%), instar 6 (15%), instar 4 (7%), and instar 3 (2%). Overall instar 3 was the most preyed upon, and a trend of smaller horseshoe crabs getting eaten by larger ones emerged. This led to the belief that cannibalism could be the cause. While it is unknown whether they were eaten while alive or dead when preyed upon, there is evidence that crabs stuck mid-molt received bites taken out of their carapace (Figure 20).

Figure 20. ***Dead Horseshoe Crab with Signs of Cannibalism****. A dead horseshoe crab stuck mid-molt has portions missing from the prosoma.*

2.4 Discussion

2.4a Summary

These experiments tested a combination of feeding regimes, water maintenance, enclosure types, and length of study on horseshoe crab growth and survival when exposed to three different temperatures. The motivation for this

research was to determine successful hatchery management strategies for less than 1 year growth to produce the largest horseshoe crabs most likely to survive in the wild. By changing multiple factors over the two-year period and keeping the temperature treatments constant, multiple culture conditions could be tested on a smaller scale with the intent of upscaling to industry standards.

The egg experiment and the egg to six-month temperature experiment are shown taking up about 6ft x 2ft of space in the aquaculture research center (Figure 21). Through both of these experiments, almost 600 horseshoe crabs were able to be cultured, including extras for other experiments. Even in limited space, a large amount of horseshoe crabs can be grown, which gives hope for the future of scaling up using the knowledge from these experiments.

Figure 21. ***Setup of Egg and Edd-Six Month Temperature Experiments***. *Space requirements for running two of these experiments at the same time. Two experiments were running in the 6'by 2' area converted into table space.*

However, while these experiments were able to test multiple factors for determining the best culturing conditions, this variability in procedures for all experiments calls into question the comparability of the temperature results. The results are compared in this thesis with the caveat that more than just the temperature could be influencing these results.

When analyzing the final sizes from all experiments, a trend of higher temperatures resulted in larger instar stages. However, when comparing the final instar stages between the six-month temperature experiments, there are some mixed results. For HT, the instar 2- six-month experiment reached instar 10 while the egg-six month reached instar 9. For MT, both experiments reached a final size of instar 8. For RT, the instar 2- six-month experiment reached instar 6 while the egg-six-month experiment reached instar 8. Not only did the egg to six-month temperature trial almost match the growth in every category of instar 2- six-month experiment, but they also exceeded this growth in RT. A potential conclusion to draw from these data is that the initial months have a significant impact on horseshoe crab growth and development. It was previously stated that the beginning months are essential to horseshoe crab growth (M. Botton et al., 2003). Adjusting the initial enclosure procedure to create favorable conditions at the beginning of their life could be more impactful.

It is important to note that in both studies, all temperature groups were able to reach instar stages higher than their wild counterparts, especially in the higher

temperatures. (Sekiguchi et al., 1988) found that the growth progression for their *L. polyphemus* experimental horseshoe crabs for the first instar was about 11-20 days, about 11 days for instar 2, about 12.5 days for instar 3, about 15.8 days for instar 4, and about 22 days for instar 5. They found that by the end of the first year, many horseshoe crabs reached instar 6, but none molted to instar 7. In year two, their horseshoe crabs reached instar 9, in the third year, they molted twice to instar 11, and in the fourth year and onward, they only molted once per year. Defining age classes and estimating growth in wild horseshoe crabs has been very difficult historically as there are no definitive methods to age a horseshoe crab (Carmichael et al., 2003), which is why estimates based on previous studies are so pivotal.

From this information, the animals in the longer-term HT experimental treatments reached sizes equivalent to approximately three years in the wild. The animals in the longer-term MT experimental treatments reached sizes equivalent to about two years in the wild. The animals in the longer-term RT experimental treatments in instar 2 to six-month experiment reached sizes equivalent to roughly one year in the wild and the animals in the egg to six-month experiment, reached sizes equivalent to approximately 2 years in the wild. All these temperatures exceeded the normal growth period for six months in the wild. Furthermore, these wild size/age estimations are based on lab-grown horseshoe crabs, which probably experienced faster molting rates than what is seen in the wild. This further emphasizes how the temperature conditions differed from the wild-grown horseshoe

crabs. While animals in all three temperatures groups exceeded the typical growth rate experienced in the wild, this could be due to the lack of predation, high-quality feed, and filtration implemented in the lab. These better conditions could have contributed to accelerated molting intervals in addition to temperature treatments.

One surprising finding from comparing the two longer studies was the difference seen in RT treatment animal growth. One potential reason for this deviation was the location in the laboratory between the two experiments. The experiments from eggs were all completed in a warmer location of the aquaculture research facility with more direct exposure to overhead lights concentrated in this area. The instar 2 setup was in a colder area of the aquaculture center with less light. Furthermore, room temperatures in the aquaculture research facility shifted throughout the year based on the season.

Other than temperature differences due to lab placement, the size differences between the studies were influenced by feeding regimens, available space, etc. Even though the difference in growth is more pronounced between temperature experiments from instar 2 vs the egg, overall, the highest temperature resulted in the largest instar stage.

In all experiments, animals in RT treatments had the highest survivorship compared to the other temperature types. Animals in RT and MT treatments had survivorships closer to each other than animals in HT treatments with lower survivorship in all studies. This lower survivorship is expected as the HT temperature

condition of 28-30 °C is included in the upper limit of what horseshoe crabs are reported surviving in (S. A. Smith & Berkson, 2005). When considering the survivorship and mortality across all three experiments, multiple factors could have contributed to the trends experienced outside of temperature treatments alone. The differences in length of study, feeding and water change schedule, horseshoe crab density, and challenges could have influenced survivorship.

In all three temperature experiments, HT treatments experienced the highest mortality. Originally, the six-week temperature experiment found that HT treatments had the highest mortalities compared to RT and MT treatments that had the same mortality. This information seemed to support the conclusion that temperature had an influence on survivorship in the first six weeks. However, when comparing the survivorship of the other two temperature studies at the six-week mark, the survivorship was more varied than originally concluded by the one two-month temperature experiment.

In the egg to two-month experiment MT treatments had the highest mortality, then RT treatments, and then HT treatments. For the instar 2-to-six-month experiment, RT and HT had the highest mortality followed by MT treatment. At the six-week mark, all three experiments had different temperature treatments with the highest mortality. This provides evidence that eggs and initial growth of horseshoe crabs may be more flexible than originally believed.

Furthermore, it has been previously reported that the high mortality in

horseshoe crabs life occurs in the first summer (M. Botton et al., 2003; Carmichael et al., 2003). However, when comparing the longer temperature experiments, it was found that almost all experiments followed general trends of steady decline in survivorships across most temperature excluding events of mortality spikes. For instance, using the six-week marker as a marker about a fourth of the way through the longer six-month experiments, if mortality followed a similar rate of steady decrease, then the mortality amount would be around 23% for each temperature treatment. These amounts are seen to be similar or a little higher for all temperature treatments except for RT treatment in instar 2-to-six-month experiment. While the amount of mortality for RT in the instar 2 to six-month experiment was higher than the expected 23% mortality, this amount far less than the 99. 999% predicted in the wild in a two month period (M. Botton et al., 2003; Carmichael et al., 2003).

Overall, the study's length initially portrays a bias that a higher survival rate occurs in the lower temperatures for shorter experiments. However, when the longer studies are dissected for comparable lengths, the temperature treatment appears to have less impact than initially seen in the egg-to-two-month experiment. The temperature mortality conclusion is not as solid as initially thought for these shorter tests.

The frequency of feedings and water changes differed from each experiment, affecting survivorship. For the experiments with smaller containers, there were more water changes to balance tank conditions and feed increases. The bigger systems were

able to have less water maintenance due to a stricter maintenance routine with filtration systems in place. The balance of feeding the necessary amount of food without creating harmful water conditions was a struggle for the longer-term experiments. This created numerous opportunities for stress as the horseshoe crabs did not have sediment on the bottom of the containers to buffer them during water changes. The constant water changes due to smaller containers in instar 2-to-six-month experiment could have contributed to the highest mortality seen in all three experiments.

For the egg to six-month experiment, the 10L containers proved to be detrimental to all temperature treatments. The rapidly growing horseshoe crabs required larger amounts of feed, worsening tank conditions and requiring more water maintenance. This created the negative cycle of constantly cleaning the tanks to maintain clean conditions, while also not providing enough nutrients to the animals in the tanks. This led to a spike in mortality as they started to cannibalize each other. Furthermore, the egg to six-month experiment density constraints compared to other experiments further intensified the chances for cannibalism opportunities. When this cannibalism was experienced in high volume, the larger instars had begun to outgrow the 10 L containers. Once they were moved to the larger area, they received increased feed, and the amount of cannibalism drastically reduced.

The combination of high density of the horseshoe crabs outgrowing their enclosure in the higher temperatures and the limited food supply was the likely cause

of the cannibalism. Cannibalism is typically seen in blue crabs and other animals classified as more aggressive than horseshoe crabs. However, (S. A. Smith & Berkson, 2005) mention that traumatic injuries in captive horseshoe crabs can result in missing appendages and dead animals from cannibalism. They mention that cannibalism typically happens during the molting process (S. A. Smith & Berkson, 2005). Wild horseshoe crabs have also been shown to be cannibalistic (Shuster & Sekiguchi, 2009). The lack of places to burrow, limited space, and overcrowding can result in traumatic injuries (Derkson et al., 2019), which can lead to further problems. The overcrowding, lack of hiding places, and insufficient food could have increased mortality in this experiment.

Furthermore, certain procedures also contributed to challenges to the horseshoe crabs in the experiments. The twisted legs and getting stuck mid-molt could have been due to stressful conditions causing problems during the molting process. Molting represents one of the riskiest times during a crab's life, when they are exposed and need to consume lots of resources. Molt death syndrome is the name that some use to refer to a condition where molting crabs cannot successfully extract themselves from their old exoskeleton. This process is commonly thought to be attributed to improper nutrition, water parameters, or potential pathogenic infections (Michael, 2023). They are unable to complete their molting due to being deprived of the necessary nutrients needed to move to the next stage. Furthermore, it is known that increased temperature conditions affected the shell hardness of *brachyuran* crabs

(Azra et al., 2019), which could be a commonality between molting stress for all aquaculture-raised molting creatures. Not only do these horseshoe crabs experience this problem due to stress, but it could be exacerbated at higher temperatures.

For the infection observed on two horseshoe crabs in the egg to six-month experiment, the exact cause was not determined as limited information on horseshoe crab diseases in captivity have been studied. However, of the studied infections in cultured horseshoe crabs, there is a focus on algal and fungal diseases (LaDouceur et al., 2019). These fungal infections have historically only been reported in captive animals, potentially due to the rarity of pathology reports on these conditions. Green algal infection is a common infection type for many horseshoe crabs with their carapace negatively impacted by this growth (Braverman et al., 2012; S. A. Smith & Berkson, 2005). Another common horseshoe crab disease is the presence of parasites covering the carapace and those in the connective tissue of many organs (LaDouceur et al., 2019). Due to the nature of the infection seen on the experimental horseshoe crabs, there is a high likelihood that this infection is a type of fungal disease that affects the carapace of the horseshoe crabs. These spots appeared on animals in the lab outside of the experiment, which leads to the belief that this fungal infection could have arisen from cross contamination.

2.4b Recommended Care Procedures

For shorter-term experiments, it is recommended to use Petri dishes/wells as they are easy to manage and allow a larger surface area for the horseshoe crabs to

move about. Aeration is recommended but not required in the initial months. MWF maintenance and water changes proved to be sufficient. This setup is ideal for approximately two months or until horseshoe crabs reach instar 3 where they need more space.

For longer-term experiments, prioritizing bottom surface area is essential. Incremental increases in enclosure sizes throughout their lives allows a closer study of individual animals while also providing the necessary living conditions. Having aeration and filtration in these systems is essential as it prevents buildup from the surplus of food required for high-density batches of horseshoe crabs. The MWF feeding and maintenance allows more flexibility in the researcher's schedule, but the weekend conditions can change rapidly.

Some culturing methods to avoid involve using many smaller containers. These containers require constant maintenance, which causes more stress to the horseshoe crabs. Also, having sediment at the bottom of the enclosures made water changes and measurements harder for research experiments.

For temperature, it is recommended to keep these horseshoe crabs at the MT conditions of 23-25 °C as they provided accelerated growth with survivorship similar to RT conditions.

2.4c Future Directions

This work could apply to industry and reintroduction programs for *L.*

polyphemus. Having tested many different types of enclosures works toward scaling up these culture experiments, which will be essential if others want to produce horseshoe crabs on an industry level. To successfully create these products on a larger scale, experiments need to be done on bigger batches of horseshoe crabs in these specified conditions.

Due to the accelerated growth in the higher temperatures with one horseshoe crab in a HT treatment reaching the size of a wild three-year-old in six months, the goal is to try and grow horseshoe crabs to adulthood in less than three years in these higher temperatures. More long-term experiments need to be done with horseshoe crabs grown to adulthood in higher temperatures.

Chapter 3: Coordination of Horseshoe Crabs in the Classroom with the Maryland Department of Natural Resources.

3.1 Overview

An experimental-based curriculum enriches classroom experience while inspiring interest and critical thinking in the sciences. The Maryland Department of Natural Resources (MDNR) sponsors a program called Horseshoe Crabs in the Classroom. Horseshoe Crabs in the Classroom focuses on introducing public elementary, middle, and high school students to the responsibility of live animal care while running experiments with these animals. This program partnered with the Institute of Marine and Environmental Technology (IMET) to create a scientist-approved aquaculture program to raise horseshoe crabs in educational environments. This chapter focuses on the collaboration between IMET and MDNR on creating an applied research project centered around adapting horseshoe crab aquaculture practices for school teachers. With input from the teachers in the program, the successes and failures of the program will be addressed.

Overall, this program reached students of diverse age groups/backgrounds while being individually tailored to the schools that participated in the program, whether through a classroom setting or an individual capstone project. While these

initial implementation years of the program have had their ups and downs, the positives have outweighed the negatives, creating the potential for a long-lasting, meaningful program.

3.2 Introduction

3.2a Importance of Hands-On Education Programs

In the United States, it has been reported that there has been a historically lower amount of public literacy surrounding ocean issues (Steel et al., 2005). As ocean issues are tied with many other global processes, a basic understanding of these issues allows better understanding of bigger challenges like climate change and other global phenomena. Most science curriculum is focused on land processes instead of the two thirds of earth covered in oceans (Madden et al., 2023). This leaves only a small fraction of the curriculum focused on ocean issues, with this topic's likelihood to be taught directly impacted by the school's distance from the coast and the socioeconomic status of the students (Madden et al., 2023). Students in higher economic status have access to better technology that allows information to be acquired at a faster rate, creating knowledge gaps between groups of students. Overall, this leads to fewer students being taught ocean science in an effective way.

While some argue that ocean issues only affect individuals who live and interact with the coast, these issues have larger repercussions worldwide. It has been shown that while people on the coasts do have more knowledge about these issues

than others, they still have trouble identifying key academic terms/themes about the ocean (Steel et al., 2005). This lack of knowledge begs the question of where people receive information on ocean issues and how to better fill these gaps to give the general population a better understanding of ocean issues.

Outside of formal education sources, the main sources of information on ocean issues are taught through media sources. While most of this knowledge comes from television and radio, these sources have been shown to not serve as effective knowledge retention sources for ocean science (Steel et al., 2005). To bridge this gap between interest and understanding, further implementation strategies are necessary to create long-lasting knowledge. One of the most founded ways to increase retention for ocean science has been hands-on experiences (Steel et al., 2005). The National Association of Biology Teachers has recommended hands-on activities in biology classes for quite some time and their mission statement highlights the importance of learner-centered practices (National Association of Biology Teachers, n.d.)

So, what is classified as hands-on learning? Hands-on learning refers to an activity that gives students physical practice/experience with equipment, giving them a more realistic and exciting introduction to whatever they are doing. While hands-on experiences have long been thought to invoke more student participation and interest, it is argued that the level of interest and relevance of the experiments to the students lives affected the success of these types of programs (Holstermann et al., 2010). To make a successful hands-on experience, it must pique students' interest, connect to

their daily lives, and provide a well-designed curriculum to implement various experiences of hands-on activities.

The sciences have always been a rapidly evolving field that relies on experimentation and adaptation. To keep up with this change, classrooms need a mixture of teaching and experiments to engage students with these shifting circumstances (Moore, 2003). These experiments teach students to incorporate multiple cross-disciplinary skills, from successfully writing a report, analyzing results, and understanding the background to what their findings mean. Students have historically been shown to have trouble connecting cross-disciplinary problems, like ocean acidification, taught in the classroom to the real world. While this problem might be taught in biology and environmental classes, to better understand the problem, an understanding of chemistry is needed. Hands-on labs that are able to incorporate multi-disciplinary problems are crucial for understanding and retention (Roche Allred et al., 2022).

By using the student's engagement and interest in the hands-on experience aspect of the curriculum, teachers are better able to mix in harder-to-understand concepts in a way that allows better retention. Furthermore, it was found that students with disabilities learn science most effectively with hands-on experiments (Scruggs et al., 2007). Not only do hands-on activities provide real world connections that create more engagement, they also provide some of the most effective ways of allowing many different types of students' exposure to concepts previously underappreciated.

There is widespread teacher interest in learning more about marine science and climate change to teach their students better (Madden et al., 2023). While this enthusiasm is appreciated, the marine sciences have been historically exclusionary to the average individual and directly impacted by many factors. Marine science as a career has not been viewed as a viable option for many due to barriers in education cost, high job competition, and low pay in the field (Windleharth et al., 2023). Certain background factors influence the likelihood of going into marine science, like proximity to the ocean, family income, gender, and ethnicity (Windleharth et al., 2023). This is why exposure is vital, especially for students who do not usually have access to the ocean.

3.2b Why the Horseshoe Crab

The American Horseshoe crab, *L. polyphemus*, inhabits the coast of North America from Maine to the Yucatan peninsula (S. A. Smith & Berkson, 2005). Horseshoe crabs have an approximately 18-22 years lifespan with reported tolerance ranges of -5-35°C and 5-30 ppt (S. A. Smith & Berkson, 2005). These creatures are arthropods that shed molts as they grow older. They have complex egg stages and have reported high resilience to many external pollutants and temperature stressors (M. Botton et al., 2003; M. L. Botton & Itow, 2009). Horseshoe crabs are hardy, long-living creatures adaptable to aquaria equipment at an early age.

The horseshoe crab is a perfect example of an animal with many interested parties effecting its conservation and survival. There are the biomedical industries

that use the horseshoe crabs' blood for LAL production, fishermen who harvest them for the whelk and eel bait industry, and conservationists asking for protective restrictions on harvesting. Non-profit, industry, and state/federal government agencies are all tied up in horseshoe crab regulations and permitting. The Atlantic horseshoe crab is also a species classified as vulnerable by the International Union for Conservation of Nature (IUCN). For a creature considered one of the oldest and unchanged evolutionary creatures, there is a very complicated network of individuals interested in their survival.

Horseshoe crabs are also a relatively unknown species for those who do not grow up surrounded by the coast/estuarine waters. This gives the perfect example of a creature that draws the students' interest due to its rarity and uniqueness. Horseshoe crabs also have enough background curricula attached to their interaction with the environment and outside organizations to create multiple lesson plans over the year. They are the perfect opportunity to create a unique hands-on project that interests students while also analyzing the political side of conservation politics in marine science.

3.3c History of the Horseshoe Crabs in the Classroom Program

The Horseshoe Crabs in the Classroom program run by the Maryland Department of Natural Resources (MDNR) initially started as "Raising Horseshoe Crabs in the Classroom," which was launched in 1999 aiming to improve the environmental literacy of students in Maryland. This program was created through a

partnership of MDNR and a workshop called “Green Eggs and Sand,” which provides curriculum and lesson plans about horseshoe crabs centered around formal and non-formal educational programming with a multi-state focus.

The program aimed to expand awareness of horseshoe crabs and their natural history/ecology by raising juvenile horseshoe crabs in middle and high school classrooms across the state of Maryland. However, this program was defunded for a period of time in the 2010s through early 2020s. This program was reintroduced in the summer of 2023 through the coordination between IMET and MDNR (**Error! R**

eference source not found.).

Figure 22. ***Logo of the Horseshoe Crabs in the Classroom Program****. Logo was created by the Maryland Department of Natural Resources.*

3.3 Restarting a Program: 2023-2025 Setup and Instructions

This chapter follows the reintroduction of “Horseshoe Crabs in the Classroom” following the 2023-2024 and 2024-2025 school years and the collaboration between IMET and MDNR.

Overall, the general timeline for the program began with IMET and MDNR

personnel traveling to Ocean City, MD, where they would collect the eggs from actively spawning horseshoe crabs at the end of June. These eggs were then grown or housed in refrigeration in the aquaculture research center at IMET through the summer. Teachers in the program received the eggs in August after a training led and run by personnel from IMET and MDNR. After receiving the eggs, teachers would begin the program at their respective schools. Halfway through the school year, representatives from IMET and MDNR visited all the schools in the program to assess how the program was running and give replacement horseshoe crabs to teachers who requested more. At the end of the year, all horseshoe crabs grown in the program were released in May at Sandy Point State Park in Maryland.

3.3a Year One: 2023-2024 School Year Setups

Horseshoe eggs were obtained from Ocean City, MD, on June 20th, 2023, from Skimmer Island and the Maryland coastal bays of northern Assateague National Park. These eggs were rinsed with filtered water and refrigerated at IMET from June until August 2023. The refrigeration slowed growth and prevented hatching. In August 2023, schools in the program received training from IMET and MDNR on how to raise the horseshoe crabs. These schools were chosen due to their previous connections to MDNR and other “in the classroom” programs.

Six public middle and high school teachers received about 100 horseshoe crab eggs to take back to their schools. The schools included Mountview Middle School, Glenelg High School, Francis Scott Key High School, Dunloggin Middle School,

Nicholas Orem Middle School, and Glenwood Middle School. These schools were mostly located in Howard County, MD with one in Carroll County and one in Prince George's County.

After receiving the eggs, they were distributed into 12-well plates labeled 1-100, with individual eggs placed in each well. After the horseshoe crabs hatched, they were transferred to the 6-well plates. As they grew, 20 horseshoe crab survivors were transferred to 1L mason jars filled with sand and an aerator with one horseshoe crab per jar (Figure 23). The remaining hatched horseshoe crabs were placed in a 10-gallon tank with sand (Figure 24). The horseshoe crabs were fed diluted frozen brine shrimp, with the amount incrementally increasing as time went on. Growth of the horseshoe crabs were tracked and monitored by teachers and students in the program.

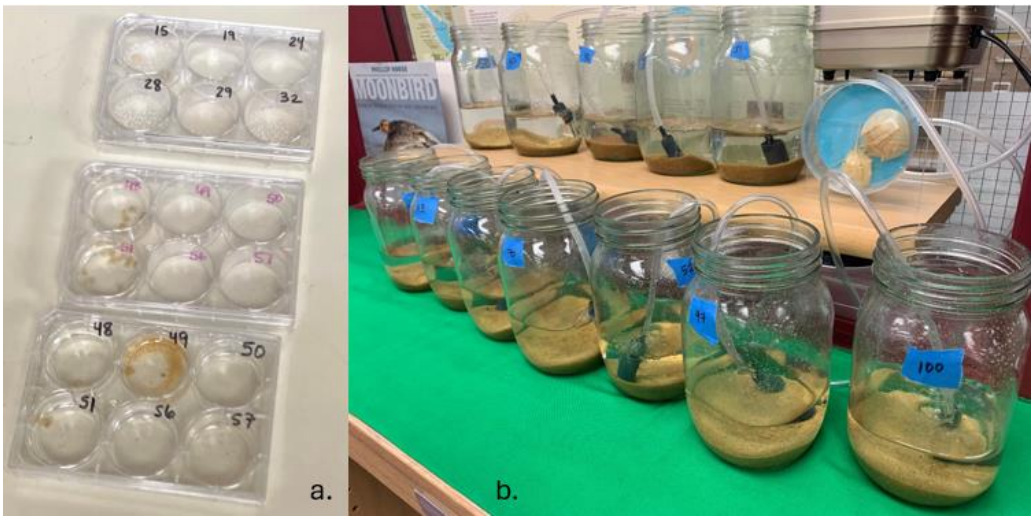

Figure 23. ***Year 1: 2023-2024 Egg and Early Juvenile Setup****. (A) 6-well dishes that the hatched horseshoe crabs were placed in and the corresponding number of horseshoe crabs from the 12-well dishes. (B) Jars for the 20 juveniles chosen to be studied and their corresponding number on the jar.*

Figure 24. ***Year 1: 2023-2024 Full setup from Glenwood Middle School.*** *Lois Han and the other library educational staff created this setup. The left side includes the twenty mason jars with sand and aeration, and on the right is the 10-gallon tank for the remaining horseshoe crabs. This setup was located in their middle school library, serving as a conversation starter with the provided posters and books about horseshoe crabs displayed.*

Five out of six teachers in the program received in-school visits in January 2024. Representatives from IMET and MDNR assessed the setup, provided recommendations, listened to teachers' feedback regarding the program, and delivered about 30 extra juvenile horseshoe crabs grown in IMET to replace the high mortality experienced in most schools. It is important to note that one of the teachers could not be contacted after the initial training and was not visited or included in the results.

At the end of the year, the horseshoe crabs grown in the classrooms were released at Sandy Point State Park in Maryland on May 30th at the MAEOE youth summit sponsored by MDNR. An end-of-the-year survey was sent out to all the teachers after this release. This survey was a Google form sent out through email that

covered student participation, ways of engagement, program improvements, teacher response, etc.

3.3b Year Two: 2024-2025 School Year Setups

New instructions and procedures were written over the summer of 2024 by Jessica Baniak (IMET) and Stephanie Tuckfield (MDNR) using the comments and new ideas from the teachers. New procedures were implemented for this school year, especially concerning instructional material and early juvenile growth enclosures. Furthermore, more schools were added to the program.

The eggs for this year's procedures were acquired on June 20th, 2025 from Skimmer Island and the Maryland coastal bays of northern Assateague National Park.by IMET and MDNR representatives. These eggs were rinsed with filtered water to remove the external debris and ensure that no bacterial infections occurred in all of the eggs. After thoroughly rinsing the eggs with 0.22 µm filtered 30 ppt artificial seawater, they were divided into groups for refrigeration or growth. For refrigeration, eggs were divided into 12-well dishes to include about 40-50 eggs for each school. The remaining eggs were stored in a heated water bath at approximately 24-25°C where they were raised until instar 3 before being given to the teachers.

About 40-50 eggs and 40-50 juveniles were distributed to the teachers in August at the 2024 workshop training conducted by IMET and MDNR. Ten schools were included in the program for this year with five returning and five new schools. These schools included Mountview Middle School, Glenelg High School, Francis

Scott Key High School, Dunloggin Middle School, Glenwood Middle School, Pointers Run Elementary School, George Washington Carver Center for Arts and Technology, Francis Scott Key Elementary/Middle School, Thomas Jefferson Elementary/Middle School, and North Caroline High School. Of the schools included in the program, five were in Howard County, two were in Baltimore City, and one was in Carroll, Baltimore, and Caroline Counties.

Since teachers received both eggs and juveniles, multiple setups were running simultaneously to allow students to always have horseshoe crabs to engage with. For the eggs, 6-well plates were used for 1-2 eggs in each well. Egg stages were tracked, and a guide for egg stages was provided. Egg development in the wells took place through the first couple of months. After the juveniles hatched from the eggs, some were moved into the "hatchery system" or 10-gallon tank. The juveniles received from IMET in August were immediately placed in the hatchery system or the 10-gallon tank.

Figure 25. ***Image of Hatchery System Setup for Year 2.*** *The hatchery system consists of a tray of 10 cups with mesh bottoms in a water bath with a heater and bubble filter. The cups are labeled with*

numbers from 1-10.

The hatchery system contains labeled cups from 1-10 with mesh bottoms (**Error! Reference source not found.**). These cups were placed in a heated water b ath with a couple of inches of water for each submerged cup. This allowed easier water maintenance for the juveniles over the twenty jars used in the previous years. The water bath included a heater for steadier temperatures and a bubble filter to provide filtration. The juveniles received from IMET were used for half of the cups and the rest contained juveniles hatched from the eggs later in the year. The remaining horseshoe crabs were moved to a 10-gallon tank with sand, a heater, and a bubble filter (Figure 26).

Figure 26. ***Year 2: 2024-2025 Setup from Pointers Run Elementary School.*** *This setup was designed by Eric Jayne, a 5th-grade teacher from Pointers Run Elementary School. On the left is the running hatchery system with the clipboards for the kids to record data. Also imaged are the tanks that help the different horseshoe crabs.*

Half-year school visits occurred in January and February 2025, during which

representatives from IMET and MDNR arrived to assess the setup, adjust to the teacher's needs, and deliver an extra about 30-40 juvenile horseshoe crabs to replace mortality and continue the program (Figure 27).

Figure 27. ***Replacement Horseshoe Crabs.*** *These horseshoe crabs were delivered to North Caroline High School on January 29th, 2025.*

After the school visits, Google Form surveys were sent out in February 2025 to all the teachers with similar questions as the previous year. The teachers released their horseshoe crabs on Thursday, May 29th, 2025, at the MAEOE youth summit sponsored by MDNR.

3.3c Survey Questions

For both years, surveys were sent out, one at the end of the year for 2023-2024 and one in February for the 2024-2025 year. These forms were made by Google Forms and sent to the teachers in the program through email. The survey questions were approved by the IRB protocol through UMBC for each year. They were deemed

"Not Human Subjects Research" as they focused on the program's successes and shortcomings.

The questions were relatively the same from year to year, with minor changes in the wording and one additional question (question 12) in the second year that allowed the teachers who participated in earlier years to give more feedback. The questions included were:

Question 1: About how many students participated in your school's Horseshoe Crabs in the Classroom program? (This includes helping out one time or heavy hands-on involvement).

Question 2: Check all that apply: Ethnicity/Race of students involved in the program:

Question 3: If you are comfortable, please answer the rough estimates/percentages of students in the race/ethnicity represented in this program. Ex) "To the best of my knowledge, about half of the students were …, while a quarter of them were …"

Question 4: What grades participated in this program at your school? Please select all that apply.

Question 5: For your school, how did you get students involved in the program (through class, a club, etc.)?

Question 6: What factors about this program made it attractive to participate in?

Question 7: Overall, did the program inspire positive and/or negative reactions from

the students participating in it? Examples are appreciated.

Question 8: What were the overarching challenges of this program?

Question 9: What do you feel was successful about this program?

Question 10: Were the school visits and check-ins by Stephanie Tuckfield and Jessica Baniak helpful to the program?

Question 11: Recommendations/tips for future years?

Question 12: If you participated in a previous year of Horseshoe Crabs in the Classroom, how did this year compare to the other years? Were this year's procedures more straightforward to follow, or are there aspects of earlier years that you would like to keep for the future?

Question 13: Would you recommend this program to other teachers?

3.4 Results

3.4a Year 1: 2023-2024 School Year

Hands-on Aspects:

Due to the flexibility in the directions, teachers had the freedom to construct their setups in several different ways (Figure 28). The left image was taken at Mountview Middle School, where the teacher Susan Mako, set it up. Susan Mako is the resource teacher for the gifted and talented program who regularly leads seminars that allow students to experience more hands-on programs. This setup was housed in

a classroom based in the library. The right image was provided by Emily Fair from Francis Scott Key High School where a student ran the setup. For their school, this program was used as a high school capstone project where, at the end of the year, they wrote a report and presented it. Emily Fair teaches Honors Biology, Honors Science curriculum, and research seminars that correspond with the fully operational aquaculture center in the basement of their school. She is involved in multiple educational aquaculture projects that her students develop and run with the help of the state department for certifications.

Figure 28. ***2023-2024 Horseshoe Crab in the Classroom Setups.*** *(A) Setup from Mountview Middle School by teacher Susan Mako. (B) Setup from Francis Scott Key High School by teacher Emily Fair with the setup being run by a student.*

Overall, the 2023-2024 program included six schools across MD (Figure 29), with five schools responding to the activities and contacting the program coordinator. Four out of the five schools were located in Howard County and one in Carroll County. Since these teachers were in the first year after restarting this program, most had previous connections to MDNR and/or had experience with aquariums. One of

the teachers had previous exposure to the original horseshoe crabs in the classroom program and most other teachers were participants in other "…in the classroom" programs sponsored by MDNR and Maryland Sea Grant.

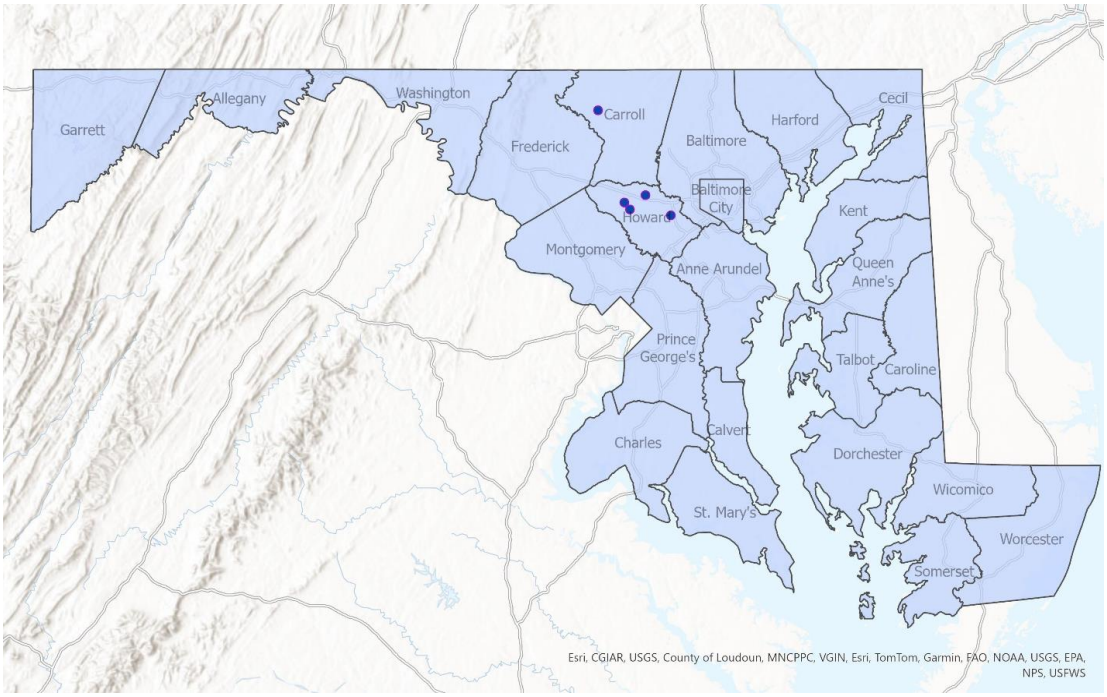

Figure 29. ***School Locations for 2023-2024.*** *Image of Maryland counties and the locations of five schools in the program. The lines are the different counties in Maryland. Created using ArcGIS Pro.*

Survey Results

Overall, approximately 71 students directly participated in the program across all schools. The total average number of students for each school was around 14, with a median of 12. However, this number varied greatly between the schools as the minimum number of students for one school was a single student, and another classroom included 30 student volunteers, with their findings reported to the whole school regularly, which exposed the program to approximately 900 students in that

school. Of the five schools followed up with, three were middle schools, and two were high schools. The middle schools each were able to incorporate all three levels of sixth, seventh, and eighth. The two high schools both focused on eleventh and twelfth graders.

For the demographic data, teachers were asked to provide the races/ethnicities of students included in the program. Since this question was optional and rooted in teacher estimates, these predictions do not give the exact diversity of students in the program, but provide an estimate of this breakdown. The ethnicity/races listed in the survey included Asian, Black or African American, Native Hawaiian or Other Pacific Islander, White, Hispanic or Latino, Middle Eastern or North African, and Other. These categories were chosen based on census ethnicity/race categories.

Overall, all of these ethnicity/race categories were represented across the schools in the program. All five schools had students in the category of White, four schools had students classified as Asian, three schools had students classified as Middle Eastern or North African, two schools had students classified as Black or African American, and one school had students classified as Native Hawaiian or Other Pacific Islander. From the provided teacher estimates of the race/ethnicity of students in the program and the number of students given for each school, for the program overall; about 46% were classified as White, 27% were classified as Asian, 10% were Middle Eastern or North African, 7% were classified as Other, 6% were classified as Black or African American, and 4% were classified as Hispanic or

Latino. Overall, this program was mostly students classified as White, Asian, and Middle Eastern or North African.

Student involvement in the program spanned many different facets. The two high schools incorporated the program into their teaching curriculum. One connected it to a science electives class called “Honors Science Research,” and another connected it through a Marine Science class offered to eleventh and twelfth-grade students. The marine science class involved about 20 students taking turns caring for the horseshoe crab during class time. The Honors Science Research class allowed one student to make this program their capstone project. Of the middle schools involved in the program, two schools gathered student participation through clubs, and one school had students sign up to participate through online applications to the whole school. One of these clubs included an environmental club responsible for all animal care. For others, students would sign up and receive SSL hours to feed the animals during off periods.

When the teachers were asked what the greatest pull of the program was for them, they all emphasized the importance of hands-on research. Due to budget constraints and ease of use, online science simulations have become increasingly popular in recent years. One teacher wanted to participate in the program because it offered, “authentic, first-hand field experience.” The teachers also emphasized the importance of working specifically with horseshoe crabs. These horseshoe crabs allow the students to learn about environmental stewardship and partner them with

MDNR, a real-world example of a job opportunity they can work for in the future. Furthermore, these creatures connect the students to the waterways around them and how what they do in these waterways might affect the creatures farther away. One teacher shared that their students had been interested in horseshoe crabs for a long time, and another school recalls students' interest in horseshoe crabs from seeing washed-up bodies on the beaches.

The program had its ups and downs, which is expected from any new program. Some of the program's successes included student engagement. Students were extremely eager to participate. One teacher reported that students felt a sense of pride in participating in the program as they cared for the creatures while working with a well-known government agency. Students didn’t have much previous knowledge about the complexities of horseshoe crabs in the wild, and this program allowed them to learn about a creature that many others do not get exposure to.

One of the program's biggest benefits was the full-year aspect of receiving the eggs in the summer and releasing them at the end of the year. While many schools had to have their horseshoe crabs supplemented after the initial batch perished, this complete cycle held their interest. The students also had the opportunity to learn how to gather data like that seen in scientific experiments. They learned to focus on animal care, as the horseshoe crabs are more demanding than some fish in the classroom programs.

On the other hand, the program had numerous problems regarding mortality

and vague directions. This program was labor intensive, with multiple water components and shifting enclosures for the horseshoe crabs as they age. Unique and new setups with significant flaws were attempted. Having 20 individual jars with aeration proved extremely difficult. Also, an important difference from growth in a lab environment included the unpredictability of school schedules and power reliability. Certain schools have A/B schedules, which meant irregular days for students to care for the horseshoe crabs. Other schools have power turned off on the weekends. Furthermore, school breaks also proved to be a hassle for the teachers as they had to maneuver enclosures back home to ensure the horseshoe crabs were cared for over break. On top of this, unpredictable snow days also factor in mortality as most teachers do not have access to their buildings during those times, which can create long periods away from the horseshoe crabs. Another constraint for the program was the necessary space requirements for the teachers. Most teachers have limited counter space in their classrooms, and finding space for these setups can be challenging.

However, the most significant challenge for the program was the high mortality experienced in almost every school. Even with culturing in consistent lab conditions, only about 40-60% of horseshoe crabs survived in temperatures comparable to the school environment. Add that survival rate to the challenges in the school environment, and that mortality increases. Most teachers had almost no surviving horseshoe crabs by halfway through the year. Even with the additional

horseshoe crabs delivered from IMET, only a select few made it to the release date at Sandy Point State Park.

The high mortality rate was disheartening to the students in the program, especially the younger students getting their first exposure to animal care. Caring for animals and having them all die is frustrating and sad. While it teaches the realities of life and animal care in science, it can dissuade people from the desire to participate in the program.

Overall, having a scientist culturing horseshoe crabs in their lab partnered with MDNR allowed a back-and-forth of knowledge that created better adjustments for the next year. The teachers provided their input and tips, which could be incorporated into the program for next year. Out of the five schools, all agreed that the in-person school visits were helpful as they got the chance to communicate with the program head and hear how other schools were doing. While the program had its challenges, almost all of the teachers recommended it to other teachers with the caveat that some problems must be fixed in the program and that the teachers must have the time and energy to devote to the program.

3.4b Year 2: 2024-2025 School Year

Hands-on Aspects

As with the previous year, teachers adapted their setups to fit the physical space constraints of their school (Figure 30). The left image was taken at Dunloggin

Middle School, where teacher Dan Blue ran the setup. Dan Blue is a middle school science teacher focusing on grades seven and eight. He runs the environmental club that participated in the program. Dan Blue was a previous participant in the program and has multiple tanks from the other, "…in the classroom" programs. The image on the right was from North Caroline High School with the setup by Kimberly Mielke. Kimberly Mielke is an ESOL teacher for 9th and 10th grade who also teaches biology to multilingual learners. She has successfully adapted this program for multilingual students. The hatchery system can be seen in the center of her setup, with everything clearly labeled for ease of use.

Figure 30. ***2024-2025 Classroom Setup.*** *(A) Setup at Dunloggin Middle School by teacher Dan Blue. (B) Setup at North Caroline High School by Kimberly Mielke.*

Overall, the hatchery system proved to be more harmful than good. The idea for individual containers to track horseshoe crabs came from the "Salamanders in the Classroom" program designed by the Maryland Sea Grant. However, for that program, salamanders were housed in freshwater for their system over six weeks compared to the horseshoe crabs requiring salt water and a longer period of time. The

water's evaporation rate proved detrimental with constantly increasing salinities. Furthermore, viewing the cups in the back with limited space was difficult. While certain teachers liked having individual cups for tracking the horseshoe crabs, the overall setup proved too much of a hassle and challenge that will not be implemented in the following years.

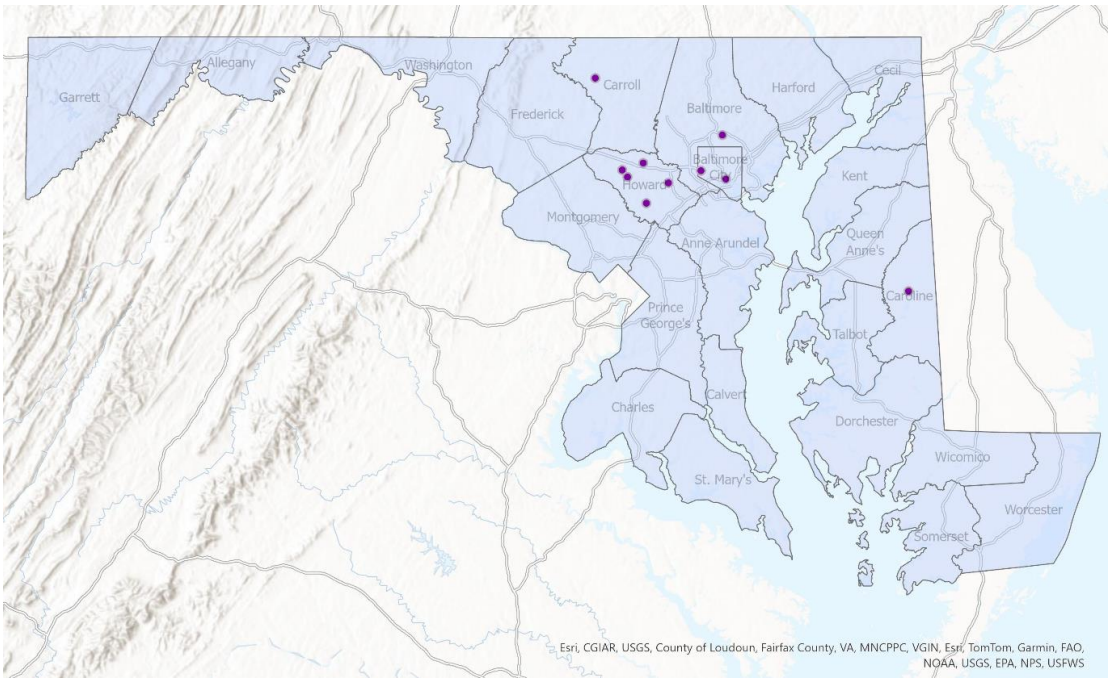

Figure 31. ***Locations of Schools for the 2024-2025 School Year.*** *The purple dots represent the location of the schools across the state of Maryland. The lines are the different counties in Maryland. Created using ArcGIS Pro.*

Overall, the 2024-2025 program participants included ten schools across Maryland (Figure 31). The five schools that participated in last year's program were included in this year's program. Of the schools included in the program, five were in Howard County, two were in Baltimore City, and one was in Carroll, Baltimore, and Caroline Counties.

Survey Results

The 2024-2025 school year survey was sent out at the beginning of February, after most of the school visits, and went until the end of March. Seven out of the ten teachers responded to the survey. The program reached approximately 200 students, averaging about 24 students per school and a median of 20. One school's smallest number of students was 10, and the largest was 40. Out of the schools involved in the program, five were middle schools, four were high schools, and one was an elementary school. In the elementary school, the students included were fifth graders chosen for an optional instructional seminar during recess/lunch break. For the middle schools, all grade levels were included. For the high schoolers, there was a focus on grades eleven and twelve.

Of the seven schools that responded, most of these ethnicity/race categories were represented across the schools in the program. Six schools had students in the category of White, four schools had students in the categories of Asian, Hispanic or Latino, and Middle Eastern or North African, and three schools had students classified as Black or African American. From the provided teacher estimates from five out of the ten schools, the race/ethnicity of students in the program included 35% of students classified as White, 31% of students classified as Hispanic or Latino, 14% of students classified as Asian, and 9% of students classified as Black or African American or Middle Eastern or North African. Overall, this program was mostly students classified as White, Hispanic, and Asian with other ethnicities/races being

less represented.

Student involvement in the program spanned from volunteer sessions during lunch breaks to organized class work. Three schools had students volunteer for the program through sign-up sheets. In some cases, these opportunities were advertised to the student TV shows for science class "pullout" seminars for students to skip their regular classes once a month to take care of the horseshoe crabs. Others had optional instructional seminars offered during recess and lunch periods. For the schools that worked the courses into the curriculum, most of them were science teachers. However, one ESOL teacher that teaches ENG 9, 10, and BIO to their students were able to incorporate the program into those sections. Lastly, other schools had school clubs, like the environmental club, to take care of animals.

When the teachers were asked to describe what attracted them to the program, the overarching reason was the draw of hands-on research. One teacher explains that, "…much of our classes in recent years are online simulations/videos…Science is messy and unpredictable. Students need to see that and understand that [most] science takes time." Others highlighted the importance of having materials provided by MDNR, allowing them to bring a program originally out of reach into their schools. They also emphasized the importance of involving live animals, which usually causes more engagement from the students.

Six out of seven teachers responded with an overwhelmingly positive message when asked if the program inspired positive or negative student reactions. Teachers

responded that they had students who usually didn't participate in class interact with the program. Whenever the tanks were displayed in public locations in the schools, students outside the program would show interest. One teacher said that the students "took pride in their role and responsibility," and another said that students "don't mind giving up their recess to learn." One teacher told how this experience motivated a student to apply for an internship at the national aquarium, located next to IMET, after being the lead caretaker for the horseshoe crabs at their school. For the one teacher who reported a more negative reaction, they remarked that the high mortality was difficult on the students.

The program held many challenges, with high mortality being a commonality between most schools. The hatchery system allowed more individual study of the horseshoe crabs but were difficult to manage for water conditions. Also, the number of snow days and breaks in the semester created a challenge for keeping the horseshoe crabs in safe condition year-round. Better containment equipment for the middle stages of the crabs is needed for future years.

The program's successes ranged from learning the intricacies of hands-on learning to maintaining student's interest. One teacher reported that, "Critical thinking skills, communication skills, and collaboration [skills] were used by all students involved." The students learned how to take observations while observing horseshoe crab development. Outside of student development, the teachers were essential to the success of the program. The teacher's problem-solved for their own

schools and devised ingenious ways to adjust the program, like creating transportation vessels to bring animals home and using plant mats as heating sources.

The school visits were helpful to those who received them as the teachers could get assurance from the visiting scientist and MDNR members. The additional horseshoe crabs were also appreciated, with one teacher remarking how the student's excitement sparked again with the larger crabs added to the tanks.

Each teacher had the opportunity to give verbal and written feedback about procedures for the following years. When asking the teachers who participated in the previous year to compare this year to the previous year, they remarked that this year's procedures were well written, but the hatchery system should change. The teachers remarked that they would like to have a way to submit their data in a streamlined way to make the program better each year. When asked if they would recommend the program to other teachers, five out of seven remarked yes, and two others remarked that the program is getting closer to what would be perfect to recommend to others.

Overall, more students were involved than the previous year, with a larger range of age groups. The program was generally well received even with the high mortality of the horseshoe crabs. Students were excited about the program and spent time outside of class to help with animal care. The hatchery system was better than the 20 jars but proved not to fit the needs of this type of program.

3.5 Summary

Overall, restarting a new program requires long periods of trial and error, even with outside help and coordination. The partnership between IMET and MDNR proved to be mutually beneficial. The scientists involved could use their knowledge of horseshoe crab aquaculture techniques from the laboratory to give more ideas to MDNR. MDNR had previous knowledge from earlier years of Horseshoe Crabs in the Classroom and served as the bridge between the scientists and the teachers. This collaboration between multiple organizations the incorporation of scientific research with outside organizations to create more meaningful end products than singular research papers.

Another success of the program was the strong connection with the teachers in all aspects of the project, from school visits that allowed them to share ideas to the surveys that provided written details about the ups and downs of the program. While these first year procedures of the program constantly shift and adapt, the overall reaction to the program was enthusiasm and excitement. The previous Horseshoe Crabs in the Classroom program had minimal interactions with the horseshoe crabs, but these new procedures created more opportunities for engagement for students. While there was the heartbreak of losing horseshoe crabs, the students and teachers still participated in the program in the following years. Most teachers were optimistic that if the survivorship was fixed than this program would be a fantastic contribution to their educational curriculum.

The kids were engaged with the horseshoe crabs and enthusiastic to participate in the program. When I visited the schools, students were excited to share horseshoe crab facts with me and describe why they liked them. The release events were bittersweet for the kids as they spent so much time/effort raising them, but they knew that the horseshoe crabs belonged in the wild. This year-round curriculum of receiving the eggs, seeing the different egg stages, and experiencing the molting of juveniles provided a very hands-on experience from start to finish.

This program was adapted to more than just science classrooms, and students could interact with the program in numerous ways. This program can be used for all ages, from elementary to high school. However, while the ages of students were from elementary to high school students, the students chosen for the program were more selective. Some of the classrooms selected only upper-level science students in programs like gifted and talented. This could result in the general student population not having as much exposure to this program. However, other schools got volunteers from the whole school and displayed the tanks in places everyone could enjoy. While certain schools had the program limited to upper-level science students, others had the opportunity to involve many different students.

The students involved in the program were predominantly White and Asian, with a mix of other demographics. While there was a larger spread of diversity in the program's second year, there needs to be a focus on diversifying the program whether through age or race/ethnicities. Programs like this are unique opportunities that

should be for all school levels and participants.

While the main challenges of the program included troubleshooting the juvenile component, trial and error is an essential component of science. The program must be flexible, allowing teachers to adapt it to their needs while also having a regimented care system. Unexpected challenges like temperature variation throughout the year, electricity being on or off, school breaks, unexpected snow days, irregular class scheduling, etc. must be considered. The procedures for the following years need to be even more thorough. This cycle of updating the program will continue, and each year will be more successful than the last.

Overall, this project used the coordination between scientific and governmental agencies to create an educational program accessible to public school teachers. These programs focused on hands-on science that will become the lifeblood of keeping students engaged in the sciences as more virtual substitutions are implemented.

Chapter 4: ICARE and Thesis Conclusions

4.1 Summary of Master's Thesis

This master's thesis included a literature review of past aquaculture studies on horseshoe crab culture conditions, three temperature experiments ranging from 6 weeks to 6 months, and two years of cooperative research with outside organizations. This work represents an applied research project incorporating scientific experiments with outside organizations to create applicable products. While chapters one and two follow a more traditional outline for the MEES graduate program, chapter three depicts the cooperative aspects of the project from a different approach. This project's motivation for integrating research with government and public organizations was due to the ICARE NSF NRT training grant (DGE 1922579) through the University of Maryland Baltimore County.

4.2 ICARE: Applied Science

The ICARE program stands for Interdisciplinary Consortium for Applied Research in the Environment, an NSF training grant awarded to Dr. Tamra Mendelson and a cohort of UMBC faculty in 2019. The ICARE program includes faculty from the University of Maryland Baltimore County (UMBC) in multiple departments, including the Department of Geography and Environmental Systems (GES), Biological Sciences, Chemical, Biochemical, Environmental Engineering,

Public Policy, and the cross-campus Marine Estuarine and Environmental Sciences (MEES) program. This training grant focuses on funding master's thesis projects centered around co-production with a partner mentor outside academia and a community stakeholder. These research projects were localized in the Baltimore/Chesapeake Region to create meaningful research for the local community without being extractive. The ICARE program strove to create research projects with more cooperative solutions and give a more diverse platform for people outside of academia to contribute while gaining valuable knowledge.

For my research, my partner mentor was Stephanie Tuckfield from the Maryland Department of Natural Resources (MDNR) and my community stakeholders were the teachers included in the Horseshoe Crabs in the Classroom program, specifically Susan Mako from Mountview Middle School in Marriottsville, MD. The project is illustrated in a concept map consisting of three parts: research, education, and outreach (Figure 32). A concept map is a visualization tool that organizes ideas on a topic in a more understandable manner. My concept map includes three main components: my research experiments, highlighted in blue; coordination with MDNR, in dark green; and external outreach components, in purple. The colors of the project change throughout time, as illustrated by the dark green and blue blending to create a lighter blue-green. This color blending represents the collaboration between aspects of the project and how they merged to be the project's next steps.

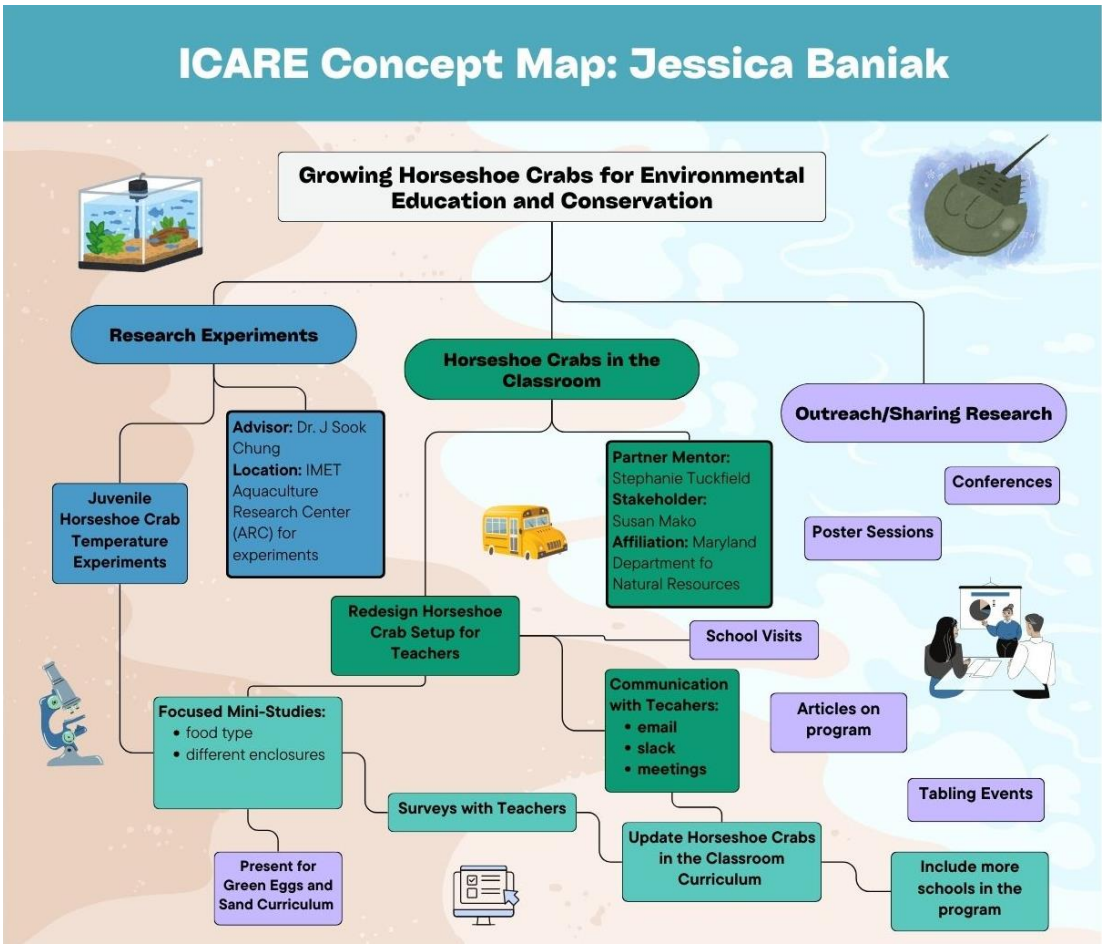

Figure 32. ***Concept Map for Research****. Included are the three main components of the research, with research experiments highlighted in blue, coordination with MDNR in dark green, and purple being the outreach added to the project. The lighter blue-green illustrates the melding of these experiments as they become more collaborative. This was created using Canvas.*

This project's first couple months was the planning and organization period during which experiments were run, and relationships between all parties started forming. One of the six-month temperature experiments was being run while communications began with Stephanie at MDNR for collaboration on a horseshoe crab research project. Dr. Chung, my advisor, assisted with Horseshoe Crabs in the Classroom for the first-year setup and procedures for the 2023-2024 school year, so

the connection between IMET, MDNR, and the schools was already in place. Through this connection, this master's project took a more integrated approach, with representatives from IMET and MDNR completing visits to schools in the program throughout the year. These visits assessed how the program was running while also delivering horseshoe crabs to the teachers. After the school visits, the collaboration between IMET, MDNR, and the teachers fully developed. This partnership led to working together to develop ideas on fixing the program for the following years.

Furthermore, the ICARE program sponsored a one-day "Co-navigator" event that brought together master's students, advisors, partner mentors, and community partners. During this training, teams were directed through hands-on exercises that encouraged planning and ideas for shaping the research projects for the upcoming months. Jessica Baniak (trainee), Stephanie Tuckfield (partner mentor), and Susan Mako (community stakeholder representative) worked together to create ideas for the future of the research and program (Figure 33).

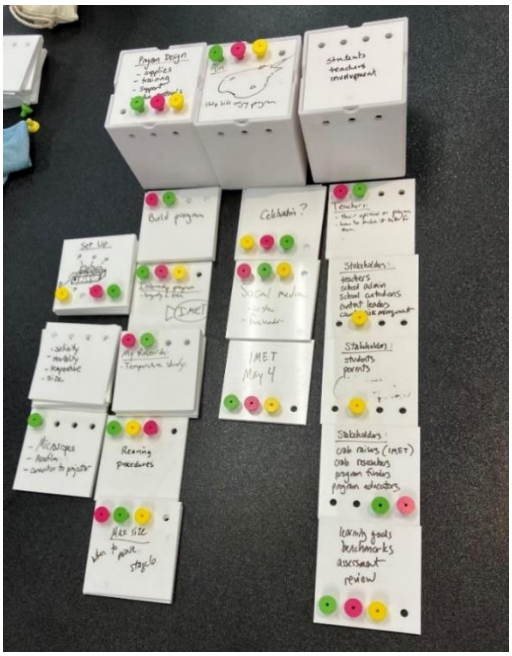

Figure 33. ***Tiles from Co-navigator.*** *These tiles were created by the collaboration between Jessica Baniak (trainee), Stephanie Tuckfield (partner mentor), and Susan Mako (community stakeholder representative) on February 23rd, 2024.*

These ideas were organized into three main categories: the overall program design, fun activities that can come from this coordination, and teacher involvement in the program. This activity allowed members involved in the research to have a clear plan for the next year.

Once program ideas were coordinated, the research could be organized into a thought-provoking, more intertwined project for the following year. Stephanie and I rewrote the instructions for the Horseshoe Crabs in the Classroom program, taking notes/directions from mini experiments completed in the lab, teacher comments from the previous year, and other "in the Classroom" programs by MDNR. Furthermore, because the ICARE traineeship is based on including community members from the

Baltimore and Chesapeake region, three new teachers from Baltimore County and Baltimore City were recruited to participate in the 2024-2025 school year.

The ICARE program also shaped this research by exposing it to different academic departments and their methodologies. Exposure to different departments, especially social science practices, created the teachers' surveys at the year's end. These surveys provided deliverables that could be analyzed and give merit to the collaboration between IMET and MDNR. Also, taking mapping and programming classes allowed better use of R Studio packages to create figures and analyze data.

Furthermore, ICARE's focus on returning science to the community motivated participation in as many public speaking and community engagement events as I could manage. These events included traditional poster sessions, lightning talks, research presentations, and outside outreach events. I presented my research as a poster at approximately six poster sessions, including the IMET poster session, two years of MEES colloquiums, the CNMS graduate student poster session, the ICARE co-navigator poster session, and the NSA poster session (Figure 34). I also gave lightning talks at these events. Furthermore, I assisted with the yearly training for Horseshoe Crabs in the Classroom and presented at the Green, Eggs, and Sand workshop on horseshoe crab education in Delaware.

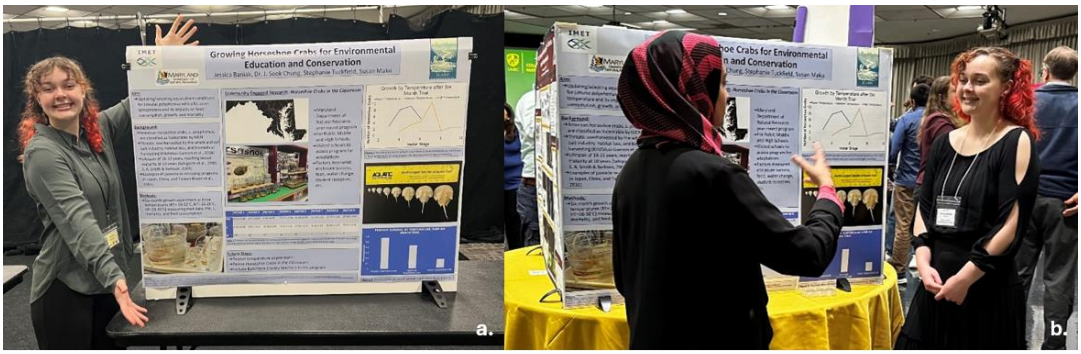

Figure 34. ***Poster Presentations****. (A) ICARE co-navigator session 02/23/2024. (B) UMBC CNMS graduate student poster session 4/12/2024.*

Other than presenting at more traditional scientific events, I became an integral part of the IMET outreach community by assisting wherever possible. This included giving tours of the aquaculture research center at IMET to visiting school groups and scientists. Some of these people included MEES curriculum staff and advisors to top university staff through the UMCES 100-year celebration.

Furthermore, I tabled at numerous events, including the IMET Open House, Masonville Cove Urban Conservation Day, STEM Unbound, etc. During these events, I would talk about the background of horseshoe crabs, how they are vulnerable, and how we use them in classroom settings to spread information to students. Outreach setups included infographics about Horseshoe Crabs in the Classroom, touch tanks of horseshoe crabs, and molting size progression (Figure 35). Most people interacting with the setups were excited about touching the horseshoe crabs. The outpouring of emotions and enthusiasm surrounding these creatures reinforced the motivation to keep working to protect these animals. The best interactions came when teachers attending these events with their students stopped to

tell me how they were in the original Horseshoe in the Classroom program. Overall, the outreach events helped communicate the science at IMET while also learning what the general public thinks about this research and what we can do to help them.

Figure 35. ***Tabling Setup.*** *(A) was taken at the STEM Unbound event hosted in the IMET building, and (B) was taken at Masonville Cove's Urban Conservation Day.*

Overall, the ICARE program acted as the motivator to create cooperative research to be used in multiple different departments, from public to governmental, while also motivating me to incorporate other elements of research into my science, creating a non-traditional master's thesis.

4.3 Conclusions on Temperature Experiments

For the three temperature experiments, overall, the temperature recommended for the highest growth with the lowest mortality was to keep the enclosures at a constant temperature within the medium temperature range of 23-25°C. The literature review in Chapter 1 of this thesis found that previous *L. polyphemus* culture studies

had an average range of 20.1 to 21.6 °C and 25.2 to 27.6 ppt. This number was comparable to the room temperature conditions in the fall and winter for my experiments. Our lab maintained the salinity for all experiments at 30 ppt throughout the experiments. By raising the temperature slightly to 23-25 °C, we saw comparable survivorships for all three experiments between room and medium temperature, but the medium temperature experiments usually reached a larger instar size. The medium temperature would allow faster growth, which gives the best chance of survival in the wild with fewer natural predators than the smaller juveniles. This is why, for studies focused on reintroducing horseshoe crabs into the wild for stock enhancement, we recommend raising them at a constant 23-25 °C.

The larger setups proved to be the most successful for longer studies as they required fewer water changes and maintenance compared to the smaller containers. The 10L containers in a water bath with a heater, aeration, and no sand were ideal for raising approximately 300 eggs/early instar juveniles for the first couple months of growth. The 10-gallon tanks with a bubble filter, heater, and no substrate proved ideal for size 4-6 instars. However, the density and space constraints became problematic when instar 7 was reached. After instar 7, they should be moved to a larger container with enough space to move around before they are released at the end of the year.

For short-term experiments, Petri dishes were ideal for the first two months. A density of 30 horseshoe crab eggs/juveniles in each dish was ideal. However, when the juveniles reach instar 2, moving them to containers with aeration proved to be

beneficial to their growth. The downside of Petri dishes was that it was harder to maintain constant temperatures with water baths as the Petri dishes would tip and spill easily.

For multi-year studies, our findings are consistent with the findings of the literature review in chapter one that a RAS/filtration system in a larger surface area is best suited to maintain the older horseshoe crabs. The longer-term experiment enclosures containing multi-year horseshoe crabs should have substrate and constant filtration for better living conditions. For shorter experiments than 6 months, sand is not recommended, as it makes it harder to find the juveniles and complete water changes.

For future directions, successful rearing practices found in this study should be performed on a much larger scale at one steady temperature to determine best practices for aquaculture centers and industries that might be interested in this investment. Also, not all horseshoe crabs responded to temperature challenges. While the majority experienced larger sizes than what would be seen in RT, a few stayed at the smaller instars of 3 and 4 throughout the experiment. This could be due to variations in plastic responses to environmental conditions. Fast-growing horseshoe crabs can be initially selected and screened using molecular markers (mitochondria cox1 and ND genes) to see if there are variations in these gene sequences in the horseshoe crab populations. This work has begun with samples from the medium temperature treatment being taken.

4.4 Conclusions on Horseshoe Crabs in the Classroom

Overall, the collaboration between IMET and MDNR for Horseshoe Crabs in the Classroom was a step in the right direction. Restarting a program always proves challenging, often in ways never expected. More work needs to be done to keep the younger juveniles alive. A focus on fixing the enclosure type and procedures for the juvenile component is needed to allow students to collect data on the horseshoe crabs.

Other than fixing some of the technical problems of the program, the students involved in the program loved being exposed to live creatures and took time out of their schedules to be involved in this program. They became connected to the animals and learned more about them because of this connection. Next year, the instructions will be rewritten to provide further clarity on setup and procedures. Additional teaching lesson plans/classroom activities will be provided to the teachers to take off some stress and allow more time to grow the horseshoe crabs. Through the information provided in the surveys, teacher visits, and online communication, the program will grow to better fit the teachers' needs and shift into a long-lasting program that will be remembered years from now.

Programs like this provide the lifeblood of keeping students interested in the sciences. With scientists receiving more scrutiny and funding cuts in recent years (Kennedy et al., 2022), it is imperative to draw interest back to the sciences and why it is so important to keep them around. Curiosity should be nurtured and encouraged, with programs like this providing an easy way to do this.

4.5 Project Conclusion

Overall, this thesis depicts an unconventional master's project that exemplifies the direction that scientific research may be heading. More and more grants are asking for applied projects that work with multiple community members. Scientific research has been viewed as an exclusive club, with only those involved in that subject ever hearing about the findings. With shifting public opinion regarding scientists (Kennedy et al., 2022), scientists must find new ways to justify their projects so that all community members can understand and appreciate them. My project incorporates a project on the conservation of horseshoe crabs into an environmental education program funded by the State of Maryland for their public schools. This allowed research that benefitted both horseshoe crabs and those who were interested in horseshoe crabs.

Future directions for reintroduction experiments include expanding on the best aquaculture practices for large stocks of horseshoe crabs for the largest crabs and the least mortality. For Horseshoe Crabs in the Classroom, future directions include perfecting the program year by year to create a steady setup that allows more and more schools to be involved in a program with a successful survival rate.

I am very grateful to be a part of a program that can draw so much interest to the sciences while also giving back to interested members. May programs like this receive the funding that they deserve in times like these.

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