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PHYSIOLOGICAL RESPONSES OF THE STATE – LISTED TEXAS PIGTOE TO ENVIRONMENTAL STRESS

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PHYSIOLOGICAL RESPONSES OF THE STATE – LISTED TEXAS PIGTOE TO ENVIRONMENTAL STRESS

by

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Marie Curie said “Nothing is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.” To those who make it their profession to teach and who taught me, you have deepened my understanding and lessened my fear – Thank you. I am grateful to those who gave of their time in helping me to understand more about the world, particularly my family who never failed to encourage me.

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Abstract

PHYSIOLOGICAL RESPONSES OF THE STATE – LISTED TEXAS PIGTOE TO ENVIRONMENTAL STRESS

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Systematic habitat destruction over the last 100 years combined with major anthropogenic stressors such as aquatic contaminants, exotic species, and economic endeavors are driving the decline in freshwater unionid species diversity. Two hundred fifty-seven individual adult Texas pigtoe (*Fusconaia askewi*) mussels (mean length, mm ± 1 SD; 58.7 ± 13.8) were collected from the upper Sabine river near Hawkins, Texas and taken to the University of Texas at Tyler to evaluate three factors likely impacting mussels in East Texas: siltation (a surrogate for bank erosion), elevated temperature and nitrogen. The impact of siltation was evaluated by burying mussels at two depths (0.25 and 0.5 meters) with a control group placed on top of the sediment. Over a 96-hour test period the resulting mortality was 15% at 0.25 meters, and 35% at 0.5 meters, with 100%

survival in the control group. In the thermal tolerance study 100% survival occurred at both the control (20°C) and the 25 °C test points. The 30°C treatment group had overall mortality of 14% and the 35°C treatment group showed a mortality rate of 43% by the end of the trial. Treatment groups for nitrogen exposure were 0, 6.25, 12.5, 25, 50, and 100 mg/L total ammonia nitrogen (TAN). Mortality resulting from nitrogen toxicity was 50% at 50.0 mg-N/L and 100% at 100 mg-N/L. There was also a significant effect of nitrogen concentration on tissue glycogen levels [$F(5, 44) = 3.370$, $p = 0.012$]. Behavioral changes in burrowing and gaping were noted in response to pollutant stress, though they were not significantly different than control mussels [$F(1, 10) = 2.966$, $p = 0.1158$], [$F(1, 10) = 3.193$, $p = 0.076$].

Chapter One

Introduction

The invertebrate phylum Mollusca is the second most diverse group of animals on the planet and consists of approximately 100,000 fresh-water, marine, and terrestrial species. About one fifth of the molluscan phylum consists of the class Bivalvia which includes marine and freshwater animals that have bodies enclosed by two hinged shells. Just over 1,200 species of freshwater bivalves are recognized (Haag, 2017) of which the largest order is Unionoida, with nearly 850 species found worldwide. Of those, over 500 species are included on the IUCN Red List of Threatened Species (IUCN, 2017). Overall, this faunal group is rapidly decreasing in numbers not only in North America where they have their greatest diversity with some 300 species, but across the world (Lydeard and Mayden, 1995; Neves, 1999; Bogan, 2008; Haag, 2012). There are 301 species listed as extinct worldwide, with 135 of those in North America (IUCN, 2017). Systematic habitat destruction over the last 100 years combined with major anthropogenic stressors such as aquatic contaminants, exotic species, and economic endeavors are driving the decline in freshwater mussel species diversity so that currently freshwater mussels make up the largest group, by percentage, of federally listed endangered or threatened invertebrates (Newton and Bartsch, 2007).

Studies designed to investigate the decline of freshwater mussels have increased as researchers have become more aware of the beneficial impacts that they contribute to aquatic habitats. The overall biomass of healthy mussel assemblages often outweighs all other benthic organisms in a given stretch of river by an order of magnitude (Layzer et al., 1993). As total mussel biomass increases so do rates of algal removal, nutrient excretion, and biodisposition of organic material (Vaughn et al., 2004). Because mussels are endobenthic and use their muscular foot to dig and burrow in the sediment, physical changes to the substrate have been observed, including sediment mixing leading to increased oxygenation and nutrient release into the water column facilitating primary production (McCall et al., 1986; Vaughn and Hakencamp, 2001). This sediment bioturbation alters chemical and microbial properties leading to changes in the abundance of co-occurring organisms (Jaramillo et al., 2017). Mussel shells can provide habitat for epibionts and refuges from predation for other invertebrates (Guiterrez et al., 2003), and for humans, they have provided cultural services such as tools, jewelry and spiritual adornments (Vaughn, 2017).

Mussels also connect to the larger stream ecosystem through their unique reproductive cycle that requires the use of a fish host. Female mussels house eggs and take in sperm released into the water column through their siphons. Once the eggs are fertilized they are known as glochidia. These glochidia are then released and must attach to a fish host, until they mature into juvenile mussels and drop off the host. Different species of mussels have specific fish hosts, and much effort is given to establishing whether mussels are generalists and can use many different fish or specialists that require a specific fish species (Haag, 2012). This parasite/host relationship with fish is an

effective method of dispersal for a mostly sessile animal and highlights an important link to understanding why stream impoundment, and the resultant separation of mussels and their fish hosts, could affect the health of mussel populations.

Surveys of Texas streams show that bank erosion and scouring of riverbeds resulting from water release from impoundments, is also problematic for lotic mussel species assemblages (Ford et al., 2009). Mussel species richness and abundance show significant declines in reaches of streams and rivers highly impacted by impoundments, and these declines decrease with increasing distance from the impoundments (Randklev et al., 2016). Rivers and streams have a natural cycle to their flows linking them to their floodplain and preventing bank erosion (Junk et al., 1989). When stream modification occurs, the natural cycle is interrupted and controlled release of large amounts of water becomes more normal rather than natural high water seasonally, causing changes in sediment load, particularly larger amounts of suspended solids, and increasingly flashy stream ecology (Landis et al., 2012). Erosion caused by agriculture, forestry and urbanization leads to a disconnect between a stream and its floodplain. This can lead to bank collapse further adding to total suspended solids which have been implicated in the decline of freshwater mussels and burying of mussels under deep sediment. (Landis et al., 2012).

One of the primary threats currently facing mussel assemblages are rising temperatures from climate change (Ganser et al., 2015). The change in average surface temperature is predicted to increase by 1.5 °C by the end of the century (IPCC, 2013). With this increase, average temperatures in water bodies will also rise tracking air temperatures (Covich et al., 1997). Mussels are considered thermoconformers whose

body temperature, and corresponding physiological processes, are regulated by the external environment. Changes in water temperatures have been shown to alter mussel metabolism (Ganser et al., 2015), and could reduce availability of energy stores, in the form of glycogen, for growth or reproduction. Losses of both protein and carbohydrates have been observed in marine mussel species exposed to increased temperatures (Gabbott and Bayne, 2009). Warmer temperatures also increase the amount of water lost through evaporation in rivers and streams, reducing flow and increasing the metabolic cost for mussels even further (Vaughn et al., 2007).

Increased levels of nitrogenous compounds in waterways because of agriculture and industrial waste are another major threat to the survival of freshwater mussels (Strayer and Malcolm, 2012; USEPA 2013). Ammonia is one nitrogen containing compound that occurs naturally in aquatic environments and which can occupy two forms; Ammonium (NH^{+}_{4}) and un-ionized ammonia (NH_3) (Haag, 2012). These two forms in equilibrium together are known as total ammonia nitrogen (TAN) and the balance is usually shifted to the non-toxic form at a neutral pH, however, there is a correlation between changes in pH and shifts in ammonia to the un-ionized form. As the pH in a water body becomes more basic, ammonia shifts to the toxic un-ionized form (Newton and Bartsch, 2006). Un-ionized ammonia is highly toxic to aquatic organisms but is usually broken down into nitrates by nitrifying bacteria. The balance of toxic ammonia can be altered by inputs of excess fertilizer runoff, or high levels of organic matter from industrial waste, making levels of nitrates dangerous (Camargo and Alonso, 2006). Mussels show a higher sensitivity to ammonia toxicity than other aquatic species, especially in the juvenile stages (Augspurger et al., 2003; Wang et al., 2007). Though

adult mussels that are capable of burrowing have an acute response to ammonia, lethal levels have not been documented for many species. Like the stress of increased temperature on metabolic activity, ammonia toxicity has been implicated in the reduction of carbohydrate (glycogen) energy stores in mussels (Chetty and Indira, 1995), a documented stress response in Unionids (Naimo et al., 1998).

Though there have been some studies which address the individual response to environmental stress (Goodchild et al., 2016; Pinkney et al., 2014) for many species no data are available. Because mussels are sessile filter feeders, and very sensitive to changes in aquatic ecosystems, they are well suited to physiological assessments in response to environmental stressors (Fritts et al., 2015).

The greatest threats facing mussels in streams in east Texas include the anthropogenic effects of pollution, temperature and flow alteration from dams and climate change. These threats can be directly lethal and have the potential to eliminate populations but are also likely to alter metabolic processes causing sub-lethal effects such as reduction in energy stores which could lead to reduced reproduction and long-term survival. The freshwater mussel used in this study, *Fusconaia askewi* (Texas pigtoe), is one species endemic to east Texas rivers and is recognized as near threatened on the IUCN red list, as special concern by the American Fisheries Society, and as threatened by the Texas Parks and Wildlife Department. The Texas pigtoe has seen a reduction in numbers but does have stable populations and is a good surrogate for less common, threatened species such as the Louisiana pigtoe and Triangle pigtoe (Howells et al., 2012). The research questions explored here are, will exposure of *F. askewi* to environmental stressors over time result in 1) increased mortality, 2) decreased tissue

glycogen and 3) an increase in avoidance behaviors? Providing data on the physiological response of this endemic, threatened species of freshwater mussel to siltation (a surrogate for bank erosion), elevated temperature and nitrogen exposure, will provide decision makers with information on the Texas pigtoe and aide in conservation planning.

Methods

Collection and laboratory housing

The Sabine River in east Texas was chosen as the collection site for this study. Texas pigtoe, while listed as threatened in Texas, is still found to be locally abundant within certain watersheds (Burlakova et al., 2012) and recent surveys assessing unionid mussels in northeast Texas rivers found Texas pigtoe to be common in many sites in the upper Sabine River and, in a wide range of age-classes suggesting active recruitment (Ford et al., 2009; Ford et al., 2014). In total, 257 individual adult mussels (mean length, mm ± 1 SD; 58.7 ± 13.8) were collected from the upper Sabine River near Hawkins, Texas on three occasions between July 2016 and September 2017. Two separate riffle areas about one half mile south of Farm-to-Market Road 14 bridge were easily accessible by boat or kayak during the summers and were shallow enough for hand collection. Water chemistry data were gathered at the time of collection (mean field parameters: pH-7.5, dissolved oxygen-6.7 mg/L, temperature-24°C, and conductivity-181 µm/cm) to allow me to closely approximate field conditions in the laboratory. Mussels were transported wrapped in towels wet with river water back to the lab at the University of Texas at Tyler within 1 hour of collection where they were weighed, measured, and tagged before being placed in temperature controlled, flow through acclimation tanks. Following methods in the most recent report by the United States Environmental

Protection Agency, data was normalized to a pH of 7 and a temperature of 20 degrees Celsius when determining the acute effects concentrations of toxic compounds for invertebrates (U.S. EPA, 2013). Specimens were acclimated to laboratory conditions for at least two weeks before testing, and fed 100 ml of phytoplankton mixture containing *nannochloropsis*, *tetraselmis*, and *isochrysis* sp. (Kent Marine PhytoPlex) every other day (ASTM, 2006; Ganser et al., 2015).

Siltation

The effect of siltation on Texas pigtoe was evaluated as a surrogate for bank collapse in field conditions. The Sabine river exhibits steep riverbanks resulting from heavy erosion with numerous bank falls reported (Ford et al., 2009). Because bivalves are filter feeders, heavy siltation is thought to result in suffocation, though the ability to unbury differs by species (Bogan, 1993).

The test chamber was a 1500 L flow-through tank with continuously flowing water to simulate a natural environment. Sand was added to the tank to completely bury the mussels to depths of 0.25 and 0.5 meters, and twenty adult individuals were buried at each depth, with an additional 20 mussels on top of the sand as a control. All mussels had individual tags on their shell, and a 1.5-meter filament line attached to a corresponding floating tag for identification. Fifteen individuals (5 from each treatment) were removed to check for mortality at 24, 48, 72, and 96 hours if they had not unburied on their own. Mussel movement was determined with a polyvinyl chloride (PVC) grid, used to note initial location and then overlaid daily to determine horizontal movement. Vertical movement was evaluated by measuring changes in filament line position above the sand (Allen and Vaughn, 2009).

Thermal Tolerance

The effects of elevated temperature on Texas pigtoe were tested through exposure of a total of 84 individuals (21 replicates per treatment) to water temperatures of 20, 25, 30, and 35 degrees Celsius over 21 days. Twenty degrees Celsius represented the baseline temperature and was also the holding temperature. The experimental units were two, large 1500 L continuous flow tanks containing approximately 15 cm of small river rock and coarse sand. Each tank was connected to a reservoir, so the water could recirculate through two loose, biological filters. De*nitrate was used to filter out nitrates, nitrites, ammonia, and organics and PhosGuard to remove phosphate and silicate. Filters were replaced before each new trial. Each unit was controlled within 1 degree Celsius by using an Arctica Titanium Chiller and a Process Technologies 1800-watt, 120-volt industrial heater, both with digital temperature control.

Mussels were acclimated to the test temperature by increasing water temperature $\leq$ 3 degrees Celsius per day (Galbraith et al., 2012). Daily water quality monitoring was done with a Hydro Tech HYDROLAB Compact DS5 for temperature, pH and dissolved oxygen following the Standard Guide for Conducting Laboratory Toxicology Tests with Freshwater Mussels (ASTM, 2006). Additionally, temperature data loggers (iButtons, Alpha Mach, Inc. Mont St-Hilaire, QC, Canada) recorded temperature every minute for the duration of each trial (Ganser et al., 2015). Every seven days, one third of the mussels per treatment were randomly selected for analysis. Tissues biopsies were collected to look for changes in glycogen as a stress indicator over time. Glycogen levels were assessed by chemical extraction from tissues followed by colorimetric absorbance on a spectrophotometer using the methods of Naimo et al. (1998). Additionally, any

mortality was recorded daily and used to determine the lethal range of temperatures for adult mussels (Pandolfo et al., 2010).

Nitrogen Toxicity

Temperature and pH change the proportion of toxic to non-toxic forms of ammonia and were therefore monitored and controlled throughout the experiment to limit their effects. Other water chemistry parameters monitored daily were dissolved oxygen and specific conductance. Because the toxic effects of ammonia are less in experiments conducted with sediment (Newton and Bartsch, 2007), all toxicity trials were conducted in eight 20-gallon tanks with 2 inches of river rocks to approximate the bottom of the Sabine River where the mussels were collected. Twelve animals (3 replicates of 4 individuals per trial) were exposed to each of six concentrations of ammonia ranging from 0-100 mg/L. Ammonium chloride was used as the source of nitrogen, and concentrations used were as follows: 0, 6.25, 12.5, 25, 50, and 100 mg/L total ammonia nitrogen (TAN) (Scheller et al., 1997).

Mussel behavior was observed in each trial once every hour to determine the amount of gaping, burrowing, moving (inactive, or relocated), and righting behaviors (realigning to a vertical position). Mussels gape when their muscles relax and their shells open. The amount of gape was determined on a scale of 0-3 (ref. figure 1).

Methods used by Bringolf et al. (2010) and Waller et al. (1999) were used and slightly modified in developing this observational format.

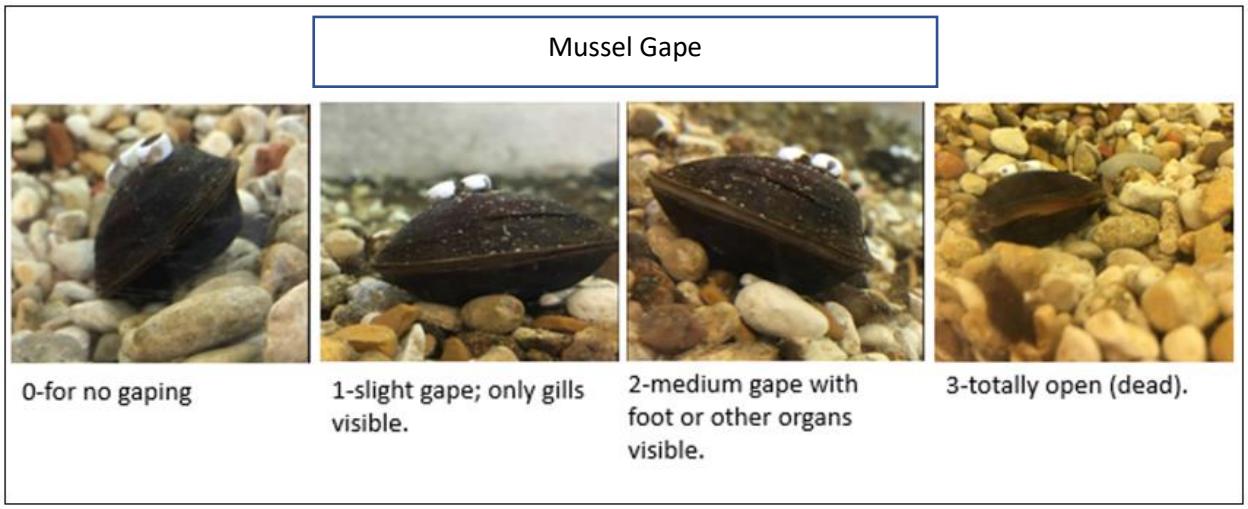

Figure 1 – image illustrating increasing mussel shell gape

Observations took place between 8 am and 5 pm for the duration of each 96-hour trial. One of the three trials in each concentration had a GoPro camera mounted in the tank recording behavior in two-hour segments. Between each two-hour segment, when the camera must be recharged, I physically observed the tank until the camera could be replaced. This video footage was in addition to observing mussels in all trials once every hour. Mortality was calculated as cumulative percent dead at 24, 48, 72, and 96 hours. Finally, tissue samples were taken for glycogen analysis at the end of each 24-hour period.

Glycogen Extraction and Analysis

Tissue samples were removed from the foot of individual mussels. Reverse pliers were inserted between the upper and lower shells to expose the tissue. A plug was removed using a pair of scissors. Both the scissors and pliers were cleaned with ethanol and allowed to air dry between each sample collection. Each sample was weighed,

labeled and stored in a minus 80-degree freezer to prevent tissue deterioration. Prior to extraction, samples were removed from the freezer and allowed to thaw. Tissue digestion and glycogen extraction were done following the methods of Naimo et al. (1998). The wet weights of the excised tissues ranged from 80 to 200 mg per mussel and were diluted twenty-fold for determination of concentration along a standard curve. Tissue samples were analyzed in triplicate with a set of standards plus a blank included on each microplate. The aqueous standard curve was linear with R^2 values exceeding 0.96. Total tissue glycogen per sample was estimated by absorbance comparison to a standard curve. A stock solution of glycogen (5000mg/L) was serially diluted to create concentrations of 1500mg/L, 1000mg/L, 500mg/L, and 150mg/L respectively. Each concentration was processed in triplicate with the same matrix and in the same manner as tissue samples. Absorbance was read on a Synergy H1 microplate reader (490 nm).

Data Analysis

A t-test was used for comparing mean size between each treatment and each replicate to ensure parity. Survivorship was analyzed with the Log-rank (Mantel-Cox) test to compare different treatments within each test. One-way ANOVA's were used to test for significant effects and analyze variability between conditions in both the temperature and nitrogen trials. If significant results were found a post hoc comparison using the Tukey HSD test was conducted.

Results

Sedimentation

The sedimentation study resulted in 15% mortality at 0.25 meters, and 35% mortality at 0.5 meters over the 96-hour test period, with 100% survival in the control group (fig 2). There was no vertical or horizontal movement in the subset buried at 0.5 meters. In the subset buried at 0.25 meters, 15% of the mussels showed vertical movement (mean 10.5 cm +/- 0.5cm) but no horizontal movement. The control group had 20% showing vertical movement downward to bury themselves (mean 8 cm +/- 5 cm), with no horizontal movement. The Log-rank (Mantel-Cox) test shows the curves are significantly different (P value = 0.0067).

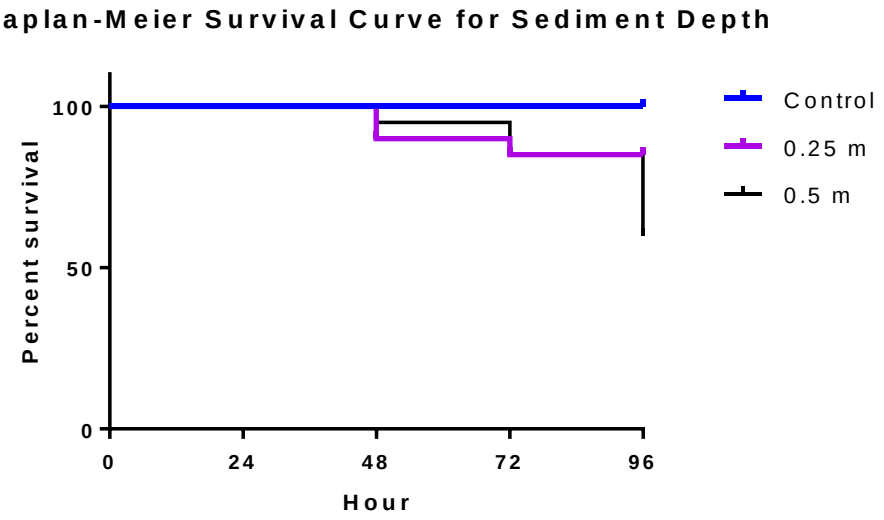

Figure 2 – Mortality in Texas pigtoes buried at different depths.

Temperature

The thermal tolerance study resulted in 100% survival at the control temperature of 20°C, and the 25°C test point. The 30°C treatment had overall mortality of 14% after

21 days, and the 35°C treatment had overall mortality of 43% by the end of the trial (Fig. 3). The Log-rank (Mantel-Cox) test shows the curves are significantly different (P value < 0.0001).

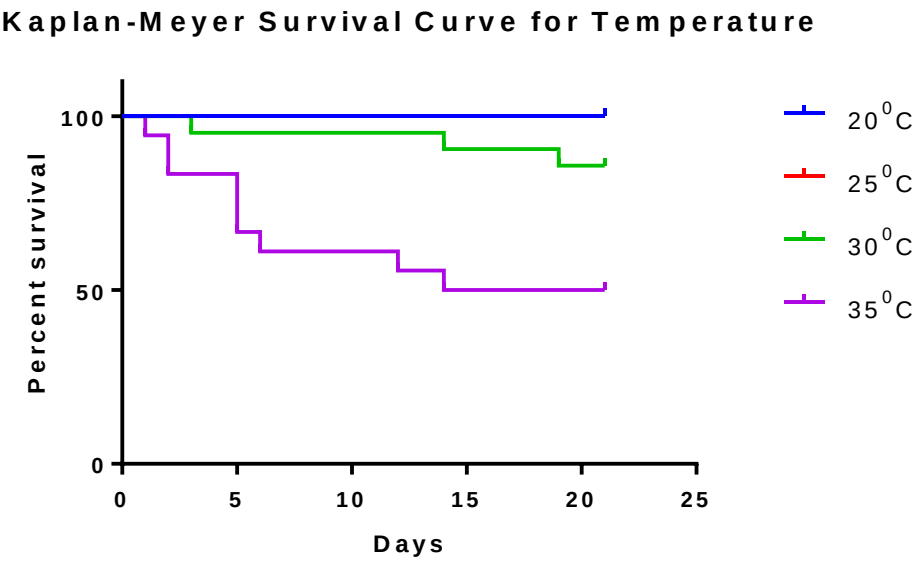

Figure 3- Mortality in Texas pigtoe at different temperatures.

Tissue Glycogen

At 20, 25, and 30°C, glycogen was not detected in more than one sample (figure 4). At 35°C, only five samples were found to contain glycogen with a mean concentration of 201.6 mg/L (range 137.3 – 268.3 mg/L).

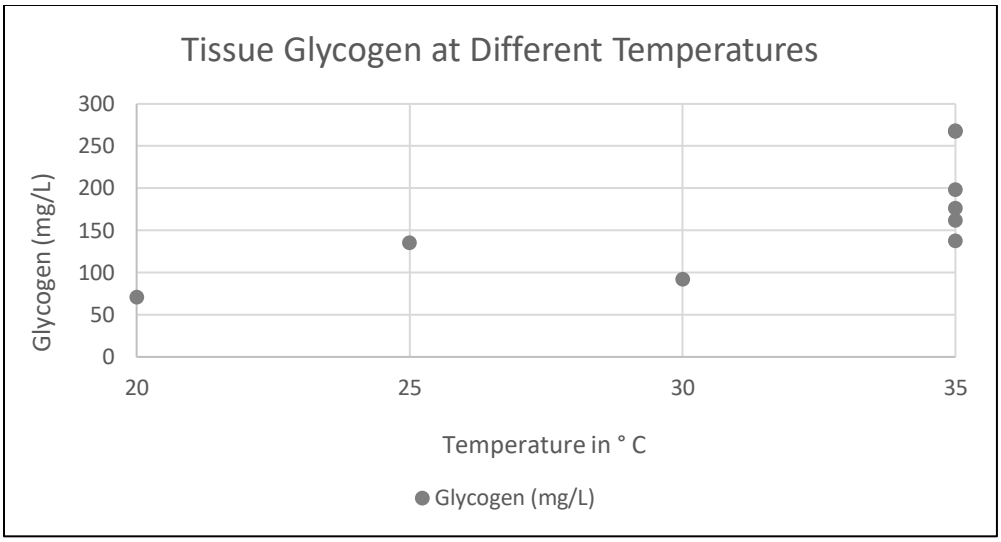

Figure 4 – Tissue glycogen concentration in Texas pigtoe at different temperatures.

Nitrogen Toxicity

No mortality occurred in the acute nitrogen exposure study, at the lowest four concentrations, apart from one individual at the 6.25 mg-N/L concentration. At 96-hours, 50% mortality was seen at a concentration of 50.0 mg-N/L and 100% mortality at 100 mg-N/L (figure 5). The Log-rank (Mantel-Cox) test shows the curves are significantly different (P value < 0.0001).

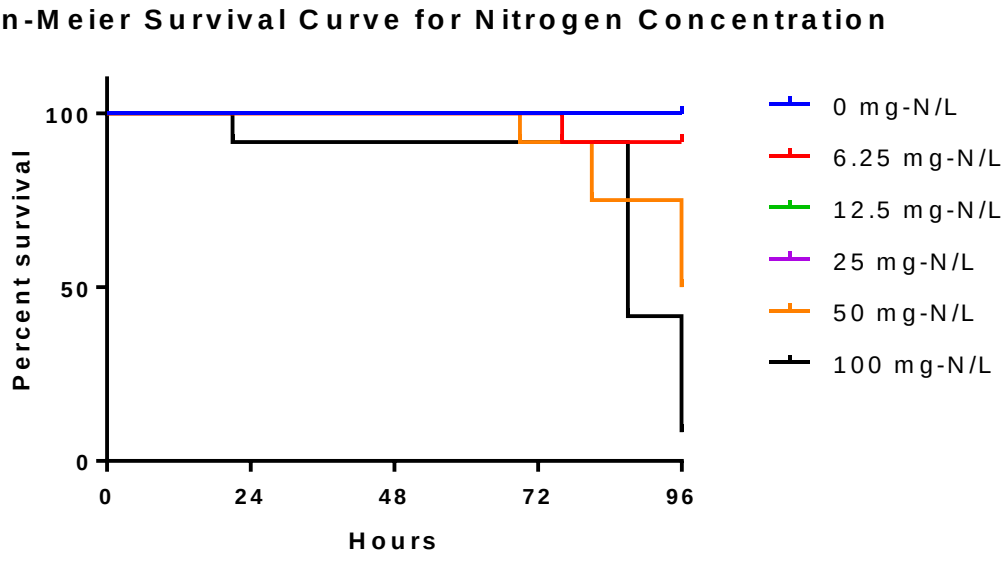

Figure 5 – Mortality in Texas pigtoe at different nitrogen concentrations.

There was a significant effect of nitrogen concentration on tissue glycogen levels [F (5, 44) = 3.370, p = 0.012] (figure 6). All samples tested had detectible levels of glycogen with a mean concentration of 458.1 mg/L (range 15.5 – 2629.3 mg/L).

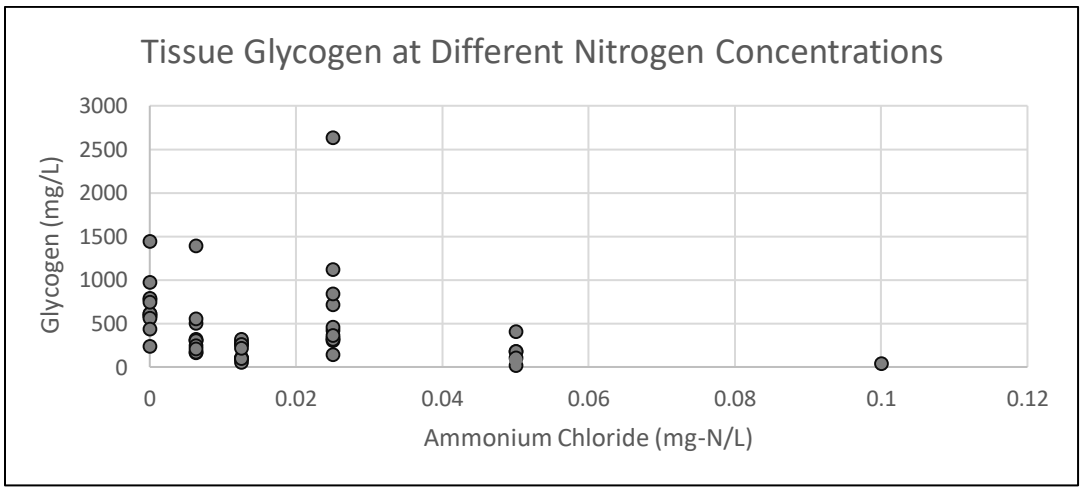

Figure 6 – Tissue glycogen concentration in Texas pigtoe at different nitrogen concentrations.

Behavior

Hourly, in-person observations were combined with video recordings to evaluate behavioral response to nitrogen toxicity. There was no effect of nitrogen exposure on the burrowing behavior of Texas pigtoe [F (1, 10) = 2.966, p = 0.1158] (figure 7).

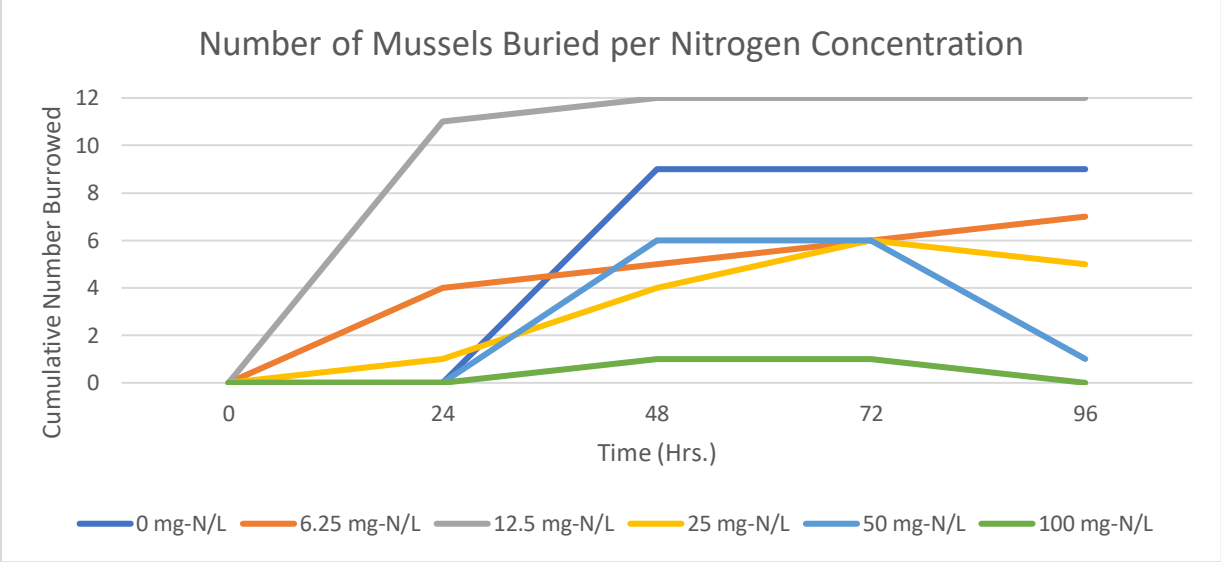

Figure 7 - Burrowing behaviors of Texas pigtoe in response to different nitrogen concentrations.

As the nitrogen concentration increased, average gape response increased; however, there was no effect of concentration on average gape [F (1, 10) = 3.193, p = 0.076] (figure 8).

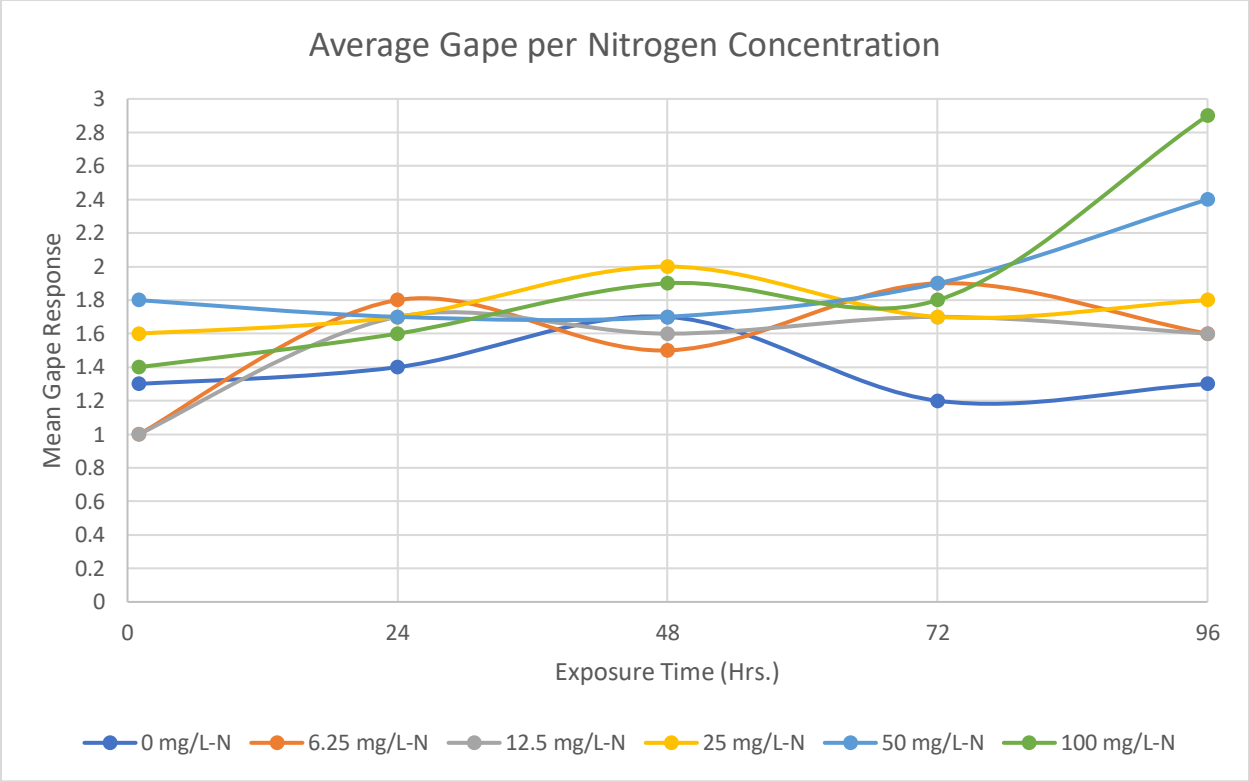

Figure 8 - Average gape measurement for Texas pigtoe exposed to different nitrogen concentrations.

Discussion

Siltation caused by bank collapse, increased water temperatures, and increased levels of nitrogen all likely affect mussels in east Texas. As mussels tend to occur in multi-species beds, the Texas pigtoe was used as a surrogate species for more endangered mussels. Mortality was positively associated with increased environmental stress in all tests.

The siltation trial as a proxy for bank collapse in the field was an acute test lasting for 96 hours. In this short amount of time none of the individual mussels were able to dig out of the sand and only 3 individuals were able to move vertically at all. This is important

because siltation has been noted as one of the leading threats to aquatic biodiversity in North America (Richter et al., 1997). In a natural setting, like the Sabine River where these mussels were taken, it is not uncommon to have banks collapse, especially below reservoirs or bridges. By burying Texas pigtoe mussels I found that overall mortality was higher in the 0.5 m treatment, however mortality at the 0.25 m treatment occurred at an earlier time point. At the 0.25 m depth, mussels tried to dig their way out of the sediment. While none reached the top, this could suggest an additional energy expense when compared to those buried at the 0.5 m depth which were not observed making any movement. Additionally, of those that did move (only in the 0.25 m group) there appeared to be no preference for directionality. One moved vertically upward and two moved downward thereby increasing the amount of sand and resulting pressure. Although overall mortality at the end of the trial was only 13.3% for all individuals, lack of movement is the more important outcome for this species. In a preliminary trial, 20 Texas pigtoe mussels were left buried and after 10 days, there was only 1 surviving mussel. If the Texas pigtoe is incapable of digging out from under the sediment this represents an important placement consideration if relocation or reintroduction efforts are needed in the future.

The mortality observed in mussels exposed to increasing thermal stress puts this species in a group of mussels which are already existing near their thermal tolerance limits. The 30°C temperature at which I began to see mortality in Texas pigtoe is noted as a summer water temperature for east Texas rivers. Summer temperatures recorded in the Neches river in 2017, a similar Texas river to the Sabine, were 30°C throughout June and July and recorded as high as 32°C in August (Hinkle, 2018). Mortality was at 14% in the

test which exposed mussels to 30°C over a 21-day period. This suggests that the typical summer temperatures in Texas streams will result in the loss of a portion of an otherwise healthy population. As the temperatures continue to increase as predicted by climate change models (IPCC, 2014), the threat this already imperiled species is under is expected to become more severe.

In addition to mortality, an analysis of sub-lethal energy use was done on mussels exposed to different temperatures. Evaluating tissue glycogen levels in the foot muscle gives an indication of the health of the mussel before a mortality event. Low levels of glycogen are an indicator of increased stress. Glycogen is the major carbohydrate storage form in animal cells and is important because it can be mobilized for energy very quickly (Engelking, 2015). Stress stimulates the expenditure of excess glycogen to maintain normal physiological processes, resulting in less available energy for growth and reproduction (Chetty and Indira, 1994). Most of the tissue samples from the mussels exposed to thermal stress did not show any detectible levels of glycogen. The length of time a specimen was held in the laboratory may have contributed to decreased tissue glycogen levels. The first collection was done in July of 2016, and the temperature trials did not begin until May of 2017. Little is known about the exact diet of different mussel species, and while only 11 individuals out of 257 died outside of testing, it is probable that over time in an unnatural setting, excess stores of glycogen that were in place upon collection were low by the time testing was conducted.

For all aquatic species, pollution comes in second only to habitat loss as a major threat to endangerment (Wilcove et al., 1998). The results of increasing nitrogen exposure on Texas pigtoe mortality indicate that while current Environmental Protection

Agency (EPA) guidelines are adequate for protecting this species under consistent conditions, a point-source interruption, even for the brief 96-hr time-period tested here, could be detrimental. East Texas has many large confined animal operations and is the corporate home of Pilgrims Pride poultry. The most up to date EPA standards require ammonia levels to be no more than 17 mg-N/L for an acute test (over a 1 hr. average) or 1.9 mg-N/L under chronic conditions (30-day averaging period) at a pH of 7 and 20°C (US EPA, 2013). Mussels were exposed to ammonium chloride as the source of nitrogen at the above-mentioned parameters and mortality was not observed until 50 mg-N/L was reached, excluding one individual that died at the 6.25 mg-N/L concentration. Because most toxicity tests on freshwater mussels are conducted on juveniles as the more sensitive life phase, this study was done on adults and is representative of a phase rarely tested but suggests that exposure in high amounts can induce stress and be lethal to adult mussels of this species.

Tissue glycogen levels were also tested in samples taken from the foot muscle of each living individual in the nitrogen toxicity test, at the end of the 96-hour trial. In this case, there was a significant effect of nitrogen concentration on tissue glycogen levels. As expected, the levels of glycogen were reduced as exposure to nitrogen was increased over time. This suggests that physiologically, more energy was needed to continue to maintain life processes under the environmental conditions of pollution, in this case excess nitrogen. The collection for this trial was done in September 2017, and the trial began in November 2017. With the nitrogen toxicity test, behavioral trends in burrowing and gaping were noted in response to pollutant stress. It is worth noting that the trend

suggests a loss of ability to effectively burrow at the higher nitrogen concentrations and that higher average gape response corresponded with increased nitrogen.

Conclusion

The long-term physiological effects of stress in mussels are strongly related to a switch in metabolic processes from aerobic to anaerobic resulting in decreased function at all levels of biological organization. I saw reductions in survivorship and a decrease in tissue glycogen levels as mussels were exposed to increasing stress, but further sub-lethal studies are needed for freshwater mussels. Physiological evaluations done on *Mytilus galloprovincialis*, a commonly studied marine species, have shown sub-lethal responses such as reduced enzyme function, increased expression of heat shock proteins and interrupted cell signaling. For freshwater mussels, more studies evaluating sub-lethal effects happening prior to mussels switching to an anaerobic metabolism are needed and can alert conservations managers to potential problems in a population before they become widespread.

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