ABSTRACT

PANDOLFO, TAMARA JANE. Sensitivity of Early Life Stages of Freshwater Mussels to a Range of Common and Extreme Water Temperatures. (Under the direction of W. Gregory Cope).

Freshwater mussels fulfill an essential role in benthic aquatic communities, but are also one of the most sensitive and rapidly declining faunal groups in North America. Rising water temperatures, caused by global climate change, industrial discharges, drought, or land development, can further challenge impaired unionid communities. The aim of this study was to determine the upper thermal tolerances of the early life stages, glochidia and juveniles, of freshwater mussels. Glochidia of eight species of mussels were tested: *Lampsilis siliquoidea*, *Potamilus alatus*, *Ligumia recta*, *Ellipsaria lineolata*, *Lasmigona complanata*, *Megalonaias nervosa*, *Alasmidonta varicosa*, and *Villosa delumbis*. Seven of these species were also tested as juveniles. Survival trends were monitored as mussels were held at three acclimation temperatures, 17°C, 22°C, and 27°C, and exposed to a range of common and extreme water temperatures (20 - 42°C) in standard acute laboratory tests. The average median effective temperature (ET50) among species in 24-h tests with glochidia was 33.7°C, ranging from 29.1 to 37.5°C. The mean ET50 in 96 h juvenile tests was 34.8°C, and ranged from 32.9 to 36.7°C. As an indicator of sublethal thermal stress, heart rate patterns for seven species of juvenile freshwater mussels were assessed visually through direct observation. Species differences were observed; *L. recta* and *V. delumbis* displayed significant changes in heart rate associated with increasing temperature at all three acclimation temperatures.

Thermal increase is almost certainly not the only stressor affecting freshwater mussels. Metals, such as copper, are a common source of toxicant exposure in aquatic

environments. The effect of a sublethal copper concentration on the upper thermal tolerance of three juvenile freshwater mussel species, *Lampsilis siliquoidea*, *Potamilus alatus*, and *Ligumia recta*, was determined. ET50s were calculated in the absence and presence of copper, and they ranged from 32.9ºC to 36.7ºC with a mean of 34.8ºC. Based on 95% confidence interval overlap, there were no differences among ET50s caused by acclimation temperature, species, or presence of copper. However, survival trends showed evidence of interactive effects between copper and temperature for all three species, suggesting this is an area that warrants further study.

In freshwater systems, the larval life stage, glochidia, of unionid mussel species develop as obligate parasites on host fish gills or fins before transforming into the juvenile life stage and dropping to the sediment to complete their life cycle. Because of the relationship between mussels and their often specific host fish species, freshwater mussels are not only potentially affected by their own variable thermal tolerance limits, but also by those of their fish hosts. Existing thermal tolerance data for eight species of freshwater mussels and their host fish were compiled and compared to determine if the community structure of these systems is at risk from rising environmental temperatures; relationships were complicated with mussels being both more and less thermally sensitive than certain host fish species. Freshwater mussels are a valuable part of aquatic ecosystems, and this study has shown that thermal stress can negatively impact these animals; therefore it is critical to maintain thermally acceptable flowing waters to protect these threatened fauna.

Sensitivity of Early Life Stages of Freshwater Mussels to a Range of Common and Extreme Water Temperatures

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the degree of
Master of Science

Toxicology

Raleigh, North Carolina

2008

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DEDICATION

To the late Dr. Richard Maas, my advisor and mentor at the University of North Carolina Asheville, who inspired me to be where I am today.

BIOGRAPHY

Tamara Pandolfo grew up in Greensboro, North Carolina. She attended the University of North Carolina at Asheville and earned her B.S. in Environmental Studies, with a concentration in Pollution Control, in 2004. Following graduation, she worked as an analyst at the Environmental Quality Institute in Asheville, where she had previously worked as an undergraduate researcher. In 2006, she joined the Department of Environmental and Molecular Toxicology at North Carolina State University as a graduate research assistant working for Dr. Greg Cope.

ACKNOWLEDGMENTS

There are many people who helped to make this research project successful. I'd like to thank Dr. Chris Barnhart and Chris Eads for providing mussels, and Shad Mosher, Erin Tracy, and Pete Lazaro for laboratory assistance. Dr. Consuelo Arellano provided statistical analysis, and Wayne Robarge at NCSU Soil Science analyzed copper samples. The Environmental Protection Agency provided funding for this project, with special thanks to Ed Hammer and Brian Thompson in Region 5. As for my committee members, Dr. Thomas Kwak was a valuable source of input and perspective, Dr. David Buchwalter was a great source of stimulating discussion and new ideas, and, of course, my committee chair and advisor, Dr. Greg Cope, was a constant source of support, advice, and encouragement. I also have to thank my family and Charlie for always standing by me.

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Chapter 1. Beating the heat: upper thermal tolerances of the early life stages of eight species of freshwater mussels (Unionidae).

(Formatted for: Ecological Applications)

Abstract

Freshwater mussels fulfill an essential role in benthic aquatic communities, but are also one of the most sensitive and rapidly declining faunal groups in North America. Rising water temperatures, caused by global climate change, industrial discharges, drought, or land development, can further challenge impaired unionid communities. The aim of this study was to determine the upper thermal tolerances of the glochidia and juvenile life stages of freshwater mussels. Glochidia of eight species of mussels were tested: *Lampsilis siliquoidea*, *Potamilus alatus*, *Ligumia recta*, *Ellipsaria lineolata*, *Lasmigona complanata*, *Megalonaias nervosa*, *Alasmidonta varicosa*, and *Villosa delumbis*. Seven of these species were also tested as juveniles. Survival trends were monitored as mussels were held at three acclimation temperatures, 17°C, 22°C, and 27°C, and exposed to a range of common and extreme water temperatures (20 - 42°C) in standard acute laboratory tests. The average median effective temperature (ET50) among species in 24-h tests with glochidia was 33.7°C, ranging from 29.1 to 37.5°C. The mean ET50 in 96-h juvenile tests was 34.8°C, and ranged from 32.9 to 36.7°C. Based on comparisons of ET50s and associated 95% confidence intervals, there were no differences among species or acclimation temperature for juvenile freshwater mussels. There were differences among some species in glochidia tests, but not among acclimation temperatures.

Acclimation temperature did not have an effect on thermal tolerance in this study, but that may be attributed to experimental design. Results indicate that freshwater mussels may already be living close to their upper thermal tolerances in some systems and thus may be at risk from rising environmental temperatures. This study provides valuable information for examining water quality criteria for temperature to ensure adequate protection of the already imperiled freshwater mussel fauna.

Keywords: freshwater mussel, Unionidae, glochidia, juvenile, temperature, thermal tolerance, ET50

Introduction

Freshwater mussels of the bivalve order Unionoida are long-lived, benthic aquatic organisms with considerable roles as nutrient processors and ecosystem engineers in the benthic aquatic environment (Vaughn et al. 2004, Howard and Cuffey 2006, Vaughn et al. 2008). Unionids are one of the most rapidly declining faunal groups in North America and elsewhere in the world. Of the estimated 840 species of freshwater mussels globally, approximately 300 are native to North America, but nearly 70% of these species are extinct or vulnerable to extinction (Graf and Cummings 2007, Bogan 1993, Williams et al. 1993) This decline has been attributed to several factors, including: habitat degradation, water withdrawal for industry, urbanization, dam construction, impoundments, sedimentation, navigation, pollution, introduction of nonindigenous

mollusks, overharvesting, and land use change (Bogan 1993, Williams et al. 1993, Strayer et al. 2004, Lydeard et al. 2004, Bogan 2008).

Freshwater mussels have a complex life history strategy (McMahon and Bogan 2001, Watters 2007). The Unionoida rely on host fish to complete their life cycle by requiring the larval life stage, glochidia, to infest the gills or fins of host fish as parasites via various strategies before transforming into the juvenile life stage and dropping to the sediment to continue their development into benthic-dwelling adults (Kat 1984, Wachtler et al. 2001, Haag and Warren 2003). The complexities of their life cycle make freshwater mussels particularly susceptible to disruption by environmental stressors, such as temperature. Therefore, rising ambient temperatures resulting from heated effluents or global climate change may pose additional risks to threatened mussel species (Hastie et al. 2003), though these risks have been largely unexplored.

Rising atmospheric carbon dioxide concentrations from anthropogenic sources have caused global average air temperatures to rise by 0.6ºC since 1900, and more accelerated effects are anticipated in the current century (IPCC 2001). Climate change has the ability to impact organisms at all levels of organization, and it has already influenced biota globally (Harley et al. 2006). There is evidence that climate change has changed the physiology, distribution, and phenology of species (Hughes 2000, Sparks and Menzel 2002, Parmesan and Yohe 2003, Berteaux et al. 2004, Harley et al. 2006). Aquatic systems are much more constrained than are terrestrial systems in the ways in which organisms can respond to warming, and therefore effects of climate change may be more pronounced (Shuter and Post 1990). Because of this, and also because changes in

temperature effects unrelated to climate change have been well documented in stream ecosystems (Feller 1981, Hewlett and Fortson 1982), aquatic systems are a valuable study system for climate change. Despite this, the effect of climate change on aquatic communities has been understudied.

The extensive literature on thermal tolerances of fish (van Dijk et al. 1999, Beitinger et al. 2000, Carveth et al. 2006, Widmer et al. 2006, Fontaine et al. 2007) and mollusks (Wolcott 1973, Al-Habbib and Grainger 1977, Ansell et al. 1980a, Ansell et al. 1980b, Matthews and McMahon 1999, Chen et al. 2007, Han et al. 2008) provides a solid background for approaching the thermal tolerance limits of freshwater mussels. Though the effect of temperature on the release, development, and viability of glochidia has been well studied (Roberts and Barnhart 1999, Jansen et al. 2001, Zimmerman and Neves 2002, Akiyama and Iwakuma 2007, Cope et al. 2008), a data gap exists in the determination of acute lethal temperature values for the early life stages of the freshwater mussel (Dimock and Wright 1993).

To address this data gap, I determined the upper thermal tolerances of glochidia of eight mussel species and seven species of juvenile freshwater mussels representing three tribes from two subfamilies of the family Unionidae (Graf and Cummings 2007). Mussels were tested at three different acclimation temperatures over a range of temperatures from 20°C to 42°C, and ET50s were calculated. These data are essential in understanding the effects of temperature on the imperiled unionids. Climate change may put mussels closer to their thermal limits, and additional heat inputs from thermal discharges, drought, or land use changes can further alter the thermal environment of

these sessile organisms. The findings of this study can be used to examine water quality criteria for temperature with a goal of protecting these valuable organisms from thermal stress.

Methods

Test organisms

To determine the temperature tolerances of selected freshwater mussel species, I exposed the glochidia and juvenile life stages of freshwater mussels to a range of common and extreme water temperatures. Each test was conducted at three acclimation temperatures: 17ºC, 22ºC, and 27ºC, and each acclimation temperature had five corresponding experimental temperatures in 3ºC increasing increments. A 20ºC reference control temperature was also assessed alongside each test (Figure 1).

Eight species representing three tribes from two subfamilies of the family Unionidae were used in this study (Graf and Cummings 2007). From the Ambleminae subfamily, five species were from the Lampsilini tribe: fatmucket (*Lampsilis siliquoidea*, Barnes, 1823), pink heelsplitter (*Potamilus alatus*, Say, 1817), black sandshell (*Ligumia recta*, Lamarck, 1819), butterfly (*Ellipsaria lineolata*, Rafinesque, 1820), and eastern creekshell (*Villosa delumbis*, Conrad, 1834); while one species was from the Quadrulini tribe: washboard (*Megalonaias nervosa*, Rafinesque, 1820). From the Unioninae subfamily, two species belonged to the Anodontini tribe: white heelsplitter (*Lasmigona complanata*, Barnes, 1823), and brook floater (*Alasmidonta varicosa*, Lamarck, 1819).

These species were chosen because they encompass a variety of life history strategies and habitats, and because of their wide geographic distribution, particularly in the central United States. The species represent three subregions of the Nearctic region (Graf and Cummings 2007): Interior Basin (fatmucket, white heelsplitter, butterfly, black sandshell, pink heelsplitter, and washboard), Gulf Coastal (white heelsplitter, butterfly, black sandshell, pink heelsplitter, and washboard), and the Atlantic Slope (brook floater and eastern creekshell). All test organisms originated from propagation facilities at Missouri State University (Interior Basin and Gulf Coastal species) and North Carolina State University (Atlantic Slope species).

Glochidia assessment

Upon arrival at the laboratory, glochidia were assessed for initial viability in accordance with American Society for Testing and Materials (ASTM) protocols (ASTM 2006). The glochidia were then acclimated to the test acclimation temperature by adjusting their temperature by no more than 1ºC per hour, with a 2 hour acclimation period once the target test acclimation temperature was reached. After this period, viability was assessed a second time and glochidia were dispensed to test chambers. Tests were conducted using glochidia of eight species that were no more than 24 hours old at the start of the test. Tests were 24 hour non-aerated static experiments conducted according to ASTM guidelines for glochidia, and viability was assessed at 24 hours by the addition of a saturated sodium chloride solution which stimulated shell closure. This response was assessed visually with an Olympus SZ61 microscope (Olympus America Inc., Center Valley, PA, USA) and QCapture Pro 5.1 digital photographic software

(Quantitative Imaging Corporation, Burnaby, BC, Canada). Assessments were also made at the 48 h time point for a time driven thermal sensitivity comparison. The observation was made, and reported here for the first time, that brook floater, unlike the other species tested, re-opened approximately one minute after initial shell closure with salt addition. To have an accurate measure of viability, it was necessary to take photographs for viability assessments before any re-opening occurred. For this reason, brook floater is not recommended as a model species in laboratory testing with glochidia unless photographs can be taken immediately.

Juvenile assessment

The same species used in glochidia tests were then used to conduct tests on newly transformed juveniles. No tests were conducted with white heelsplitter, and brook floater was not tested at the 17ºC acclimation temperature. Experiments were conducted with fatmucket, pink heelsplitter, and black sandshell; these mussels ranged in age from 3 to 8 weeks, and the average size for fatmucket was 1,386 µm, pink heelsplitter was 1,377 µm, and black sandshell was 947 µm. The remaining species (butterfly, washboard, brook floater, and eastern creekshell) ranged in age from less than 1 week to 4 weeks old and the average size for butterfly was 335 µm, washboard was 364 µm, brook floater was 398 µm, and eastern creekshell was 363 µm. Upon arrival at the laboratory, viability of juveniles was assessed; mussels were then acclimated to the test acclimation temperature by adjusting their temperature by no more than 2.5ºC per day, with at least a 24 hour acclimation period once the target acclimation temperature was attained. After this period, viability was assessed a second time and organisms were distributed to test

chambers. These were 96 hour non-aerated static renewal tests, with 100% water renewal at 48 hours; tests were conducted according to ASTM guidelines for juveniles (ASTM 2006). Juvenile mussel viability was assessed visually using an Olympus SZ61 microscope to detect foot movement outside of the shell, foot movement within the shell, or the presence of a heart beat. Assessments were also made at the 48 h time point for a time based thermal sensitivity comparison.

Thermal tolerance

Survival data from both glochidia and juvenile tests were used to generate median effective temperatures (ET50s) using the trimmed Spearman-Karber method with ToxCalc v.5.0.26 toxicity data analysis software (Tidepool Scientfic Software, McKinleyville, CA, USA). ET05 data were generated using the Maximum Likelihood Regression probit analysis via the same software. An ET50 is defined as the temperature at which 50% of the exposed population exhibits some predefined effect; for glochidia this effect was loss of viability determined via shell closure response with the addition of saturated salt solution, and for juveniles this effect was loss of viability determined by lack of foot movement within or outside of the shell, and/or lack of a heart beat. An ET05, similarly, is the temperature at which 5% of the exposed population exhibits the previously defined effects; because it is protective of 95% of a population, it is typically a more sensitive measure. Comparisons of ET50s and ET05s were made using 95% confidence interval overlap. Intervals that did not overlap were considered to be significantly different, whereas ET50s with overlapping intervals were determined to be similar.

Quality assurance

Quality assurance and control were ensured by conducting all tests according to the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM 2006). Control viability was deemed acceptable if it did not decrease from initial viability at the start of the test. Duplicate counts were made for 10% of photographs taken during glochidia viability assessments (Appendix Table 1 and Table 2). All tests were conducted in light and temperature controlled environmental chambers (Precision Model 818, Thermo Electron Corp., Marietta, OH, USA, and Isotemp Model 146E, Fisher Scientific, Dubuque, Iowa, USA). National Institute of Standards and Technology (NIST) certified thermometers were used for daily temperature monitoring. Water quality conditions, including alkalinity, hardness, conductivity, pH, temperature, and dissolved oxygen, were measured at the start of each test and again at the 48 hour time point. Alkalinity and hardness were measured by titrametric procedures with standard methods (APHA 1995). Conductivity, temperature, pH, and dissolved oxygen were measured with a calibrated meter (YSI model 556 MPS multi-probe, Yellow Springs Instrument Co., Yellow Springs, OH, USA). For all tests, alkalinity ranged from 92 to 110 mg CaCO3/L with a mean of 103.9 mg CaCO3/L, hardness ranged from 138 to 162 mg CaCO3/L with a mean of 149.6 mg CaCO3/L, conductivity ranged from 472 to 717 µs/cm with a mean of 564.2 µs/cm, pH ranged from 6.87 to 8.96 with a mean of 8.44, and dissolved oxygen ranged from 4.61 to 9.73 mg/L with a mean of 7.28 mg/L (n=27 for alkalinity and hardness, n=223 for all other parameters). Test temperatures had a

maximum of 2ºC departure from the target temperature, with only 1.4% of temperatures exceeding a 1ºC departure (n=866).

Statistical analysis

Results were analyzed with SAS® Proc Mixed (SAS Institute Inc, 2006). Survival data were arcsin transformed. Acclimation temperature and experimental temperature were considered fixed effects, while repetitions within each acclimation temperature were considered as random effects. Acclimation temperatures were considered crossed effects, and experimental temperature was taken as a nested effect within each acclimation temperature. Significance level was established at $p \leq 0.01$ for model effects, while means differences were analyzed by a t-test for pairwise least squares mean differences with Tukey's adjusted p-value and 0.01 significance level to control type I error. Significance level was established at $p \leq 0.01$ in order to ensure significance was assigned only to responses beyond those associated with variation in natural mussel populations.

When significance was detected, pairwise mean comparison was used to analyze mean differences between acclimation temperatures. Significance of experimental temperatures was studied through the analysis of simple effects for experimental temperatures within each acclimation temperature and when necessary, the effect differences of experimental temperatures were analyzed within each acclimation temperature with a t-test for pairwise least squares mean differences with Tukey's adjusted p-value and 0.01 significance level. Signficance level for each pairwise mean

comparison was calculated using the distribution of the studentized range (Steel et al. 1997).

Results

Glochidia

Viability trends were monitored for glochidia of the eight species tested at all three acclimation temperatures (Figure 2). For comparisons across overall viability at each acclimation temperature (Figure 3), the mean viability shown in the graphs refers to the average viability of all temperatures over the acclimation temperature in question, e.g. the 22ºC acclimation value is the mean survival for 22, 25, 28, 31, 34, and 37ºC. For this reason, we would expect the 22ºC and 27ºC acclimations to have lower average viability because they included temperatures that produced total mortality whereas the 17ºC acclimation did not. This graph is useful for comparing broad responses to increasing temperature regimes, but is not applicable for determining effects of acclimation on thermal tolerance. Fatmucket experienced significant changes in overall viability at only the 27ºC acclimation, which was different from the control, 17ºC, and 22ºC acclimations (all $p<0.0001$). Pink heelsplitter, butterfly, washboard, and eastern creekshell all had similar viability in controls and the 17ºC acclimation temperatures, but the 22ºC and 27ºC acclimations were significantly different from the control, and the latter two acclimations also differed significantly from each other (all pairwise comparisons $p<0.0001$). For black sandshell, control viability differed from neither the 17ºC acclimation nor the 22ºC acclimation, though 17ºC and 22ºC differed from each other, and the 27ºC acclimation

differed from all of the others (all $p<0.0001$). Brook floater had an identical response to black sandshell, though for the difference between 17ºC and 22ºC acclimation, $p=0.0024$. White heelsplitter had a unique response in that the 17ºC acclimation was different from the control ($p=0.0021$) and from the 22ºC acclimation ($p<0.0001$). The control and 22ºC were not significantly different, but the 27ºC acclimation was significantly different from the others ($p<0.0001$).

At the 17ºC acclimation temperature (Figure 4), only glochidia of eastern creekshell were adversely affected by temperature, mortality at 32ºC was significantly greater than at any other temperature (all $p<0.0001$, except at 29ºC $p=0.0006$). All other species did not have significant mortalities at these temperatures. At the 22ºC acclimation temperature (Figure 5), fatmucket showed no significant differences from the control at these temperatures (all $p>0.01$). Pink heelsplitter control viability was significantly higher than viability at 28ºC ($p=0.0003$), 31ºC, 34ºC, and 37ºC ($p<0.0001$). The 34ºC treatment also had significantly lower viability than 22ºC and 25ºC ($p<0.0001$), as well as 28ºC ($p=0.0002$). In addition to the control, the 37ºC treatment also differed significantly from 22ºC ($p<0.0001$) and 25ºC ($p=0.0004$). For black sandshell, 37ºC was the only temperature with significant effects, differing from the control and 22ºC ($p<0.0001$), 25ºC ($p=0.0009$), and 28ºC ($p=0.0003$). Butterfly had similar viability in control through the 31ºC temperature, but the 34ºC and 37ºC treatments had significantly lower viability than all lower temperatures (all $p<0.0001$). For white heelsplitter and brook floater, 37ºC had significantly lower viability than at any other temperature (all $p<0.0001$). Washboard control viability was significantly higher than at 34ºC or 37ºC

($p<0.0001$), and 37ºC viability was lower than at any other temperature (all $p<0.0001$). The 34ºC treatment differed significantly from every other temperature except for 31ºC ($p\leq0.0001$), and 31ºC may have been the threshold temperature for this species because it had significantly lower viability than at 22ºC ($p=0.0013$) or 25ºC ($p=0.0055$), but not from 28ºC or the control ($p>0.01$). Eastern creekshell control viability was significantly higher than viability at 31ºC, 34ºC, or 37ºC ($p<0.0001$). Viability at 37ºC was significantly lower than at any other temperature (all $p<0.0001$), and while the 34ºC temperature did not differ from 31ºC, it was significantly different from all others ($p\leq0.0001$, except for 22ºC $p=0.0020$). Viability at 31ºC did not differ from viability at 22ºC, but was significantly lower than viability at 25ºC ($p=0.0001$) and 28ºC ($p=0.0017$).

At the 27ºC acclimation temperature (Figure 6), fatmucket viability at 39ºC and 42ºC was significantly lower than at any other temperature ($p<0.0001$), with both temperatures causing near total mortality. Pink heelsplitter was the most thermally sensitive species at these temperatures, with all temperatures, including 27ºC, causing near total or total mortality when compared with the control ($p<0.0001$). For black sandshell control viability was significantly higher than at 33ºC, 36ºC, 39ºC, and 42ºC (all $p<0.0001$). Viability at 39 ºC and 42 ºC was significantly lower than at any other temperatures, with total mortality at these temperatures (all $p<0.0001$). Viability at 36ºC was significantly different than any temperature except 33ºC ($p<0.0001$), and 33ºC viability was significantly lower than 27ºC ($p<0.0001$). There were distinct levels of viability for butterfly, control viability was higher than all others, viability at 27- 36ºC was lower than control but higher than the two highest temperatures, and viability at 39ºC

and 42ºC was lower than at all other temperatures (all $p<0.0001$). For white heelsplitter, viability at 39ºC and 42ºC was significantly lower than viability at all other temperatures ($p<0.0001$). Washboard control viability was higher than at 33ºC, 36ºC, 39ºC, and 42ºC ($p<0.0001$). Viability at 36ºC, 39ºC, and 42ºC was not significantly different, though viability at these temperatures was significantly lower than at all other temperatures (all $p<0.0001$, except 33ºC: $p=0.0002$ for 39ºC, and $p=0.0003$ for 42ºC). Brook floater control viability was significantly higher than viability at 36ºC ($p=0.0046$), 39ºC and 42ºC ($p<0.0001$). Viability at 39ºC and 42ºC was significantly lower than all other temperatures (all $p<0.0001$). Eastern creekshell control viability differed significantly from all temperatures except 30ºC ($p<0.0001$), and 39ºC and 42ºC had lower viability than all other temperatures ($p<0.0001$). Viability at 36ºC was significantly different from all other temperatures except 33ºC ($p<0.0001$), while viability at 33ºC was significantly lower than at 30ºC ($p=0.0004$) but not than 27ºC ($p=0.2024$).

Juveniles

Viability trends for juvenile freshwater mussels at all three acclimation temperatures were monitored (Figure 7). In overall comparisons of viability across acclimation temperatures (Figure 8), there were no differences between the control and the 17ºC acclimation test for any species (all $p>0.01$). Fatmucket showed that both the 22ºC acclimation and 27ºC acclimation were different from the controls and 17ºC acclimation, as well as different from one another (all $p<0.0001$). Washboard exhibited the same pattern, with the control being significantly different from both the 22ºC acclimation ($p=0.0074$) and the 27ºC acclimation ($p<0.0001$), and the 22ºC and 27ºC

acclimations differing from each other ($p<0.0001$). Pink heelsplitter also showed a similar response though 22ºC acclimation was not significantly different from the 17ºC acclimation test ($p=0.4551$), while it was different from the control ($p=0.0066$). For black sandshell, the 22ºC and 27ºC acclimations were not different from each other ($p=0.1249$), but both were different from control and 17ºC acclimation ($p<0.0001$). Butterfly had significantly different results only at the 27ºC acclimation, as did brook floater and eastern creekshell ($p<0.0001$ for all species).

For all species of juvenile freshwater mussels at the 17ºC acclimation temperature (Figure 9), there were no significant differences among experimental temperatures ($p>0.01$). In the 22ºC acclimation test (Figure 10), there were no significant differences among the experimental temperatures for butterfly ($p>0.01$). For fatmucket, pink heelsplitter, washboard, brook floater, and eastern creekshell the highest temperature, 37ºC, was the only temperature with a significant effect, with near total or total mortality exhibited for all species ($p<0.0001$ for all species). Black sandshell showed significant difference from the control at both 34ºC ($p=0.0020$) and 37ºC ($p<0.0001$). At the 27ºC acclimation temperature (Figure 11), all species had total mortality at the two highest temperatures, 39ºC and 42ºC ($p<0.0001$ for all species). Black sandshell and brook floater showed significant differences from the control only at these temperatures, while fatmucket, pink heelsplitter, butterfly, washboard, and eastern creekshell also showed a significant effect of temperature at 36ºC ($p<0.0001$ for all species except brook floater $p=0.0120$).

ET50s/ET05s

ET50s for glochidia (24 h) and juvenile (96 h) freshwater mussels were calculated for the 22ºC acclimation and 27ºC acclimation (Table 1). Overall ET50s ranged from 29.1°C to 37.5°C with a mean of 34.2°C. Glochidia ET50s ranged from 29.1°C to 37.5°C with a mean of 33.7°C. Only one ET50 was generated for the 17ºC acclimation for eastern creekshell glochidia at 24 hours (ET50 = 31.68 ºC, 95% confidence interval = 27.55- 36.43ºC). At the 22ºC acclimation, pink heelsplitter glochidia had a significantly lower ET50 (i.e. was less thermally tolerant) than both white heelsplitter and brook floater. At the 27ºC acclimation, white heelsplitter glochidia had a significantly higher ET50 (i.e. was more thermally tolerant) than washboard and eastern creekshell. There were no differences among acclimation temperatures for a given species where data existed for comparison. There were also no differences between the 24 h and 48 h time point for a given species at a particular acclimation temperature where data existed for comparison (Appendix Table 3). Juvenile ET50s ranged from 32.9°C to 36.7°C with a mean of 34.8°C. There were no differences among juvenile ET50s at 22ºC or 27ºC acclimations for any species. There was also no difference between acclimation temperatures for the 6 species with data available for comparison. Both fatmucket and eastern creekshell juveniles had lower ET50s at 96 h than at 48 h for the 27ºC acclimation (Appendix Table 4). Life stage comparisons found that at the 22ºC acclimation, pink heelsplitter glochidia have a lower ET50 than juveniles of that species, while at the 27ºC acclimation, fatmucket glochidia have a higher ET50 than juveniles.

Overall ET05s for glochidia (24 h) and juvenile (96 h) mussels at the 22ºC and 27ºC acclimation temperatures ranged from 21.55ºC to 35.28ºC, with a mean of 29.49ºC (Table 2). Glochidia ET05s ranged from 21.55ºC to 35.28ºC with a mean of 28.26ºC. Only one ET05 was generated for the 17ºC acclimation for eastern creekshell glochidia (ET05 = 26.96ºC, 95% confidence intervals = 11.11- 28.97ºC). There were no differences among species for the glochidia life stage at the 22ºC acclimation, but at the 27ºC acclimation, fatmucket and brook floater had significantly higher ET05s than black sandshell, washboard, and eastern creekshell. There were no differences between acclimation temperatures for the two species having data for comparison. At the 27ºC acclimation, fatmucket had a lower ET05 at 48 h than at 24 h (Appendix Table 5). Juvenile ET05s ranged from 29.13ºC to 33.21ºC with a mean of 31.95ºC. There were no differences among species for either the 22ºC acclimation or the 27ºC acclimation, though data were limited. Only eastern creekshell had ET05s for both acclimation temperatures, and these were not different. No time point comparisons could be made with the juvenile ET05s (Appendix Table 6). At the 27ºC acclimation, eastern creekshell juveniles had a higher ET05 than glochidia of that species. The two other life stage comparisons, eastern creekshell at the 22ºC acclimation, and brook floater at the 27ºC acclimation, showed no differences.

Discussion

Thermal tolerance studies with fish and mollusks have shown that temperature can affect basic physiological processes such as growth, metabolism, and immune

condition (Schulte 1975, Newell et al. 1977, Widmer et al. 2006, Chen et al. 2007, Fontaine et al. 2007, Petes et al. 2007, Han et al. 2008). Temperature has also been shown to drive many behaviors in the freshwater mussel including burrowing, seasonal migration, filtration rates, and distribution (Amyot and Downing 1997, Waller et al. 1999, Bartsch et al. 2000, Watters et al. 2001, Loayza-Muro and Elias-Letts 2007). Despite the abundance of data regarding thermal tolerances of other organisms and the effect of temperature on various processes of freshwater mussels, this study was the first to determine acute lethal thermal tolerances for the two early life stages of several species of freshwater mussels.

The results obtained in this study are comparable to acute thermal tolerance studies with other mussel species. The ET50s in my study ranged from 29.1°C to 37.5°C with a mean of 34.2°C for all acclimation temperatures and both life stages. The 24 h LT50 for ten species of marine mussels ranged from 23.7°C to 30.6°C, depending on acclimation, with the animals acclimated to a higher temperature, 16°C, exhibiting higher tolerances than mussels acclimated to 13°C (Urban 1994). The invasive zebra mussel, *Dreissena polymorpha*, has an upper lethal temperature limit of 30°C (Iwanyzki and McCauley 1993). The European marine mussel *Tellina fabula* has an LT50 ranging from 24.6°C to 30°C over acclimation temperatures ranging from 5°C to 24°C, whereas the species *T. tenuis* has LT50s ranging from 29.6°C to 31.7°C over the same acclimation temperatures (Ansell et al. 1980a). The only acute thermal tolerance study to date with early life stage freshwater mussels reported a 96 h LT50 of 31.5°C for one week old juvenile *Utterbackia imbecillis* (Say, 1829), and 33°C for one week old *Pyganodon*

cataracta (Say, 1817) (Dimock and Wright 1993). These results fall directly in line with the results from the juvenile portion of my study.

My results did not show a clear relationship between acclimation temperature and thermal tolerance for freshwater mussels. In other studies, thermal testing on mollusks has shown mixed responses to acclimation (Wolcott 1973, Al-Habbib and Grainger 1977, Ansell et al. 1980a, Ansell et al. 1980b, Matthews and McMahon 1999). However, acclimation temperature may be the most important factor relating to thermal tolerance in fish, and in most cases, acclimation temperature directly relates to thermal tolerance (Newell et al. 1971, Ansell et al 1980a, Ansell et al 1980b, Urban 1994, Beitinger and Bennett 2000, Carveth et al. 2006), though in some cases a neutral relationship exists (Widmer et al. 2006).

Acclimation can relate to thermal tolerance in a number of ways: first, there can be no effect from acclimation, second, thermal tolerance can increase with increasing acclimation temperature, and third, thermal tolerance can decrease with increasing acclimation temperature (Precht et al. 1973). The second response is the most common in poikilotherms (e.g. Newell et al. 1971, Ansell et al. 1980a, Ansell et al. 1980b, Urban 1994, Beitinger et al. 2000, Carveth et al. 2006) whereas the first and third are rare (Al-Habbib and Grainger 1977, Widmer et al. 2006).

Comparisons of thermal tolerances as a function of acclimation in my study must be done through comparisons of ET50s, and as there were no differences among ET50s for the species tested at both the 22ºC and 27ºC acclimation temperatures, there were no observed effects of acclimation on thermal tolerance for mussels in this study. However,

ET50s may not be the best way to compare thermal tolerances when mortality changes from close to zero to almost 100% over a very small temperature span (Wolcott 1973, Hines et al. 1980).

Acclimation effects are difficult to distinguish in this study because the experimental temperatures were not the same at each acclimation temperature, and there were 3ºC increments between temperatures where lethality occurred. It is also possible that there was no clear acclimation/thermal tolerance relationship in this study because the acclimation period was shorter than in some previous studies due to practical necessity of working with mussel early life stages. The nature of the early life stages of freshwater mussels makes it necessary to use a short acclimation period, especially for glochidia. Glochidia are not viable for an extended period of time after release from a gravid female (see Cope et al. 2008), therefore a test with a 24 h duration and a 2 h acclimation period may be relevant to situations experienced environmentally.

It has been observed that short term laboratory acclimation over a few days can not alter previous acclimatization to an animal's seasonal natural environment (Ansell et al. 1980b). A review of acute temperature studies with 50 aquatic species, including fish, mollusks, crustaceans, a medusozoa, and an annelid, found in most cases an acclimation period exceeding 96 h (de Vries et al. 2008), while other studies have used even longer periods of time from 14 to 34 days (Newell et al. 1971, Tomanek and Somero 1999, Carveth et al. 2006, Widmer et al. 2006). A review of the thermal tolerance data for 21 fish species found that it took from 1 to 20 days for the fish to reacclimate and adapt their thermal tolerances (Beitinger and Bennett 2000). According to ASTM protocol,

acclimation for mussel early life stage tests should occur over 2 hours for glochidia and for at least 24 hours for juveniles, with temperature increases of no more than 3ºC/h (ASTM 2006). The methods used in this study exceeded these minimum recommendations by using these time recommendations with a maximum temperature change of 1ºC/h for glochidia and 2.5ºC/day for juveniles. In the future, thermal tolerance tests with juvenile mussels with a longer acclimation period would be valuable for comparison with the results of this study in order to determine any latent effect of acclimation on thermal tolerance.

Different species often have different thermal tolerances, and these differences can be caused by genetic factors or through acclimation to a species's natural habitat (Wolcott 1973, Urban 1994, Tomanek and Somero 1999, Beitinger and Bennett 2000, Petes et al. 2007). My results showed significant differences in ET50s in glochidia among several species, but no differences among juveniles. This may be attributed to the fact that glochidia viability is highly variable among species, even at common temperatures (Cope et al. 2008); some species may be more sensitive to stressors of any kind, but once glochidia have transformed into juveniles, they exhibit similar viabilities and sensitivities to stressors because only the healthiest individuals survive the transition. As adults, morphological characteristics may play a role in the different thermal tolerances among species. The shape, thickness, and size of a mussel's shell can all contribute to regulation of internal temperature (Bartsch et al. 2000). Elliptical shells have larger surface area per volume and can cause a mussel's temperature to rise faster than one with a more spherical shape, as do shells smaller in size; in addition, thinner

shelled species, such as Anondontines, may be less thermally tolerant than thicker shelled species, such as Amblemines (Bartsch et al. 2000).

Thermal tolerances also may differ among life stages, with younger life stages tending to be more sensitive than adults (Loosanoff et al. 1951, Ansell et al. 1980b), and for freshwater mussels, the free living glochidial life stage tends to be most vulnerable (Bauer 2001). The ET50s that were determined for glochidia and juveniles of the same species in my study demonstrated that the two early life stages of pink heelsplitter and fatmucket had different thermal tolerances, though the relationship was not the same. Pink heelsplitter glochidia were more sensitive to thermal stress, whereas for fatmucket, juveniles were more sensitive. Other species in this study, black sandshell, butterfly, washboard, brook floater, and eastern creekshell, did not have differences in ET50s between life stages. Laboratory testing of chemical exposures with freshwater mussels have found that while glochidia sensitivity can vary with species, the response is generally similar to that of newly transformed juvenile mussels (Ingersoll et al. 2007, Keller et al. 2007). In a laboratory environment, the same may be true for thermal exposures, however in the mussel's natural environment, the different life stages will be exposed to thermal stress differently. Glochidia are primarily exposed to stressors, including temperature, through surface water while in the free-living stage in the water column (Cope et al. 2008); juvenile mussels, however, remain burrowed in sediment for the first 2 to 4 years of life after transformation and therefore sediment may provide a thermal buffer (Strayer et al. 2004, Schwalb and Pusch 2007).

Warm temperatures, even when not lethal, create a higher demand on metabolic energy and therefore, can interfere with behavior, maintenance, and reproductive processes (Barnett 1972, Ansell et al. 1980a, Ansell et al. 1980b, Dudgeon and Morton 1984, Parker et al. 1984, Weaver et al. 1991, Urban 1994, Roberts and Barnhart 1999, Bartsch et al. 2000). The ET05s generated in this study represent temperatures that are most likely high enough to cause sublethal effects which can weaken or otherwise disrupt populations of freshwater mussels. ET05s ranged from 21.6ºC to 35.3ºC with a mean of 29.5ºC overall, and these temperatures are a more environmentally relevant benchmark to use in determining thermal stress because while these temperatures may be lethal to only 5% of the exposed population, they may have other adverse effects. For instance, the timing of reproduction can be altered by changes in temperature (Barnett 1972). This can lead to decreased fertilization and recruitment success (Walther et al. 2002, Philippart et al. 2003). Shifts in reproductive periods can also lead to asynchrony, where, in an attempt to give early life stages optimal environmental conditions, reproductive timing is altered and there is a mismatch with other environmental conditions, such as nutrient or habitat availability (Visser and Holleman 2001, Philippart et al. 2003). Rising temperatures have already been shown to cause a mismatch in relationships between early life stages of marine bivalves and the phytoplankton and shrimp they rely on for nourishment (Philippart et al. 2003). A similar mismatch in relationship could inhibit the freshwater mussel from reproducing if the timing of gravidity and glochidial release becomes asynchronized with the presence and distribution of the necessary host fish, as

changes in temperature can alter suitable thermal habitat, leading to shifts in species distributions (Eaton and Scheller 1996, Daufresne et al. 2003, Mohseni et al. 2003).

Because acclimation to environmental conditions occurs over time, normal temperatures in an animal's natural habitat are rarely harmful (Ansell et al. 1980b). However, changes in temperature extremes do not contribute to acclimating animals to higher thermal tolerances, but increases in extreme temperatures have more of an adverse effect than gradual changes (Newell et al. 1971, Stachowicz et al. 2002, Hastie et al. 2003). The chances of negative thermal effects on aquatic organisms increase in the summer when the natural heat load may be increased to threatening levels by heated inputs (Durrett and Pearson 1975, Ansell et al. 1980a, Parkin and Stahl 1981). Summer water temperatures in the United States can range from 25ºC in the Midwest to 34ºC in Texas (Wellborn and Robinson 1996, Wright et al. 1999). As demonstrated here, these temperatures are very close to the upper thermal tolerances for the early life stages of several species of freshwater mussels, and species living closest to their thermal limits may be most susceptible to changes in environmental temperatures (Tomanek and Somero 1999, Stillman 2003). In Texas, the summer water temperature of 34ºC rose to 38- 42ºC in a heated effluent discharge pond (Wellborn and Robinson 1996). My study has shown that temperatures above 37ºC cause total mortality in juvenile freshwater mussels, and temperatures from 37- 39ºC cause total mortality in glochidia, with significant mortality for both life stages occurring at lower temperatures.

Freshwater mussels are likely to encounter rising environmental temperatures whether from climate change or directly from heated effluents, drought, or land use

changes. Because this group of organisms is already among the most imperiled in the world, it is crucial to identify the factors that are contributing to population declines. I have demonstrated that temperatures sometimes encountered in a summer stream or aquatic environment can be lethal. Sublethal effects occur before acute lethality, and these effects may also contribute to population declines. Shifts in reproductive schedules in freshwater mussels could be detrimental if a mismatch between the mussels and their host fish were to occur. Water quality criteria that take into account the fact that thermal stress can come from several sources at the same time must consider that while one heat source may not be detrimental to aquatic organisms, a combined input might be. It is also important to consider that freshwater mussels are constantly exposed to a range of other stressors, both chemical and nonchemical, and that a bivalve that has already been weakened by thermal stress may be more susceptible to other adverse conditions (Sokolova 2004).

Freshwater mussels are a valuable part of the aquatic ecosystem, but they are also the most threatened. The results of this study indicate that there is a narrow range where acute thermal stress becomes lethal for freshwater mussels, and it is necessary to keep rivers and streams thermally acceptable for these sensitive animals. By incorporating my findings to review water quality criteria for temperature, we can ensure the protection of freshwater mussels from rising environmental temperatures. Future studies on temperature with native freshwater mussel early life stages should investigate longer term or chronic exposure to elevated water temperatures, the influence of burrowing in

sediment on temperature sensitivity of juveniles, and mesocosm-based or in situ caging exposures to heated waters.

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Table 1. ET50s and 95% Confidence intervals (in parentheses) for glochidia (24 h) and juvenile (96 h) mussels at the 22ºC and 27ºC acclimation temperatures. ND indicates ET50s outside of tested temperature range, or unable to be determined, *white heelsplitter juveniles not tested. All ET50s reported as ºC.

	22ºC Acclimation		27ºC Acclimation	
Species	Glochidia	Juveniles	Glochidia	Juveniles
Fatmucket	ND	35.54 (35.14- 35.96)	36.92 (35.29- 38.62)	34.31 (33.50- 35.14)
Pink heelsplitter	29.06 (25.55- 33.06)	34.79 (33.12- 36.54)	ND	34.60 (33.36- 35.90)
Black sandshell	ND	32.90 (29.58- 36.59)	33.89 (30.40- 37.79)	36.74 (34.37- 39.27)
Butterfly	33.65 (31.17- 36.32)	ND	30.64 (18.48- 50.79)	34.21 (33.20- 35.25)
White heelsplitter	35.99 (34.28- 37.79)	*	37.51 (36.94- 38.09)	*
Washboard	32.38 (29.58- 35.45)	34.16 (32.26- 36.18)	32.44 (29.23- 36.01)	34.98 (33.51- 36.52)
Brook floater	35.80 (34.58- 37.07)	35.05 (33.77- 36.39)	36.85 (35.28- 38.49)	35.29 (32.79- 37.99)
Eastern creekshell	32.87 (29.63- 36.47)	34.60 (32.75- 36.54)	31.43 (27.60- 35.79)	34.72 (33.19- 36.32)

Table 2. ET05s and 95% Confidence intervals (in parentheses) for glochidia (24 h) and juvenile (96 h) mussels at 22ºC and 27ºC acclimation temperatures. ND indicates ET05s unable to be determined, *no test run for white heelsplitter juveniles. All ET05s reported as ºC.

	22ºC Acclimation		27ºC Acclimation	
Species	Glochidia	Juveniles	Glochidia	Juveniles
Fatmucket	ND	ND	35.28 (33.99- 35.79)	ND
Pink heelsplitter	21.69 (12.41- 24.79)	ND	ND	33.21 (31.61- 33.96)
Black sandshell	ND	29.13 (23.22- 30.92)	28.37 (18.96- 31.00)	ND
Butterfly	30.73 (24.76- 32.16)	ND	ND	ND
White heelsplitter	ND	ND	ND	ND
Washboard	28.18 (19.97- 30.38)	ND	27.60 (21.07- 29.61)	ND
Brook floater	ND	ND	35.26 (32.94- 35.76)	32.33 (27.17- 33.74)
Eastern creekshell	26.94 (17.73- 29.51)	32.52 (27.58- 33.35)	21.55 (6.71- 25.75)	32.56 (30.57- 33.46)

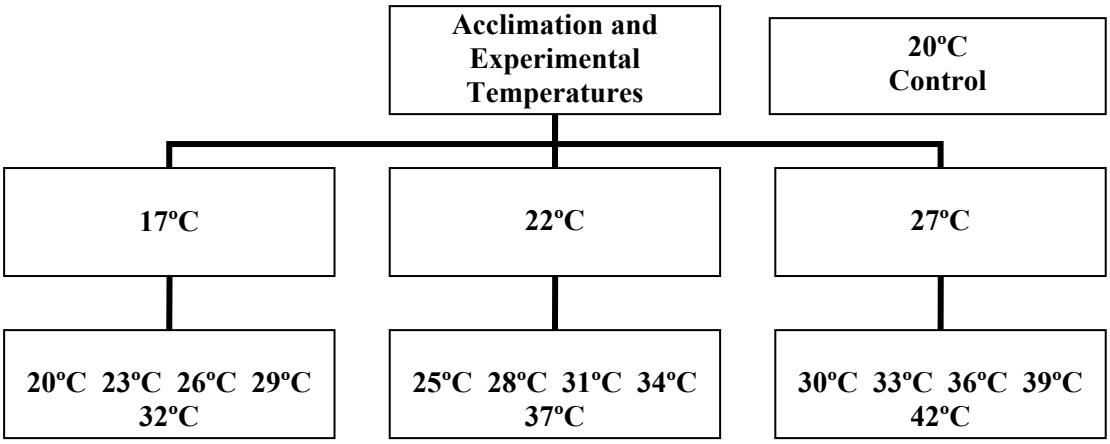

Figure 1. Experimental design showing acclimation and experimental temperature schemes for the early life stages tests with freshwater mussels.

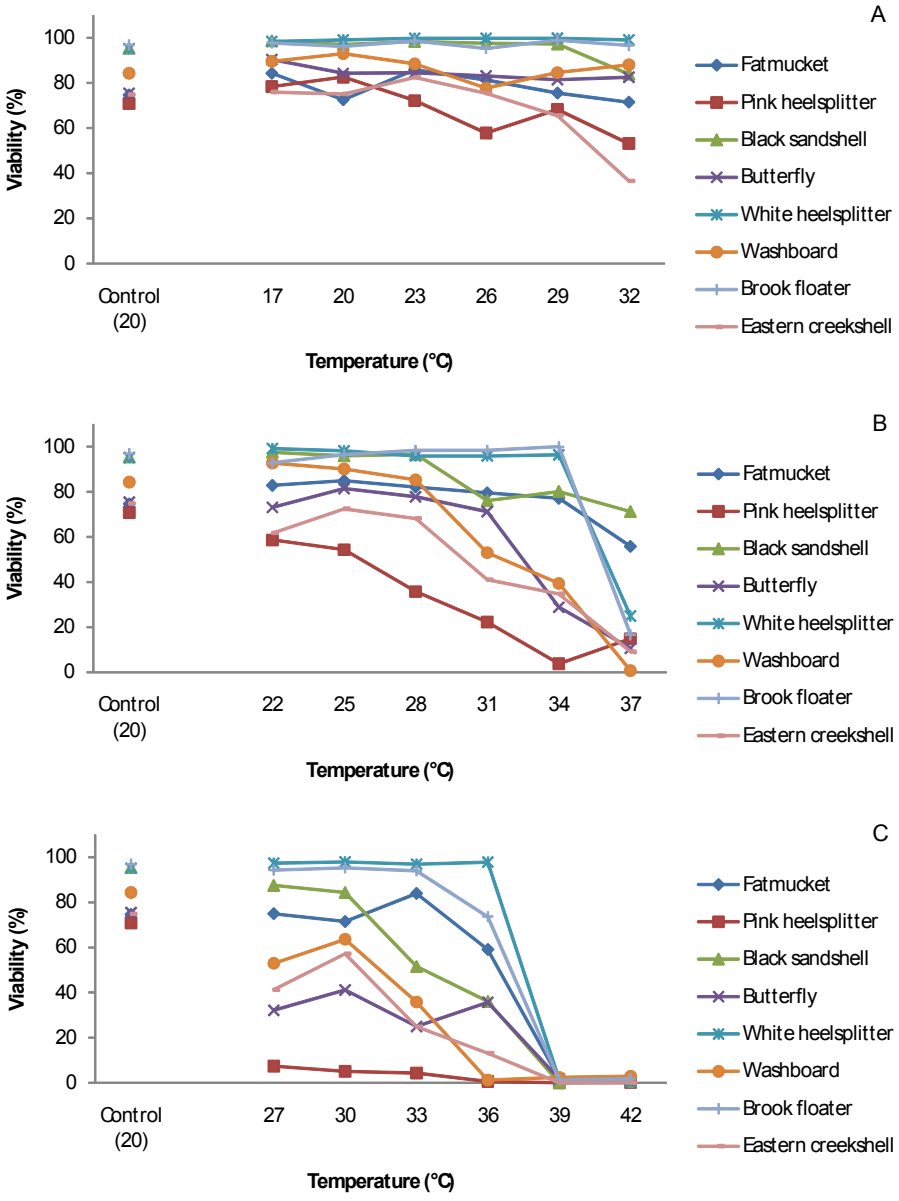

Figure 2. Viability trends for glochidia of eight species of mussel at three acclimation temperatures; 17°C (A), 22°C (B), 27°C (C).

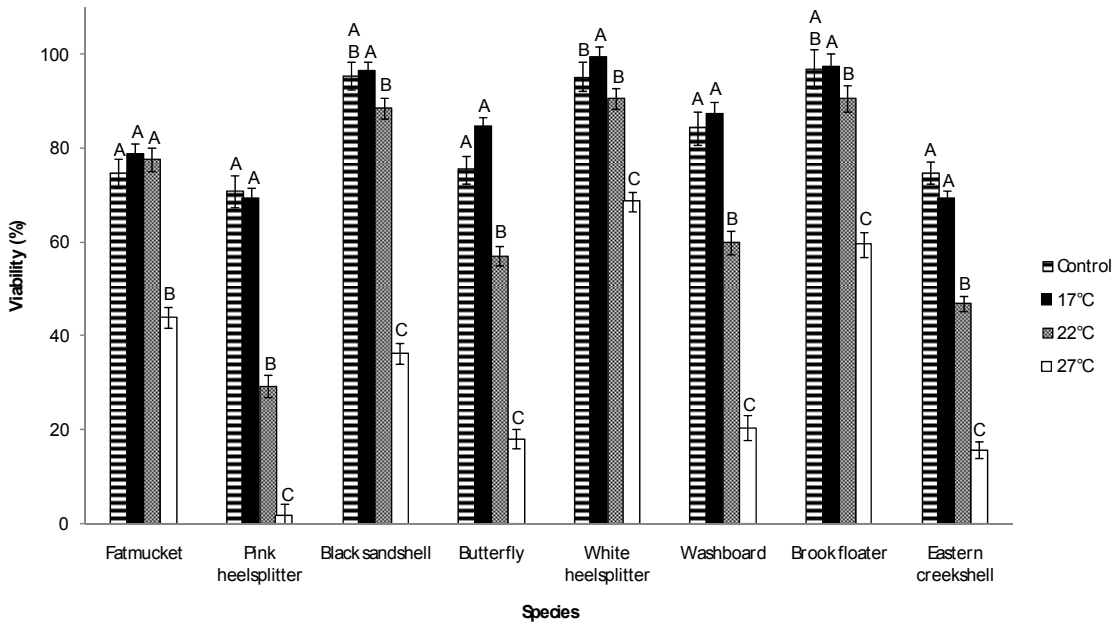

Figure 3. Comparisons of mean viability over all experimental temperatures at three acclimation temperatures for glochidia of eight species of freshwater mussel. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

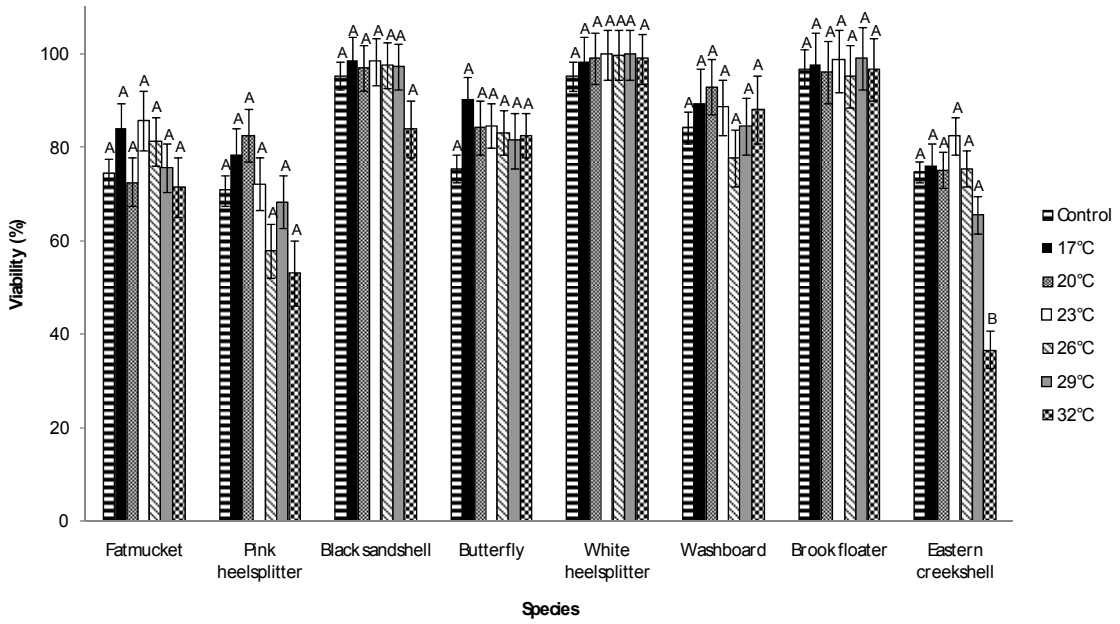

Figure 4. Thermal tolerances of glochidia of eight mussel species at the 17°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

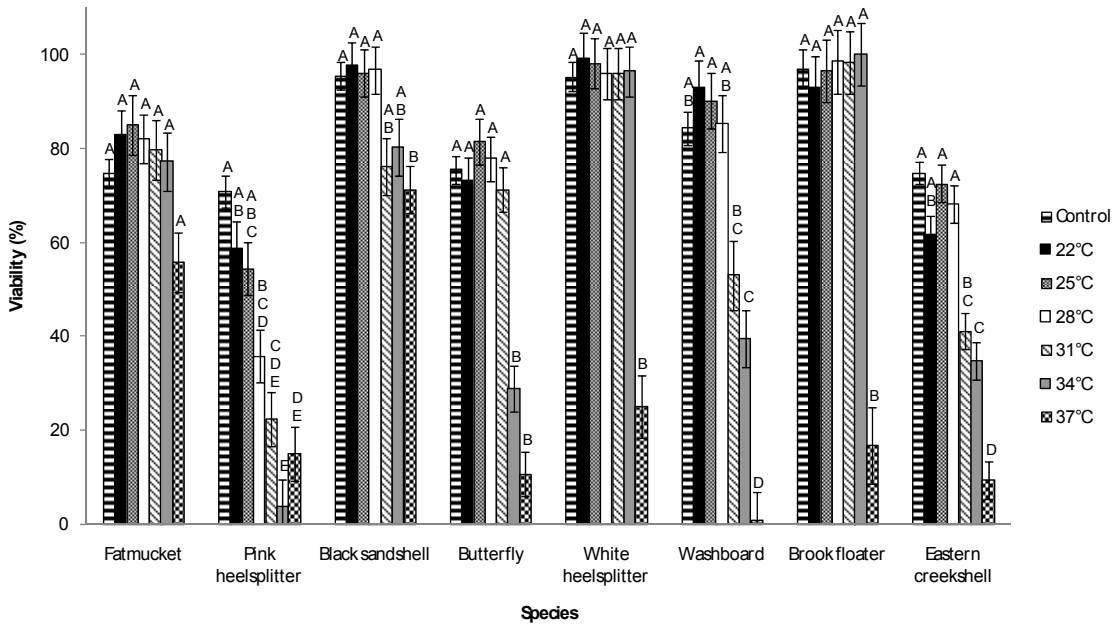

Figure 5. Thermal tolerances of glochidia of eight mussel species at the 22°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

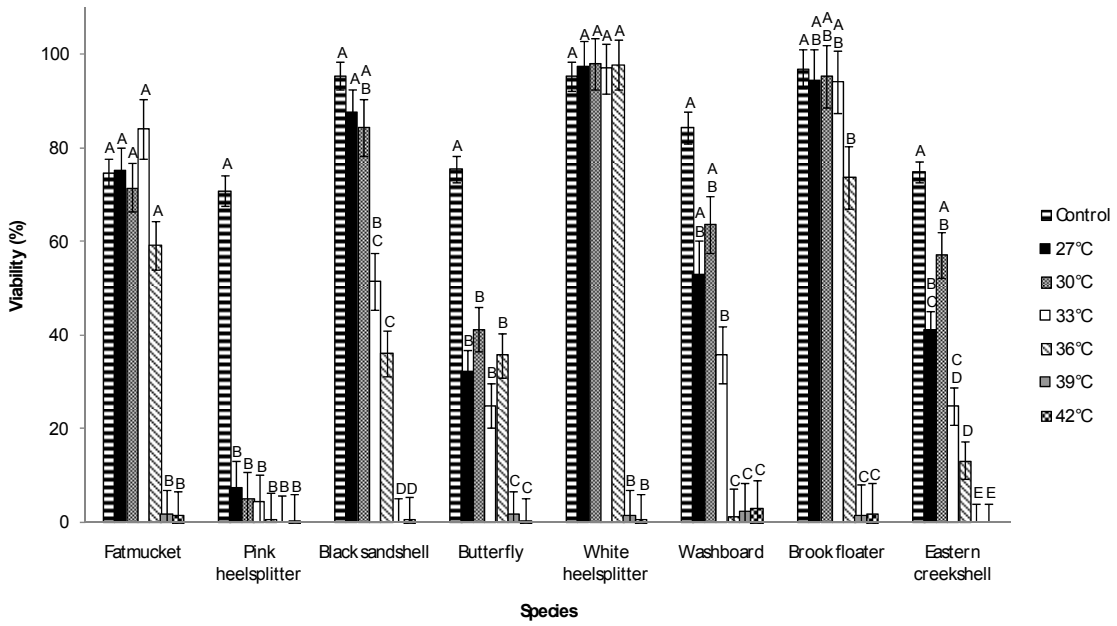

Figure 6. Thermal tolerances of glochidia of eight mussel species at the 27°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

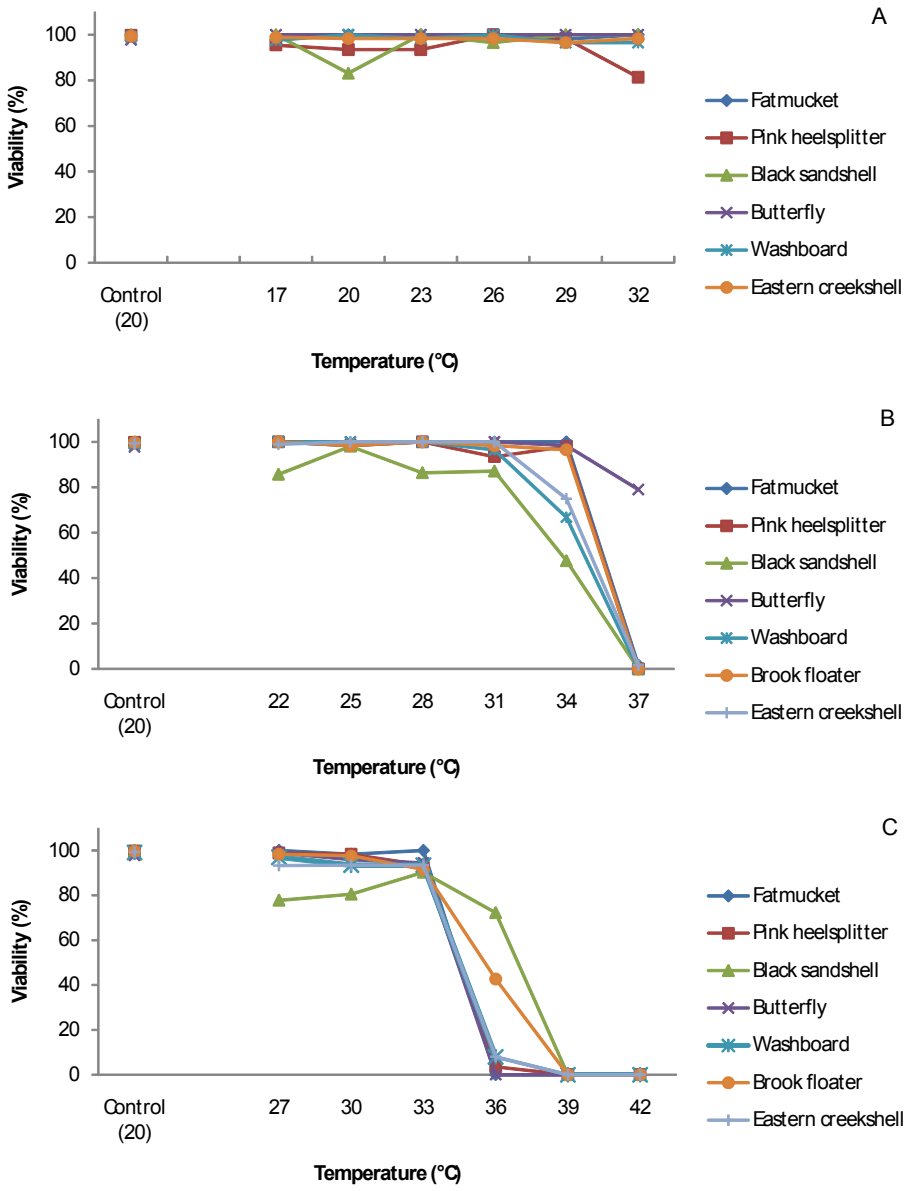

Figure 7. Viability trends for seven species of juvenile freshwater mussels at three acclimation temperatures; 17°C (A), 22°C (B), 27°C (C).

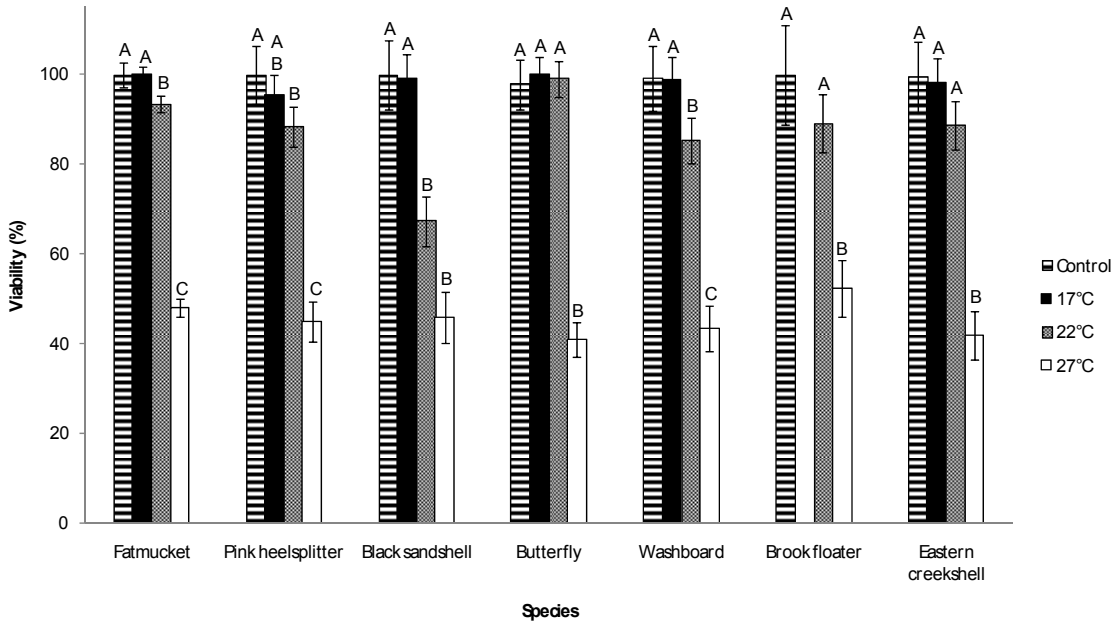

Figure 8. Comparisons of mean viability over all experimental temperatures at three acclimation temperatures for seven species of juvenile freshwater mussels. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the $\alpha=0.01$ level. Letters refer to significance within a species not among species.

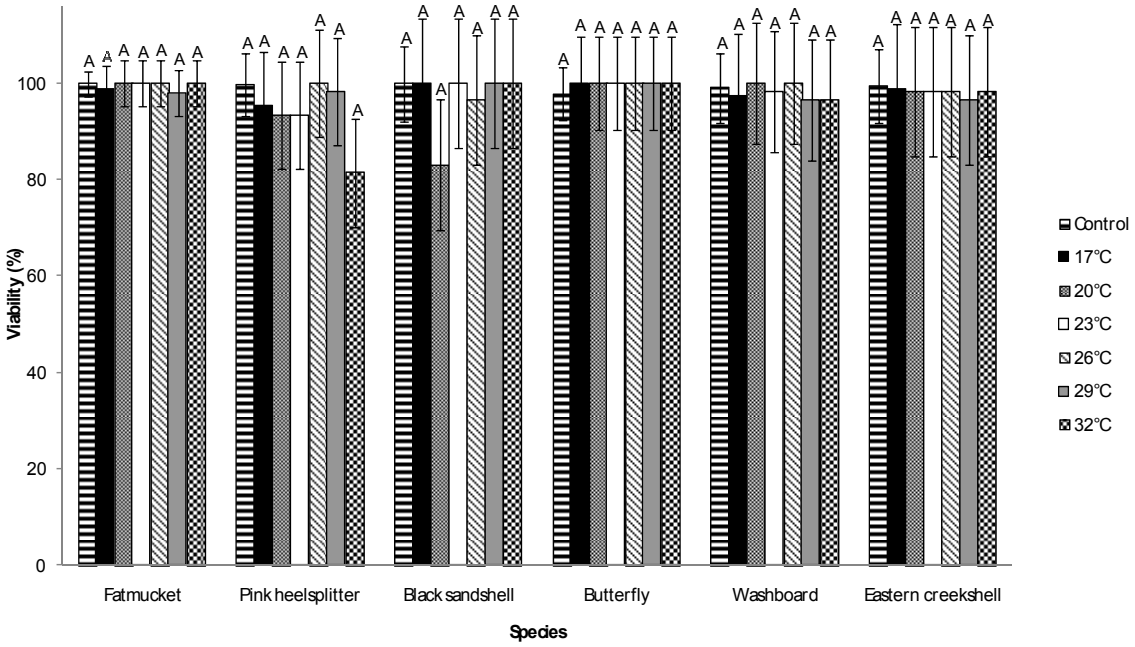

Figure 9. Thermal tolerances of six species of juvenile freshwater mussels at the 17°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

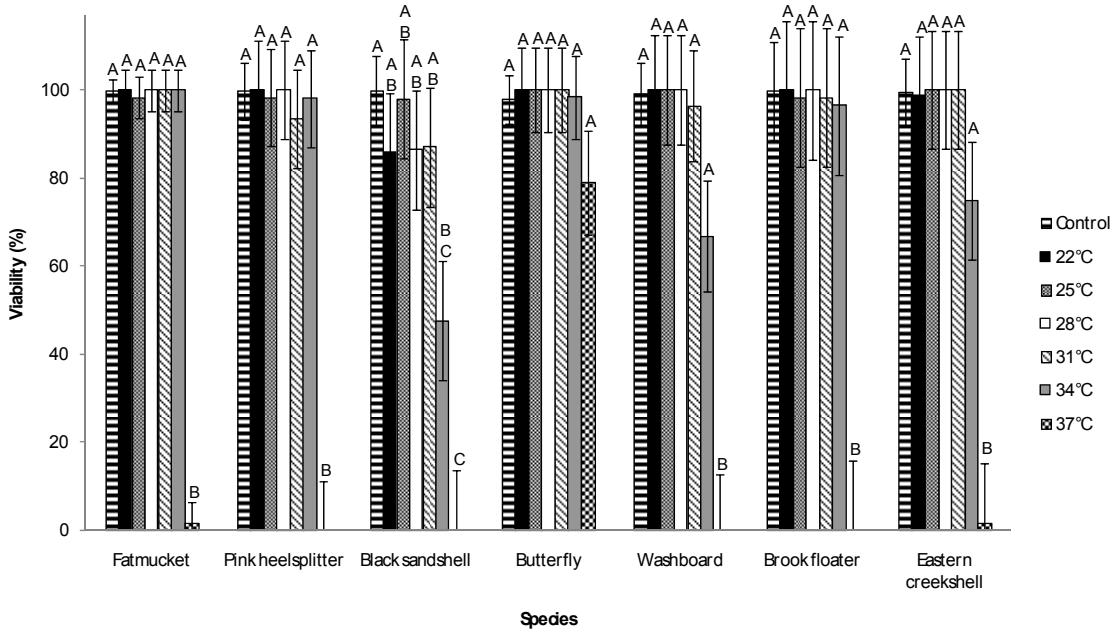

Figure 10. Thermal tolerances of seven species of juvenile freshwater mussels at the 22°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

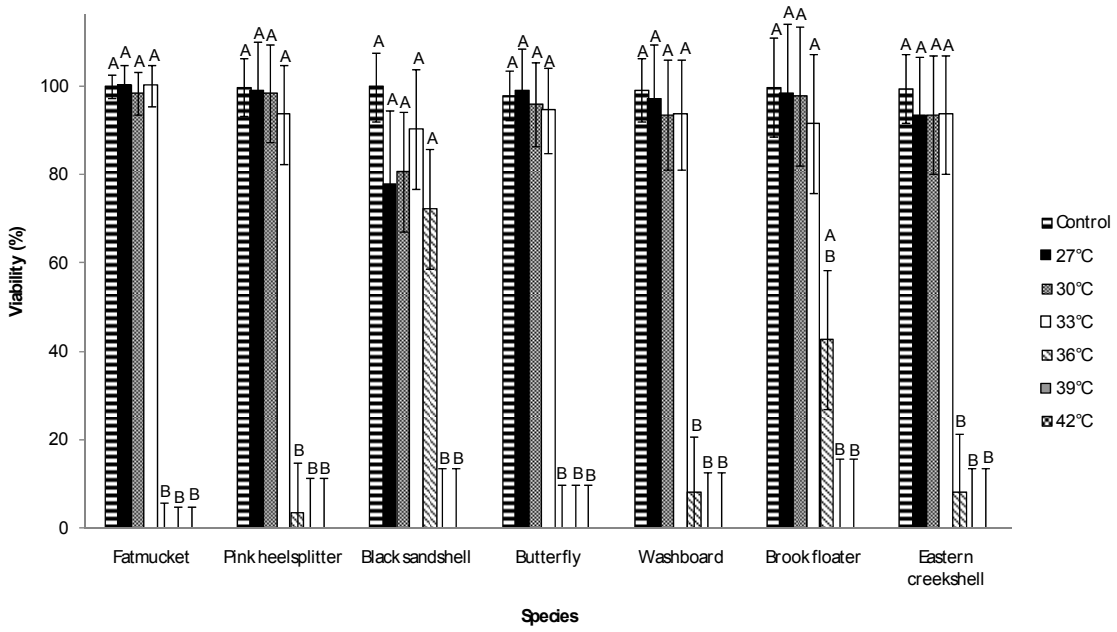

Figure 11. Thermal tolerances of seven species of juvenile freshwater mussels at the 27°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

Chapter 2. Heart rate as a sublethal indicator of thermal stress in juvenile freshwater mussels.

(Formatted for: Comparative Biochemistry and Physiology Part A)

Abstract

Freshwater mussels belonging to the order Unionoida fulfill an essential role in aquatic communities, but are also one of the most sensitive and rapidly declining faunal groups in North America. Rising water temperatures, caused by global climate change, industrial discharges, drought, or land development, can further challenge impaired unionid communities. Because of a direct relationship between heart rate and temperature in poikilotherms, heart rate has been used as an indicator of whole animal thermal stress. The purpose of this study was to determine heart rate patterns for seven species of juvenile freshwater mussels (*Lampsilis siliquoidea*, *Potamilus alatus*, *Ligumia recta*, *Ellipsaria lineolata*, *Megalonaias nervosa*, *Alasmidonta varicosa*, and *Villosa delumbis*) in response to a range of experimental temperatures at three different acclimation temperatures. Heart beat was assessed visually through direct observation, and species differences were observed; *L. recta* and *V. delumbis* displayed significant changes in heart rate associated with increasing temperature at all three acclimation temperatures. However, no acclimation effects were detected. The use of heart rate appears to be a suitable indicator of thermal stress in unionid mussels.

Keywords: freshwater mussel, Unionidae, heart rate, temperature, thermal tolerance

Introduction

Freshwater mussels of the bivalve order Unionoida are long-lived, benthic aquatic organisms with considerable roles as nutrient processors and ecosystem engineers in the aquatic community (Vaughn et al. 2004, Howard and Cuffey 2006, Vaughn et al. 2008). Unionids are one of the most sensitive and rapidly declining faunal groups in North America and the world. Of the estimated 840 species of freshwater mussels globally, approximately 300 are native to North America, but nearly 70% of these species are extinct or vulnerable to extinction (Graf and Cummings 2007, Bogan 1993, Williams et al. 1993) This decline has been attributed to several factors, including: habitat degradation, water withdrawal for industry, urbanization, dam construction, impoundments, sedimentation, navigation, pollution, introduction of nonindigenous mollusks, overharvesting, and land use change (Bogan 1993, Williams et al. 1993, Lydeard et al. 2004, Strayer et al. 2004, Bogan 2008). Though the mussel's life cycle makes it susceptible to disruptions, large data gaps exist regarding the effects of both chemical and non-chemical stressors, such as temperature, on freshwater mussels. Rising water temperatures caused by heated effluents or global climate change may pose an additional risk to already threatened mussel species (Hastie et al. 2003).

Changes in temperature can affect various metabolic physiological functions such as oxygen consumption and heart rate. In poikilotherms, heart rate increases with temperature within physiological limits, and because it often reflects metabolic rate, heart rate has been used as an indicator of whole-animal thermal stress (Helm and Trueman

1967, Harrison 1977a, Polhill and Dimock 1996, Braby and Somero 2006). Because heart rate is so closely linked with metabolism, it is a standard response in poikilotherms for heart rate to increase with increasing experimental temperature; this has been demonstrated time and again for various species, including pulmonates (Harrison 1977a, Harrison 1977b), marine bivalves (Trueman and Lowe 1971, Lowe and Trueman 1972, Braby and Somero 2006), and freshwater mussels (Dietz and Tomkins 1980, Polhill and Dimock 1996). In addition to this general positive relationship between temperature and heart rate, it has also been observed that at a point, this relationship breaks and there is a critical high temperature where heart rate plateaus or begins to decrease (Lowe and Trueman 1972, Harrison 1977a, Harrison 1977b, Braby and Somero 2006).

The purpose of this study was to determine the applicability of heart rate as a measure of sublethal thermal stress in juvenile freshwater mussels. I monitored the heart rate of seven species of juvenile freshwater mussels representing three tribes from two subfamilies of the family Unionidae (Graf and Cummings 2007) via direct visual observation in response to different temperature treatments. Mussels were tested at three different acclimation temperatures over a range of temperatures from 20°C to 42°C, and heart beats were counted, compiled, and compared among species.

Materials and methods

Test organisms

The seven species of juvenile freshwater mussels used in this study represented three tribes from two subfamilies of the family Unionidae (Graf and Cummings 2007) From the Ambleminae subfamily, five species were from the Lampsilini tribe: fatmucket (*Lampsilis siliquoidea*, Barnes, 1823), pink heelsplitter (*Potamilus alatus*, Say, 1817), black sandshell (*Ligumia recta*, Lamarck, 1819), butterfly (*Ellipsaria lineolata*, Rafinesque, 1820), and eastern creekshell (*Villosa delumbis*, Conrad, 1834); while one species was from the Quadrulini tribe: washboard (*Megalonaias nervosa*, Rafinesque, 1820). One species, brook floater (*Alasmidonta varicosa*, Lamarck, 1819) belonged to the Anodontini tribe of the Unioninae subfamily.

Fatmucket, pink heelsplitter, and black sandshell ranged in age from 3 to 8 weeks, and the average size for fatmucket was 1,386 µm, pink heelsplitter was 1,377 µm, and black sandshell was 947 µm. The remaining species (butterfly, washboard, brook floater, and eastern creekshell) ranged in age from less than 1 week to 4 weeks old and the average size for butterfly was 335 µm, washboard was 364 µm, brook floater was 398 µm, and eastern creekshell was 363 µm. At the 17ºC acclimation temperature, brook floater was not tested due to insufficient numbers of test organisms, and heart rate measurements for washboard could not be made because heart beat was difficult to observe.

Heart rate assessment

Juvenile freshwater mussels were exposed to a range of common and extreme water temperatures and their heartbeats recorded. Each test was conducted at three acclimation temperatures: 17ºC, 22ºC, and 27ºC, and each acclimation temperature had five corresponding experimental temperatures in 3ºC increasing increments. There was also a 20ºC reference control temperature that was assessed alongside each test (Figure 1). Temperatures above 37ºC resulted in total mortality for all species, thus no heart rate data was available for those temperatures.

Heart rate was assessed visually using an Olympus SZ61 microscope. Heart beats were counted for 15 seconds per mussel, using 1 to 4 mussels from each of 3 replicates per treatment. Duplicate heart beat counts were performed periodically using an additional researcher for quality assurance of heart rate determination (Appendix Table 7). Heart rate is expressed in all figures as mean heart beats counted in a 15 second time interval. For comparison purposes, some values were converted to beats per minute (bpm).

Quality Assurance

Other quality assurance and control procedures were maintained by conducting all tests according to the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM 2006). All tests were conducted in light and temperature controlled environmental chambers (Precision Model 818, Thermo Electron Corp., Marietta, OH, USA, and Isotemp Model 146E, Fisher Scientific, Dubuque, Iowa, USA),

and National Institute of Standards and Technology (NIST) certified thermometers were used for daily temperature monitoring. Water quality conditions, including alkalinity, hardness, conductivity, pH, temperature, and dissolved oxygen, were measured at the start of each test and again at the 48 hour time point. Alkalinity and hardness were measured by titrametric procedures with standard methods (APHA 1995). Conductivity, temperature, pH, and dissolved oxygen were measured with a calibrated meter (YSI model 556 MPS multi-probe, Yellow Springs Instrument Co., Yellow Springs, OH, USA). For all tests, alkalinity ranged from 92 to 110 mg CaCO3/L with a mean of 103.7 mg CaCO3/L, hardness ranged from 140 to 162 mg CaCO3/L with a mean of 150.7 mg CaCO3/L, conductivity ranged from 472 to 684 µs/cm with a mean of 562.3 µs/cm, pH ranged from 7.08 to 8.87 with a mean of 8.43, and dissolved oxygen ranged from 4.61 to 9.73 mg/L with a mean of 7.16 mg/L (n=15 for alkalinity and hardness, n=127 for all other parameters). Test temperatures had a maximum of 1.5ºC departure from the target temperature, with only 0.5% of samples exceeding a 1ºC departure (n=546).

Statistical analysis

Results were analyzed with SAS® Proc Mixed (SAS Institute Inc, 2006). Acclimation temperature and experimental temperature were considered fixed effects, while repetitions within each acclimation temperature were considered as random effects. Acclimation temperatures were considered crossed effects, and experimental temperature was taken as a nested effect within each acclimation temperature. Significance level was established at $p \leq 0.01$ for model effects, while means differences were analyzed by a t-

test for pairwise least squares mean differences with Tukey's adjusted p-value and 0.01 significance level to control type I error. Significance level was established at $p \leq 0.01$ in order to ensure significance was assigned only to responses beyond those associated with variation in natural mussel populations.

When significance was detected, pairwise mean comparison was used to analyze mean differences between acclimation temperatures. Significance of experimental temperatures was studied through the analysis of simple effects for experimental temperatures within each acclimation temperature and when necessary, the effect differences of experimental temperatures was analyzed within each acclimation temperature with a t-test for pairwise least squares mean differences with Tukey's adjusted p-value and 0.01 significance level. Significance level for each pairwise mean comparison was calculated using the distribution of the studentized range (Steel et al. 1997).

Results

Heart rate at the control temperature of 20ºC averaged 55 bpm among all species. Black sandshell had the lowest control heart rate of 38 bpm, butterfly, washboard, and eastern creekshell all had an average control heart rate of 53 bpm, fatmucket control heart rate was 58 bpm, brook floater's was 62 bpm, and pink heelsplitter had the highest control heart rate at 65 bpm. Trends of all heart rate responses over experimental temperature at all three acclimation temperatures were observed (Figure 2). Fatmucket,

butterfly, washboard, and brook floater had no significant differences across overall mean heart beat per acclimation temperature (Figure 3). Pink heelsplitter had a significantly lower mean heart rate at the control ($p<0.0001$) than at the 22ºC acclimation, but not at the 27ºC acclimation ($p=0.0243$). The 17ºC acclimation mean heart rate was also different from the 22ºC acclimation ($p=0.0092$) for pink heelsplitter. The control heart rate for black sandshell was significantly lower than for 17ºC, 22ºC, and 27ºC acclimations ($p<0.0001$). For eastern creekshell, the control was significantly lower than the 22ºC acclimation ($p=0.0018$) and the 27ºC acclimation ($p<0.0001$), but was not different from the 17ºC acclimation ($p=0.0365$). The 17ºC acclimation was significantly lower than the 27ºC acclimation ($p=0.0004$), but not the 22ºC acclimation ($p=0.4514$); the 22ºC and 27ºC acclimations were not different ($p=0.0156$).

At the 17ºC acclimation temperature (Figure 4), butterfly and pink heelsplitter did not exhibit any significant changes in heart rate as a response to increasing temperature ($p>0.01$). There appeared to be an upward trend of heart rate with temperature for pink heelsplitter, but it was not statistically significant. Fatmucket had a significant increase in heart rate from the control associated with the 26ºC experimental temperature ($p=0.0023$), but not with the two highest temperatures, 29ºC and 32ºC. The 17ºC acclimation temperature was also significantly lower than 26ºC ($p=0.0082$). Black sandshell exhibited significant increases in heart rate from the control at 26ºC, 29ºC, and 32ºC (all $p<0.0001$), though the control was not different from any of the other temperatures ($p>0.01$). Heart rate at 23ºC, 26 ºC, and 29ºC was not different ($p>0.01$),

and 29ºC was also similar to 32ºC ($p>0.01$). However, 32ºC was different from all other temperatures ($p<0.0001$ for all comparisons, except $p=0.0041$ for 26ºC). For eastern creekshell, the control ($p=0.0002$), 17ºC ($p=0.0014$), and 20ºC ($p=0.0018$) experimental temperatures were all different from the 32ºC treatment. All other temperature treatments were not different.

Fatmucket, pink heelsplitter, washboard, and brook floater all showed no significant change in heart rate associated with increasing temperatures at the 22ºC acclimation temperature (Figure 5). For black sandshell, the control was significantly different from both 31ºC and 34ºC ($p<0.0001$), no other differences were observed. Butterfly control heart rate was significantly lower than at 34ºC ($p<0.0001$). The 34ºC heart rate was also different from 25ºC ($p=0.0044$) and 28ºC ($p=0.0008$), but not the 22ºC treatment ($p=0.0109$). Eastern creekshell control heart rate was significantly lower than at 34ºC ($p=0.0018$), and this was the only significant difference for this species at the 22ºC acclimation.

At the 27ºC acclimation temperature, there are heart rate data for at most 5 temperatures because total mortality occurred at the two highest temperature treatments, and therefore no heart rate analysis could be performed (Figure 6). Fatmucket, pink heelsplitter, butterfly, washboard, and brook floater all exhibited no significant differences among temperatures at this acclimation (all $p>0.01$), though there were some upward trends in heart rate associated with increased temperature. Black sandshell had significantly lower heart rate at the control temperature when compared with both 33ºC

and 36ºC ($p<0.0001$). The 36ºC heart rate was similar to the 33ºC treatment ($p=0.9252$), though it was different from 27ºC and 30ºC ($p<0.0001$). Eastern creekshell had a significantly lower heart rate at the control temperature than at 33ºC ($p<0.0001$), but all other treatments were not different.

Black sandshell and eastern creekshell were the only two species to show significant effects of temperature on heart beat at all three acclimation temperatures. For these two species mean heart rate was plotted against all experimental temperatures at all three acclimation temperatures. For black sandshell (Figure 7), increasing temperature accounted for 80.3% of the variation in heart rate, whereas increasing temperature accounted for 82.7% of the variation in heart rate for eastern creekshell (Figure 8). Although pink heelsplitter did not exhibit significant effects of temperature when broken down by acclimation temperature, temperature accounted for 75.2% of the increase in heart rate when all experimental temperatures were combined (Figure 9).

Discussion

Studies that examined heart rate in mollusks have employed several methods including impedance pneumography (Trueman 1967, Trueman and Lowe 1971, Braby and Somero 2006), plethysmography (Bahkmet and Khalaman 2006), photocells (Dietz and Tomkins 1980), and direct visual observation (Harrison 1977a, Harrison 1977b). The majority of methods are useful only in adult specimens that are large enough to accommodate the attachment of required equipment; therefore, for heart rate observations

of newly transformed juvenile freshwater mussels with transparent shells, direct visual observation, as used in this study, is the preferred method.

Because of shell thickness, age, and other morphological factors, not all species of juvenile freshwater mussels are as amenable to heart beat assessment as others, and a large sample size may be needed to achieve results beyond natural variability. With species that are difficult to assess, this may not be possible. In this study, for some species, heart rate can be used reliably to monitor thermal stress at increasing temperatures, but in others there appears to be no correlation between increasing temperature and heart rate.

Black sandshell and eastern creekshell were the only two species in this study to show significant effects of temperature on heart beat at all three acclimation temperatures. When all experimental temperatures are combined for one species for all three acclimation temperatures, there is a clear relationship between increasing temperature and increasing heart rate for the two species, black sandshell and eastern creekshell, which exhibited significant effects of temperature at all three acclimation temperatures. For these two species, increasing temperature contributed to about 80% of the change in heart rate (Figures 7 and 8). Pink heelsplitter, though it did not have significant effects of temperature at any single acclimation temperature, did show an R^2=0.7515 when heart rate was plotted against all temperatures (Figure 9). This suggests that while temperature clearly affects heart rate for pink heelsplitter, the natural variability in the mussels tested was too great for significance to be assigned. If the same

mussel cohort was followed through increases in temperature, significant results may have been obtained for this species. The other species used in this study did not show relationships that were as clear, for butterfly R^2=0.4487, fatmucket R^2=0.3769, washboard R^2=0.0.0331, and brook floater R^2=0.0596.

The differences in the heart rate-temperature relationship may in part be due to the relative ease of detecting heart beat in some species compared with others. The two species with the most significant heart rate trends, black sandshell and eastern creekshell, were also the species with most easily observed heart beats due to shell and anatomical characteristics, therefore more measurements could be made per replicate, and the count was most likely more robust. Fatmucket, butterfly, and brook floater heart beats were notably difficult to find at times, and washboard heart beats were so difficult to observe at the 17°C acclimation that no data were collected at these temperatures.

Heart rate can be an extremely variable response, both among species and individuals. While some species exhibit no correlation between heart rate and animal size (Harrison 1977a, Harrison 1977b, Braby and Somero 2006), body mass has been inversely related to heart rate in freshwater mussels (Polhill and Dimock 1996). Different species of the same genus can have significantly different baseline heart rates, as demonstrated for three *Mytilus* (Linnaeus, 1758) species (Braby and Somero 2006), and earlier life stages can have different basal heart rates than adults of the same species (Polhill and Dimock 1996). A recent study has shown that freshwater mussels belong to distinct thermal guilds that influence ecosystem services through different rates of

filtration, biodeposition, and nutrient excretion in response to thermal stress (Spooner and Vaughn 2008). Mussels in the "thermally tolerant" guild increased ecosystem services, i.e. energy and nutrient transfer, with increasing temperature, while the "thermally sensitive" guild displayed various responses (Spooner and Vaughn 2008).

In this study, the average heart rate for all seven species of juvenile freshwater mussels at a control temperature of 20°C was 55 bpm, with a range from 38 bpm (black sandshell) to 65 bpm (pink heelsplitter). These heart rates are comparable to the heart rates of other juvenile freshwater mussels at 22°C, 54 bpm and 40 bpm for *Utterbackia imbecillis* (Say, 1829) and *Pyganodon cataracta* (Say, 1817) respectively (Polhill and Dimock 1996). Adults of the freshwater mussel species *Ligumia subrostrata* (Say, 1831) had a baseline heart rate of 16-19 bpm at 23°C (Dietz and Tomkins 1980); this rate is lower than the rates observed in this study, but that is to be expected as juvenile mussels have higher metabolic rates than adults (Polhill and Dimock 1996, Sukhotin and Portner 2001, Sukhotin et al. 2002). Juvenile animals can also be more sensitive than adults to environmental stressors (Precht 1973, Dimock and Wright 1993), and therefore, heart rates may be more easily influenced by temperature.

In the Polhill and Dimock (1996) study with *U. imbecillis* and *P. cataracta,* the same mussels were monitored at all temperatures and heart rates were determined after 15 minutes of exposure. In contrast, for this study, different mussel cohorts were used at each temperature, and heart rate is a mean from 3 to 10 mussels from a total of 3 replicate treatments after 96 h of exposure. Because of these conditions, the results from this study

include a larger amount of natural variation. More significance may have been observed with heart rate changes at each temperature increase if the same mussels had been followed through the temperature changes, but the experimental design of this study aimed to target heart rate patterns of mussel populations rather than individuals. In addition, heart rates were calculated over 15 second time intervals, and these counts were extrapolated to bpm values for comparison with similar studies. It is worth noting however, that the heart rate of freshwater mussels is rarely constant and is subject to changes in rate in short amounts of time. The mussels that were chosen for heart rate count in this study were selected based on clarity and regularity of beat, therefore, the bpm counts are not expected to differ greatly from 15 second rate counts.

The highest heart rate observed in this study was 135 bpm for eastern creekshell at 33°C in the 27°C acclimation, though all of the other species, except for brook floater, also reached heart rates greater than 100 bpm at some point in the study. Most often (67% of instances), these heart rates were reached at temperatures exceeding 30°C, with the other instances occurring at temperatures equal to or exceeding 25°C. Adult *L. subrostrata* reached a maximum heart rate of only 52 bpm at 35°C (Dietz and Tomkins 1980), but as with baseline heart rate, juvenile measurements are expected to be higher (Polhill and Dimock 1996, Sukhotin and Portner 2001, Sukhotin et al. 2002).

Size and age of juvenile mussels in the ranges used in this study did not clearly influence ease of heart rate monitoring, though in some studies both age (Sukhotin and Portner 2001, Sukhotin et al. 2002) and size (Polhill and Dimock 1996, Sukhotin et al.

2002) have been shown to influence metabolic processes; other studies have found no such correlation (Sukhotin et al. 2002, Braby and Somero 2006). Black sandshell and eastern creekshell do not fall on the same side of the age and size range of mussels used in this study, though they were the species with the most reliable heart beat counts. The age of all juvenile mussels ranged from less than 1 week old up to 8 weeks old; black sandshell was between 3 and 8 weeks old during testing, whereas eastern creekshell was only one week old. In regard to size (shell length), the averages for all species ranged from 335 µm to 1,386 µm, with eastern creekshell on the smaller end at 363 µm and black sandshell on the larger end at 947 µm. These observations indicate that there may not be one set of conditions that is optimal for viewing heart beats, and that this must be determined on a species basis, but as long as juveniles are young enough to be transparent, but large enough for a clear view of heart beat, visual heart rate assessment can be considered.

Though the ability for accurate heart rate measurement is a factor in any observed heart rate-temperature relationship, it is also true that differences among thermal tolerances exist in species. Three *Mytilus* species under common acclimation and assessment conditions had significantly different heart rates, which the authors suggested were genetically fixed (Braby and Somero 2006). Braby and Somero (2006) go on to conclude that differences in survival caused by genetically determined thermal tolerances among species have the potential to outweigh the ability of the species to physiologically adapt to changing temperatures. This suggests that a species living close to its thermal

limits may be able to adapt heart rate to increasing temperatures, unless the temperature exceeds the upper thermal tolerance of the animal. For this reason, it is important to have survival data corresponding to heart rate data; these data are provided for the seven species used in this study in a companion paper (Pandolfo 2008). For instance, black sandshell was the only species to show significant decreases in survival at 34°C in the 22°C acclimation, which could explain why it was also one of the only species to show significant heart rate effects. Somewhere between 34°C and 37°C, we could expect heart rate to slow because at 37°C all species, except butterfly, experienced total mortality.

As with thermal tolerance in general, acclimation has sometimes been shown to affect heart rate responses to changing temperatures. While some poikilotherms exhibit increased thermal tolerance in heart rate response with increasing acclimation temperature (Segal 1956, Braby and Somero 2006) others do not (Ahsanullah and Newell 1970, Widdows 1973). Acclimation responses can not always be assumed based on prior studies with a certain test organism. In a study with juvenile and adult life stages of the freshwater mussels *Utterbackia imbecillis* and *Pyganodon cataracta*, adults did not experience acclimation effects, but juvenile *P. cataracta* experienced normal acclimation of heart rate while juvenile *U. imbecillis* exhibited inverse acclimation (Polhill and Dimock 1996).

Seasonal acclimation effects, more appropriately termed acclimatization, have been shown to affect thermal tolerance of heart rate. With the pond snail *Lymnaea stagnalis* (Linnaeus, 1758), summer acclimated animals exhibit lower heart rate at warm

temperatures, and thus increased heat tolerance, when compared with winter acclimated animals (Harrison 1977a). Because seasonal acclimation can include other factors such as photoperiod and reproductive state, a follow-up study with the same species tested the effects of temperature alone. Similarly, thermal tolerance increased with acclimation temperature (Harrison 1977b). Because acclimation effects on heart rate can be so variable, generalizations regarding the factors controlling these responses should not be applied to all species (Harrison 1977b), in fact differences in evolutionary adaptation temperatures play a role in heart rate responses to changing temperatures among different species (Braby and Somero 2006).

Because of experimental design and overall study objectives, it was difficult to examine the effects of acclimation on heart rate in this study. For example, the experimental temperatures were different for each acclimation in order to assess thermal sensitivity. In addition, the acclimation period used in the study may have been too short (temperature adjustment of 2.5°C/d with 24 h at final temperature) to establish a true acclimation (a necessity of working with mussel early life stages), as short term laboratory acclimation is not always sufficient for overcoming an animal's previous acclimation (Ansell et al. 1980). A review of acute temperature studies with 50 aquatic species, including fish, mollusks, and crustaceans, found in most cases an acclimation period exceeding 96 h (de Vries et al. 2008), whereas other studies have used even longer periods of time from 14 to 34 days (Newell et al. 1971, Tomanek and Somero 1999, Carveth et al. 2006, Widmer et al. 2006).

Another common effect of temperature in poikilotherms is a plateau of heart rate after a peak rate (Harrison 1977a, Harrison 1977b, Lowe and Trueman 1972), such as in *L. subrostrata*, which demonstrated a high correlation between heart rate and temperature until a peak at 32°C followed by a plateau (Dietz and Tomkins 1980). In this study, it was observed that at 36°C, the threshold temperature for total mortality at the 27°C acclimation, heart beats were very hard to find and may have been too slow or too weak to count in some of the mussels that were still alive. This phenomenon may indicate, if freshwater mussels follow the commonly observed pattern, that the peak or plateau occurred somewhere before 36°C but that the 3°C intervals between assessment temperatures may have prevented this threshold from being obvious. Also, the plateau effect may be short-lived in juvenile mussels, with a small threshold between peak heart rate and sharp declines. For juvenile mussels, there is a small range of temperatures where mortality increases from almost zero to nearly 100% (Pandolfo 2008); a further analysis of heart rate at the temperatures in this range, broken down by at most 1°C increments might more clearly identify the point where heart rate slows or plateaus after the initial increase.

Bradycardia is a commonly observed phenomenon in mollusks, but decreasing heart rate at high temperatures may not be attributed to the direct effects of temperature alone. Bradycardia may be part of a more complicated behavioral response, typically involving valve closure which limits aerobic metabolism (Braby and Somero 2006). Adult *L. subrostrata* heart rate dropped to 4-8 bpm, and sometimes stopped completely,

when its valves were closed (Dietz and Tomkins 1980). During this study, sometimes heart beat would appear to stop in an unpredictable manner, and then start beating again in a very random fashion. In fact, observations made throughout the study indicate that heart rate can be very erratic in freshwater mussels.

Observations of the marine mollusk *Isognomon alatus* (Gmelin, 1791) indicated that occasionally the heart would stop beating for 2-3 hours at a time, and this behavior was not connected with environmental changes (Trueman and Lowe 1971). Heart rate fluctuations in bivalves clearly exist, and they are a highly individual and often an unattributable occurrence (Bakhmet and Khalaman 2006). Erratic heart rate and natural fluctuations can not be ignored when using heart rate as a measure of physiological condition (Bakhmet and Khalaman 2006). Erratic heart behavior can increase variability in data and even make some organisms unfit for experimentation (Harrison 1977a, Braby and Somero 2006).

Thermal stress has critical physiological implications for the well-being of an animal. While increased temperatures can increase productivity, there is a thermal limit above which the positive relationship between temperature and physiological function plateaus or becomes negative (Schulte 1975, Newell et al. 1977, Buxton et al. 1981). The additional energy cost associated with increased metabolism indicated by increased heart rate can translate into reductions in energy reserves allocated to maintenance, growth, and reproduction; this can lead to impaired fitness and consequences for population survival. However, it is not always clear what the end result of changing heart rate in response to

thermal stress will be. Some have found that in animals with a stable baseline heart rate and a traditional heat response, declining heart rate can be a temporary nonlethal response to temperature (Braby and Somero 2006), whereas others suggest that bradycardia as a result of thermal stress may indicate future mortality (Polhill and Dimock 1996).

This study has demonstrated that heart rate in juvenile freshwater mussels can be used as an indicator of thermal stress, but that some species (e.g. black sandshell and eastern creekshell) are more amenable to this purpose than others. Some common temperature responses were documented in this study, including a general increase in heart rate associated with increased experimental temperature. However, no acclimation effects were demonstrated, though this may have been due to a priori constraints associated with the experimental design and the duration of the acclimation period. Juvenile mussels demonstrated erratic heart beat at times, and additional studies are needed to assess results from direct visual observation of heart beat in juvenile freshwater mussels.

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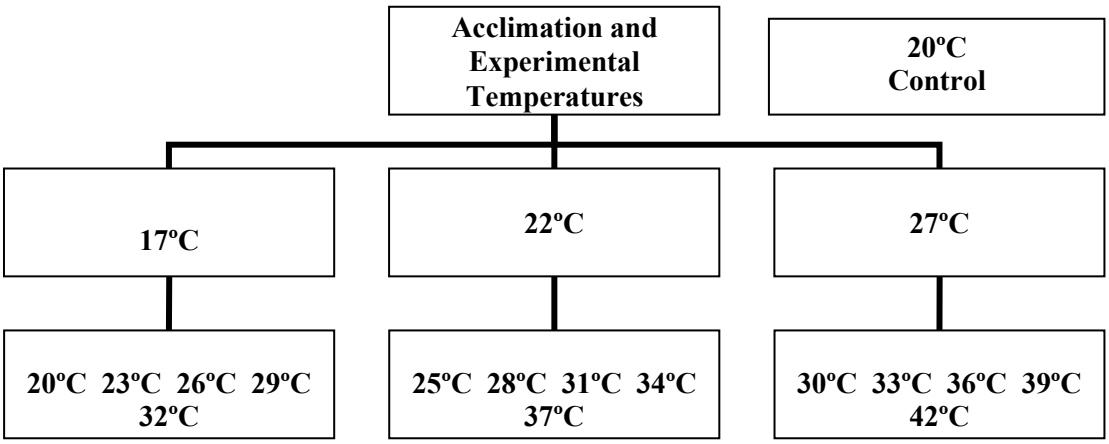

Figure 1. Experimental design showing acclimation and experimental temperature schemes for juvenile freshwater mussel tests.

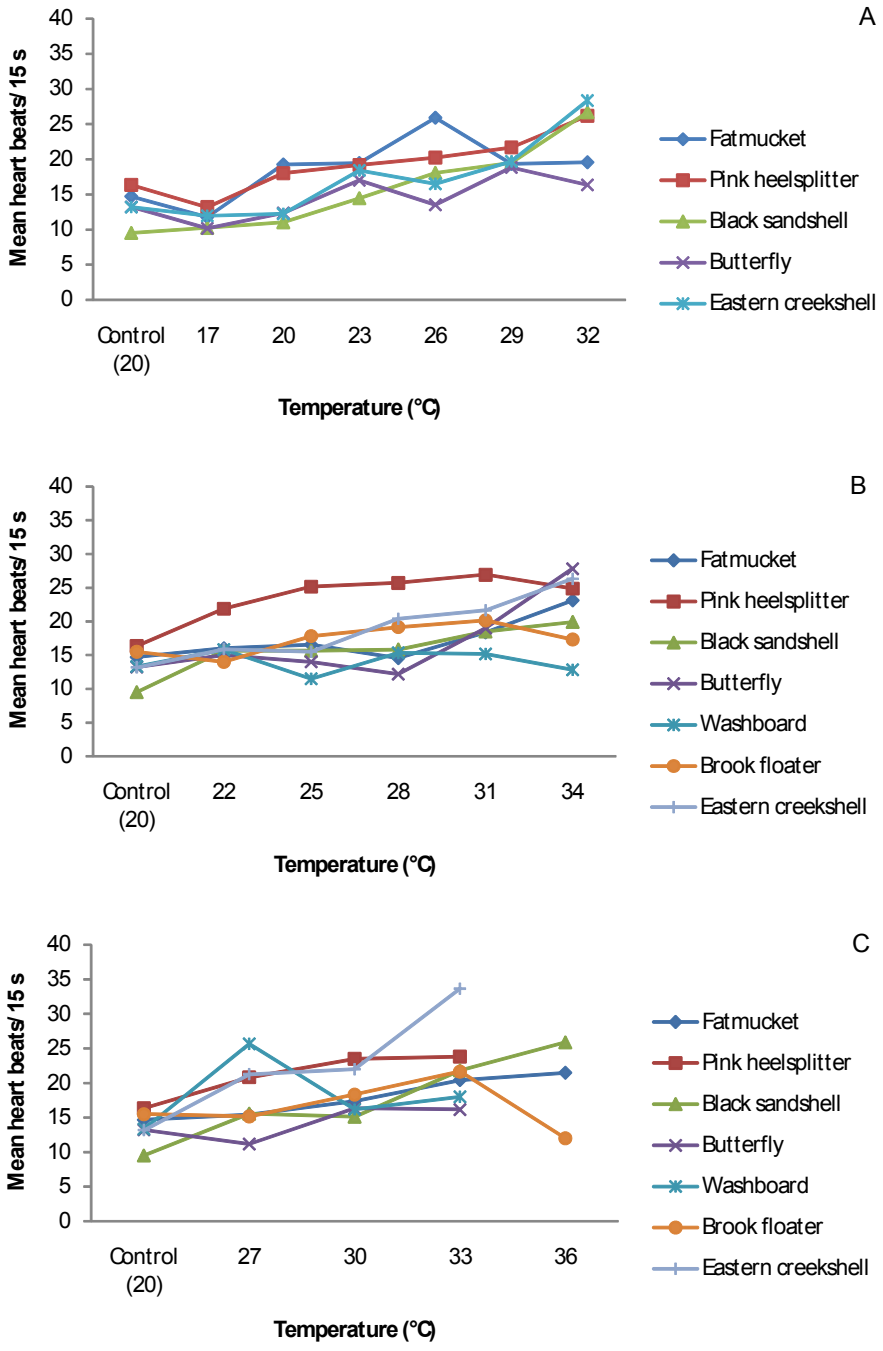

Figure 2. Heart rate trends for seven species of juvenile freshwater mussels with increasing experimental temperature at three acclimation temperatures; 17°C (A), 22°C (B), and 27°C (C).

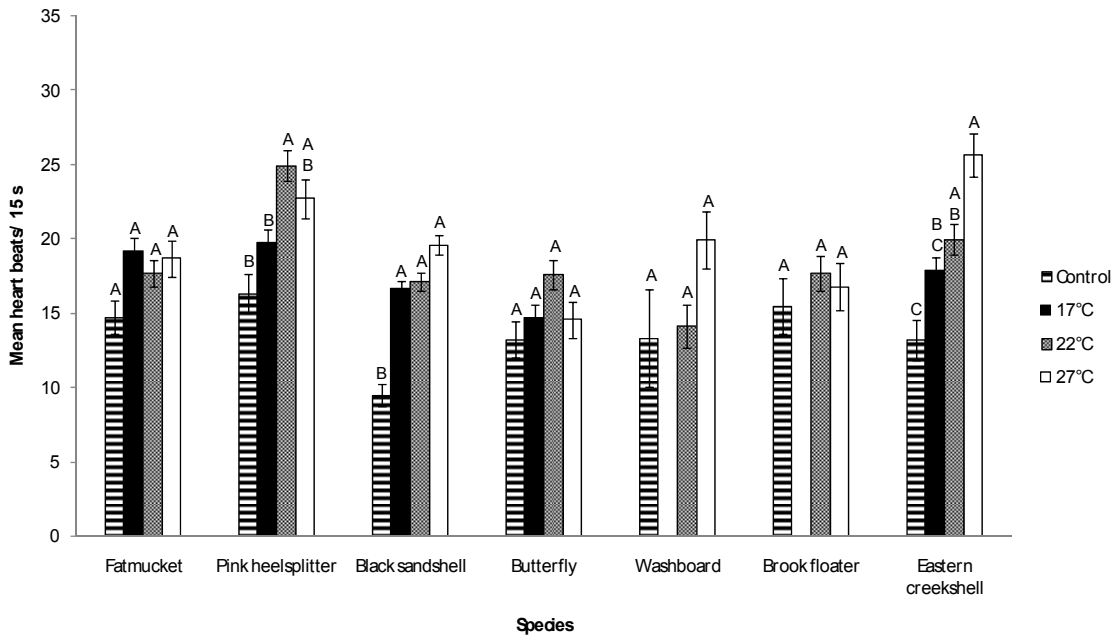

Figure 3. Mean heart rate in seven species of juvenile mussels in response to experimental temperatures at three different acclimation temperatures. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the $\alpha=0.01$ level. Letters refer to significance within a species not among species.

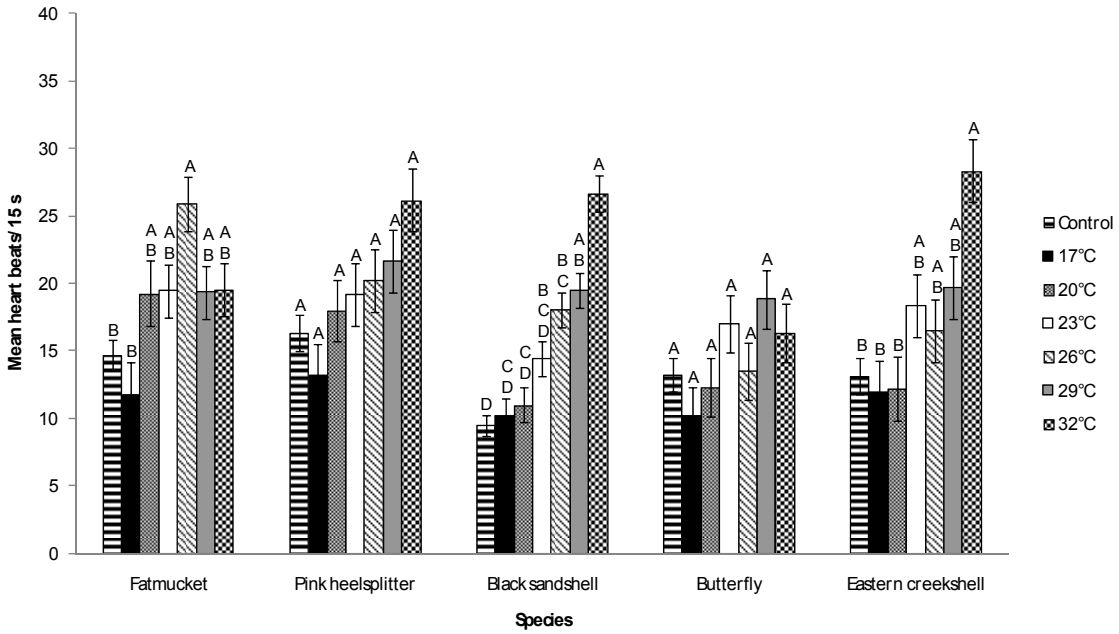

Figure 4. Heart rate in five species of juvenile mussels in response to increasing experimental temperature at the 17°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

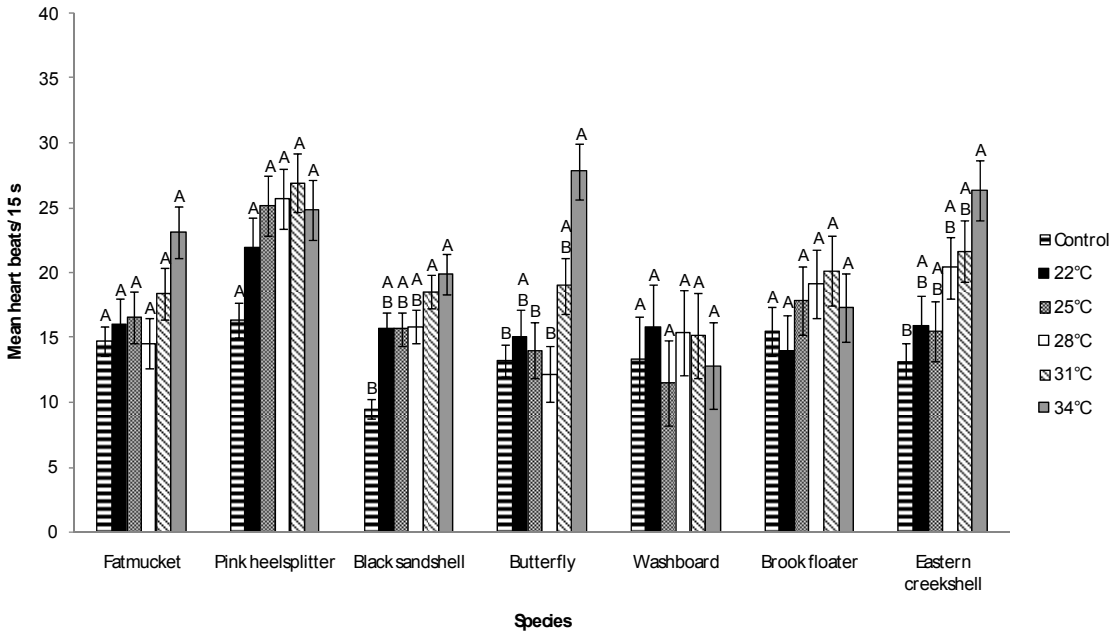

Figure 5. Heart rate in seven species of juvenile mussels in response to increasing experimental temperature at the 22°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

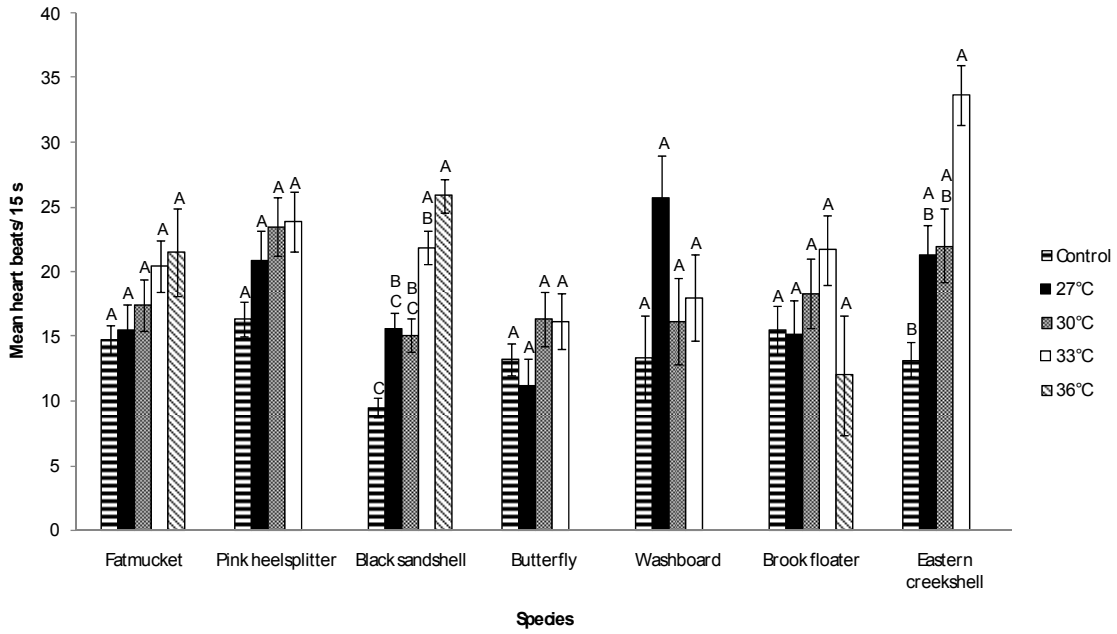

Figure 6. Heart rate in seven species of juvenile mussels in response to increasing experimental temperature at the 27°C acclimation temperature. Error bars represent standard error. Bars accompanied by the same letter were considered not significantly different at the α=0.01 level. Letters refer to significance within a species not among species.

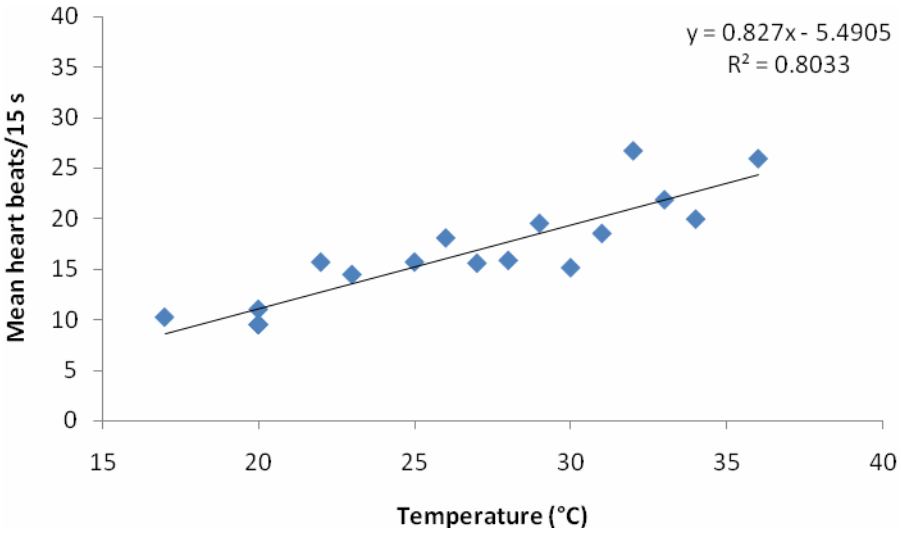

Figure 7. Mean heart rate of juvenile black sandshell mussels over all acclimation and experimental temperatures.

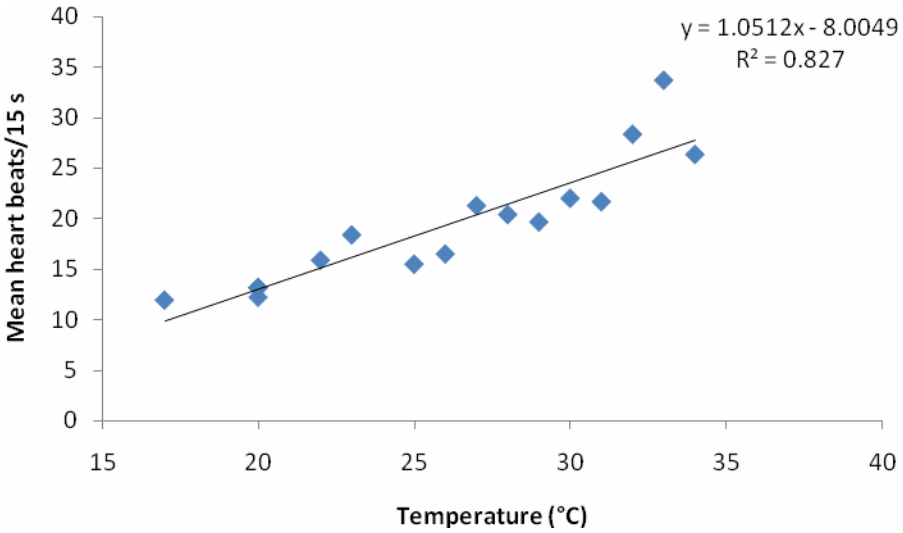

Figure 8. Mean heart rate of juvenile eastern creekshell mussels over all acclimation and experimental temperatures.

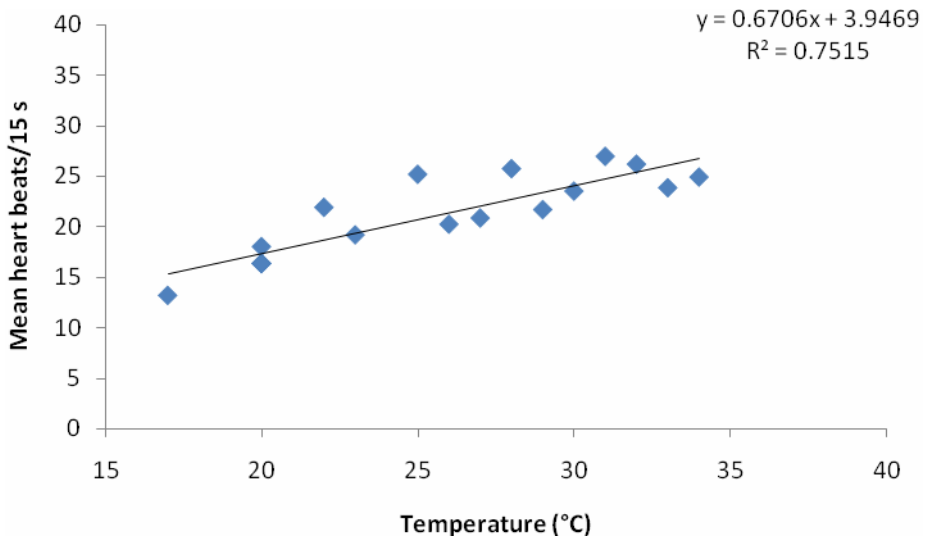

Figure 9. Mean heart rate of juvenile pink heelsplitter mussels over all acclimation and experimental temperatures.

Chapter 3. Thermal tolerance of juvenile freshwater mussels (Unionidae) under the additional stress of copper

(Formatted for Environmental Toxicology and Chemistry)

Abstract

Freshwater mussels fulfill an essential role in aquatic communities, but are also one of the most sensitive and rapidly declining faunal groups in North America. Rising water temperatures, caused by global climate change or industrial discharges, can further challenge impaired unionid communities, but thermal stress is almost certainly not the only stressor affecting freshwater mussels. Metals, such as copper, are a common source of toxicant exposure in aquatic environments. The toxic effects of copper on the early life stages of freshwater mussels have been well-studied, and freshwater mussels are more sensitive to copper than most aquatic organisms. The purpose of this study was to determine the effect of a sublethal copper concentration on the upper thermal tolerance of three species, *Lampsilis siliquoidea*, *Potamilus alatus*, and *Ligumia recta*, of juvenile freshwater mussels.. Thermal tolerance was determined over a range of experimental temperatures (20 - 42°C) at three acclimation temperatures (17°C, 22°C, and 27°C). Median effective temperatures (ET50s) were calculated in the absence and presence of copper, and they ranged from 32.9ºC to 36.7ºC with a mean of 34.8ºC. Based on 95% confidence interval overlap, there were no differences among ET50s caused by acclimation temperature, species, or presence of copper. However, survival trends

showed evidence of interactive effects between copper and temperature for all three species, suggesting this is an area that warrants further study.

Keywords: Unionidae, freshwater mussel, juvenile, early life stage, copper, thermal tolerance, multiple stressors, metal toxicity

Introduction

Freshwater mussels of the bivalve order Unionoida are long-lived, benthic aquatic organisms with considerable roles as nutrient processors and ecosystem engineers in the aquatic environment (Vaughn et al. 2004, Howard and Cuffey 2006, Vaughn et al. 2008), but unionids are also one of the most sensitive and rapidly declining faunal groups in North America. Nearly 70% of North America's 300 species are extinct or vulnerable to extinction (Graf and Cummings 2007, Bogan 1993, Williams et al. 1993). This decline has been attributed to several factors, including habitat degradation, water withdrawal for industry, urbanization, dam construction, impoundments, sedimentation, navigation, pollution, introduction of nonindigenous mollusks, overharvesting, and land use change (Bogan 1993, Williams et al. 1993, Strayer et al. 2004, Lydeard et al. 2004, Bogan 2008). Despite their vulnerable state, data gaps still exist regarding the effect of chemical and nonchemical stressors, such as temperature, on freshwater mussels. Increasing water temperatures due to heated effluents and global climate change may pose additional risks to threatened mussel species (Hastie et al. 2003).

Thermal increase is almost certainly not the only stressor affecting freshwater mussels in most situations, as it is probably more common for organisms in their natural environment to be impacted by multiple stressors than single stressors (Folt et al. 1999, Merovich and Petty 2007). In poikilotherms, because temperature controls physiological processes, any toxicant that acts on the same processes can be expected to have an interactive effect (Heugens et al. 2001). The effects of temperature in combination with toxicants and other stressors have been well-studied (see Cairns et al. 1975, Heugens et al. 2001 for reviews). A review of 151 combined stress experiments involving temperature revealed that 70% of studies found a positive relationship between temperature and toxicity, 10% found a negative relationship, and 20% found no correlation (Heugens et al. 2001). Thus, in general, it is expected that chemical toxicity increases with increasing temperature.

Metals are a common source of exposure in freshwater mussels, and they can alter growth, filtration efficiency, enzyme activity, and behavior (Naimo 1995). The toxicity of copper to freshwater mussels has been well established under a number of test conditions (Keller and Zam 1991, Huebner and Pynnonen 1992, Jacobson et al. 1993, Jacobson et al. 1997, Wang et al. 2007a, Wang et al. 2007b, Wang et al. 2007c, Gillis et al. 2008). Results of these studies indicate that the early life stages of freshwater mussel are more sensitive to copper than most other aquatic organisms (Huebner and Pynnonen 1992, Jacobson et al. 1997, March et al. 2007, Wang et al. 2007b). The toxicity of copper to freshwater mussels is a relevant concern because of its widespread presence in aquatic

systems. For instance, copper concentrations in 50% of samples from three North Carolina streams exceeded the ecological screening values for acute and chronic exposures (Ward et al. 2007).

Because copper has well-known toxic effects on freshwater mussels and is ubiquitous in the aquatic environment, it is reasonable to suspect that some populations of freshwater mussels will find themselves under the combined stress of both copper exposure and increased water temperature via heated effluent, climate change, or other factors. The purpose of this study was to test the upper thermal tolerances of three species of juvenile freshwater mussels under the influence of a sublethal concentration of copper. Mussels were exposed to a range of common and extreme water temperatures in the presence and absence of copper and their survival responses were compared among treatments.

Methods

Test organisms

Three species of freshwater mussels were exposed to a range of common and extreme water temperatures, with and without copper as a secondary stressor. Each test was conducted at three acclimation temperatures: 17ºC, 22ºC and 27ºC, and each acclimation temperature had five corresponding experimental temperatures in 3ºC increasing increments. There was also a 20ºC reference control temperature that was assessed alongside each test (Figure 1).

Three species representing the Lampsilini tribe from the Ambleminae subfamily of Unionidae were used in this study: fatmucket (*Lampsilis siliquoidea*, Barnes, 1823), pink heelsplitter (*Potamilus alatus,* Say, 1817), and black sandshell (*Ligumia recta,* Lamarck, 1819) (Graf and Cummings 2007). These species were chosen because they encompass a variety of life history strategies and habitats, and because of their wide geographic distribution, particularly in the central United States. The species represent two subregions of the Nearctic region (Graf and Cummings 2007): Interior Basin (fatmucket, black sandshell, pink heelsplitter) and Gulf Coastal (black sandshell, pink heelsplitter). All test organisms came from propagation facilities at Missouri State University. Test organisms were newly transformed juveniles and ranged in age from 3 to 8 weeks. The average size for fatmucket was 1,386 µm, pink heelsplitter was 1,377 µm, and black sandshell was 947 µm.

Viability assessment

Upon arrival at the laboratory, viability of juveniles was determined to be 90% or greater in order to initiate testing; mussels were then acclimated to the test acclimation temperature by adjusting their temperature by no more than 2.5ºC per day, with at least a 24 hour acclimation period once the target temperature was attained. After this period, viability was assessed a second time and organisms were distributed to test chambers. These were 96 hour non-aerated static renewal tests, with 100% water renewal at 48 hours; tests were conducted according to ASTM guidelines for juvenile mussel toxicity testing (ASTM 2006). Juvenile mussel viability (survival) was assessed visually using an

Olympus SZ61 microscope to detect foot movement outside of the shell, foot movement within the shell, or the presence of a heart beat. The target copper concentration was 10 µg/L (Copper (II) sulfate pentahydrate, >98%, ACS grade, Acros Organics/Fisher Scientific, Pittsburgh, PA, USA) for these tests and was chosen to be less than published ET50s for early life stages of freshwater mussels, as we were targeting sublethal effects (Keller and Zam 1991, Huebner and Pynnonen 1992, Jacobson et al. 1993, Jacobson et al. 1997, Wang et al. 2007a, Wang et al. 2007b, Wang et al. 2007c, Gillis et al. 2008). Copper solutions were renewed at 48 h, and water samples were taken at the start of each test and at 48 h to determine actual exposure concentrations.

Thermal tolerance

Survival data were used to generate median effective temperatures (ET50s) with the trimmed Spearman-Karber method using ToxCalc v.5.0.26 toxicity data analysis software (Tidepool Scientfic Software, McKinleyville, CA, USA). An ET50 is the temperature at which 50% of the exposed population exhibits some predefined effect; for this experiment, this effect was loss of viability determined by lack of foot movement within or outside of the shell, and/or lack of a heart beat. Comparisons of ET50s were made using 95% confidence interval overlap. Intervals that did not overlap were considered to be significantly different.

Quality assurance

Quality assurance and control were maintained by conducting all tests according to the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater

Mussels (ASTM 2006). All tests were conducted in light and temperature controlled environmental chambers (Precision Model 818, Thermo Electron Corp., Marietta, OH, USA, and Isotemp Model 146E, Fisher Scientific, Dubuque, Iowa, USA), and National Institute of Standards and Technology (NIST) certified thermometers were used for daily temperature monitoring.

Water quality conditions, including alkalinity, hardness, conductivity, pH, temperature, and dissolved oxygen, were monitored at the start of each test and again at the 48 hour time point. Alkalinity and hardness were measured by titrametric procedures with standard methods (APHA 1995). Conductivity, temperature, pH, and dissolved oxygen were measured with a calibrated meter (YSI model 556 MPS multi-probe, Yellow Springs Instrument Co., Yellow Springs, OH, USA). For all tests, alkalinity ranged from 92 to 110 mg CaCO3/L with a mean of 101.7 mg CaCO3/L, hardness ranged from 146 to 162 mg CaCO3/L with a mean of 153.7 mg CaCO3/L, conductivity ranged from 526 to 684 µs/cm with a mean of 582.4 µs/cm, pH ranged from 7.08 to 8.76 with a mean of 8.31, and dissolved oxygen ranged from 4.50 to 9.73 mg/L with a mean of 6.61 mg/L (n=6 for alkalinity and hardness, n=90 for all other parameters). Test temperatures had a maximum of 1.5ºC departure from the target temperature, with only 1.3% of samples exceeding a 1ºC departure (n=231).

Copper concentrations were verified with Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) in the Department of Soil Science at North Carolina State University using standard methods and approved quality assurance protocols.

Copper exposure concentrations averaged 108% of the target concentration of 10 µg/L, with a mean concentration of 10.8 µg/L (n= 57; range= 7.4 to 13.7 µg/L).

Statistical analysis

Results were analyzed with SAS® Proc Mixed (SAS Institute Inc, 2006). Viability data were arcsin transformed. Water, acclimation temperature and experimental temperature were considered fixed effects, while repetitions within each water and acclimation temperature were considered as random effects. Water and acclimation temperatures were considered crossed effects, and experimental temperature was taken as a nested effect within each combination of water and acclimation temperature. Significance level was established at 0.01 for model effects, while means differences were analyzed by a t-test for pairwise least square mean differences with Tukey's adjusted p-value and 0.01 significance level to control type I error. Significance level was established at $p \leq 0.01$ in order to ensure significance was assigned only to responses beyond those associated with variation in natural mussel populations.

Interaction effects of water and acclimation were analyzed through the testing of simple effects for acclimation temperature within each type of water, and comparing both types of water within each acclimation temperature. When adequate, pairwise mean comparison was used to analyze mean differences between acclimation temperatures. Significance of experimental temperatures was studied through the analysis of simple effects for experimental temperatures within each water and acclimation temperature and when necessary, the effect differences of experimental temperatures was analyzed within

each acclimation temperature and water type with a t-test for pairwise least square mean differences with Tukey's adjusted p-value and 0.01 significance level. Significance level for each pairwise mean comparison was calculated using the distribution of the studentized range (Steel et al. 1997).

Results

For fatmucket, pink heelsplitter, and black sandshell at 96 h, water type alone (i.e. no copper vs. copper) did not significantly affect viability (p=0.1147, 0.0927, and 0.6148 respectively). The interaction between acclimation temperature and water type was also not significant for fatmucket (p=0.7351), pink heelsplitter (p=0.1722), or black sandshell (p=0.0663). However, while the [water x experimental temperature (acclimation temperature)] interaction was not significant for fatmucket (p=0.4398) or black sandshell (p=0.6399), the interaction was significant for pink heelsplitter (p=0.0076). In this study, the only significant difference at 96 h caused by the addition of copper was for pink heelsplitter at the 34ºC experimental temperature within the 22ºC acclimation test. At this temperature, viability was significantly reduced with the addition of copper (p=0.0004).

Mean viability over all experimental temperatures was compared at three acclimation temperatures with and without treatment of 10 µg/L of copper for all species with no significant differences at 96 h caused by the addition of copper (Figure 2). Differences in viability observed among acclimation temperatures were not related to

acclimation but rather to increasing experimental temperatures associated with the different acclimation temperature schemes. For example, because the two highest test temperatures at the 27ºC acclimation, 39ºC and 42ºC, caused total mortality in all cases, they directed the overall viability at that acclimation lower than the control or 17ºC acclimation where no mortality was caused by temperature. Therefore, these results are useful for comparing viability trends broadly among species and between temperature and copper treatments, but not for assessing acclimation effects.

At the 17ºC acclimation temperature, there were no significant reductions in viability for fatmucket, pink heelsplitter, or black sandshell at any temperature in the presence or absence of copper (all $p>0.01$). All three species maintained control viability levels at temperatures up to 32ºC, with and without copper. At the 22ºC acclimation temperature, fatmucket viability patterns remained identical with and without the addition of copper (Figures 3 and 4). For black sandshell, viability patterns shifted slightly at 34ºC, however this difference was not significant ($p=0.9999$). In contrast, viability of pink heelsplitter at 34ºC decreased significantly with the addition of copper ($p=0.0004$). These results indicate that the addition of 10 µg/L of copper decreased the threshold temperature of effect for pink heelsplitter. At the 27ºC acclimation temperature, fatmucket and pink heelsplitter did not exhibit any changes in viability associated with the addition of copper (Figures 5 and 6). Once again, black sandshell viability trends changed slightly with the addition of copper, but the difference, at 36ºC, was not significant ($p=1.0000$).

At the 48 h (non-ASTM) time point, effects of copper exposure on viability were seen for all three species. The [water x experimental temperature (acclimation temperature)] interaction was significant for fatmucket, pink heelsplitter, and black sandshell ($p=0.0007$, 0.0047, <0.0001 respectively). Water type alone was also significant for pink heelsplitter ($p=0.0045$), and black sandshell also exhibited a significant interaction between acclimation temperature and water ($p=0.0092$). Mean viability over all experimental temperatures at the 22ºC acclimation for pink heelsplitter was also significantly reduced with the addition of copper ($p=0.0048$) (Figure 7).

In the 22ºC acclimation test at 48 h, the copper effect for pink heelsplitter was similar to 96 h, with viability significantly reduced by copper at 34ºC ($p<0.0001$) (Figure 8). In addition, both fatmucket and black sandshell experienced a significant reduction in viability caused by copper at 37ºC ($p<0.0001$ for both). An apparent increase in black sandshell viability at 39ºC in the 27ºC acclimation test was not significant ($p=0.0387$) (Figure 9).

ET50s (96 h) for all species with and without copper at the 22ºC and 27ºC acclimation temperatures ranged from 32.90ºC to 36.74ºC with a mean of 34.75ºC (Table 1). No ET50s were calculated for the 17ºC acclimation because there were no significant mortalities at those temperatures (Appendix Table 8). Based on 95% confidence interval overlap, there were no differences among ET50s caused by acclimation temperature, species, or presence of copper.

Discussion

The results of this study show that copper has an interactive effect with elevated temperature for some freshwater mussel species, including fatmucket, pink heelsplitter, and black sandshell. At the 96 h time point, thermal tolerance, as measured by ET50s, did not change for any species with the addition of 10 µg Cu/L; however, the [water x experimental temperature (acclimation temperature)] interaction was significant for pink heelsplitter.

The 17°C acclimation revealed no differences in viability trends for any species caused by the addition of copper. This suggests that if copper has an effect, it does not act acutely at temperatures up to 32°C at 96 h. At the 22°C acclimation, only pink heelsplitter showed a significant decrease in viability associated with copper. At the 27°C acclimation temperature, there were no significant decreases in viability with the addition of copper for any species, though all the test temperatures at this acclimation elicited an "all or nothing" viability response, masking any threshold effects. Results at 96 h indicate that mortality was caused by high temperatures before any copper interaction effects could be observed.

At 48 h, these threshold effects were more apparent, as the [water x experimental temperature (acclimation temperature)] interaction was significant for all three species. Pink heelsplitter experienced a significant decrease in viability attributed to copper at 34°C in the 22°C acclimation test, as it did at 96 h; fatmucket and black sandshell also exhibited reduced viability caused by copper at 37°C in the same test. The effects of

copper were not evident for fatmucket and black sandshell at 96 h because by that time, thermal stress caused total mortality at the highest temperatures (37-42°C). However, at 48 h, the interaction between copper and thermal stress became clear for these species as interaction effects (at 37°C) were not masked by total, or near total, mortality.

This is not the first study to assess the combined effects of temperature and copper on freshwater mussels. A study involving *Actinonaias pectorosa* (Conrad, 1834) glochidia found a positive linear relationship between temperature and copper toxicity with an LC50 of 132 µg Cu/L at 10°C and an LC50 of 42 µg Cu/L at 25°C (Jacobson et al. 1997).

The mussels used in my study ranged in age from 3 to 8 weeks old. Because these were not newly transformed juveniles, they may have been more tolerant of copper exposure. Copper EC50s for 2 month old juveniles (average of 37 µg/L) were shown to be higher than for newly transformed juveniles (average of 22 µg/L) (Wang et al. 2007b). Jacobson et al. (1993) found that juvenile mussels appeared to avoid copper at low concentrations (24-30 µg/L) by closing their valves, though they also pointed out that this response can be only temporary because valve closure also inhibits feeding. A longer test duration in my study may have overcome the valve closure response (e.g., Cope et al. 2008), if it occurred.

My study also used reconstituted ASTM hard water (average hardness 153.7 mg $CaCO_3$/L) for all treatments, this water was chosen based on conditions used during mussel propagation. Metals become less toxic in hard water because they complex with

carbonates and become less soluble; in addition, calcium and magnesium ions may decrease membrane permeability (Keller and Zam 1991). Calcium, magnesium, and sodium may also reduce metal toxicity in aquatic organisms by competing for binding sites (Gillis et al. 2008). The toxicity of copper to freshwater mussels has indeed been shown to decrease with increasing water hardness (Keller and Zam 1991, Jacobson et al. 1993, Jacobson et al. 1997, Gillis et al. 2008), thus copper effects may have been more evident if the tests were conducted in soft water.

The copper concentration used in this study was chosen to be non-acutely toxic because pilot experiments with glochidia using a higher copper concentration caused mortality that masked any thermal effects (Pandolfo, unpublished data). A review of copper EC50s (or LC50s) for the early life stages of freshwater mussels revealed variation among species, though it was largely noted that glochidia and juvenile sensitivities were similar (Jacobson et al. 1997, Wang et al. 2007a). Acute copper toxicity data were available for juvenile fatmucket and black sandshell glochidia, but not for pink heelsplitter. Copper EC50s for 2 month old fatmucket juveniles were between 32-60 µg/L and 18-25 µg/L for newly transformed juveniles at 96 h in hard water (Wang et al. 2007a, Wang et al. 2007b). An EC50 of 34.8 µg/L was determined for black sandshell glochidia at 24 h in soft water (Gillis et al. 2008). Chronic toxicity tests with 2 month old juvenile *Lampsilis siliquoidea*, *Villosa iris* (I. Lea, 1829), and *Epioblasma capsaeformis* (I. Lea, 1834) found chronic values in hard water ranging from 8.5 to 9.8

µg Cu/L for survival and 4.6 to 8.5 µg Cu/L for growth (Wang et al. 2007c). The 10 µg/L copper concentration used in this study compares well with these values.

Because temperature regulates physiology in poikilotherms, increased temperatures can change the toxicokinetics of toxicants through increased metabolism, activity, or feeding (Cairns et al. 1975). These changes can cause increased uptake or detoxification of toxicants, and as a result, an organism's thermal tolerance can be altered by the toxicant, or increased temperature could affect an organism's tolerance to the toxicant (Heugens et al. 2001). A study with the marine mussel *Mytilus edulis* (Linnaeus, 1758) found that while accumulation of nonessential metals showed a positive relationship with temperature, copper accumulation was inversely related to temperature (Mubiana and Blust 2007). Also, while temperature had little or no influence on the elimination of most metals, copper elimination was not independent of temperature. From these results, Mubiana and Blust (2007) suggested that while higher intake with increased temperatures is expected, at the whole-organism level physiological processes are complicated and unpredictable.

Interaction between two stressors is expected when they act on the same physiological processes (Heugens et al. 2001), therefore any toxicant that interferes with metabolism or respiration may interact with thermal stress. However, interactive effects among multiple stressors do not always have an obvious and/or positive relationship. A study assessing combined impacts of acid mine drainage and thermal effluents on benthic macroinvertebrate communities found that the effects varied with season and that

interaction was most evident in the failure for communities to recover from the combined stress, whereas recovery occurred rapidly downstream of single stressors (Merovich and Petty 2007). Also, a study involving copper, temperature, and pathogen exposure as stressors to *Mytilus edulis* found that the interactions were complex and results from single stressor experiments do not necessarily translate to natural situations (Parry and Pipe 2004). A study of the interactions among thermal stress, food availability, and surfactant exposure in *Daphnia pulex* (Leydig, 1860) and *D. pulicaria* (Forbes, 1893) found that while combined stresses were usually worse than the individual stressors, some scenarios existed where antagonism between stressors occurred and interaction effects were less stressful than the individual stresses (Folt et al. 1999).

Additional studies have demonstrated a lack of interactive effects among multiple stressors. In a study involving four species of freshwater fish, while endosulfan and chlorpyrifos reduced the critical maximum temperature for all species, exposure to phenol did not (Patra et al. 2007). A study involving the freshwater fish *Lepomis cyanellus* (Rafinesque, 1819) found that exposure to cadmium did not reduce the critical thermal maximum of the fish; the authors suggested the lack of interaction may be attributed to low cadmium uptake or to detoxification processes (Carrier and Beitinger 1988).

Other studies have demonstrated that the combined effects of increased temperature and cadmium exposure resulted in synergistic effects on mitochondrial respiration, aerobic energy production, and survival in the marine oyster *Crassostrea*

virginica (Gmelin, 1791) (Sokolova 2004, Lannig et al. 2006a, Lannig et al. 2006b), while the isopod *Porcellio scaber* (Latreille, 1804) showed significant interactive effects of temperature and zinc on growth (Donker et al. 1998). In a study of the early development of *Mytilus trossulus* (Gould, 1850), toxicity of copper was greater at higher temperatures, with only 5% of larvae reaching the next stage of development at 20°C, compared with 69% at 7°C and 84% at 15°C at the same copper concentration (Yaroslavtseva and Sergeeva 2007).

The multiple stress scenario presented here for freshwater mussels is highly relevant as multiple stressors are probably more common than single stressors (Folt et al. 1999, Merovich and Petty 2007). Not only is copper present in surface waters (Ward et al. 2007), but juvenile release from host fish occurs largely in mid to late summer when water temperatures are elevated (Jacobson et al. 1993). If newly transformed juveniles are released as water temperatures are at, or approaching, their highest, toxicants in the water column may be more likely to have an adverse effect (Jacobson et al. 1993). Also, because juvenile mussels spend the first 2 to 4 years of their lives burrowed in sediment, they may also be exposed to toxicants associated with the sediments (Jacobson et al. 1997, Cope et al. 2008). A companion study of the thermal tolerances of the early life stages of eight species of freshwater mussels (Pandolfo 2008) found that mussels may already be living close to their thermal limits, and in general, organisms living closest to their thermal limits experience the greatest effects from toxicants (Heugens et al. 2001).

The results that were observed in this study indicate that freshwater mussel species may differ in their responses to combined stressors. All three species showed a significant interaction between elevated temperatures and sublethal copper exposure at 48 h, whereas only pink heelsplitter showed evidence of this interaction at 96 h. The results presented here are intriguing enough to warrant further study. Freshwater mussel populations are deteriorating rapidly, and because they are routinely exposed to multiple stressors it is crucial to identify interactions that may be contributing significantly to their decline, especially in the context of increasing temperatures and toxicant exposure.

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Table 1. ET50s with 95% confidence intervals (in parentheses) in 96 h juvenile mussel tests in the presence and absence of 10 µg/L of copper. *ET50 determined graphically, no confidence interval provided.

	22ºC Acclimation		27ºC Acclimation	
Species	No copper	Copper	No copper	Copper
Fatmucket	35.54 (35.14- 35.96)	35.47*	34.31 (33.50- 35.14)	34.47*
Pink heelsplitter	34.79 (33.12- 36.54)	32.99 (30.67- 35.47)	34.60 (33.36- 35.90)	34.45 (34.25- 34.65)
Black sandshell	32.90 (29.58- 36.59)	34.73 (32.91- 36.66)	36.74 (34.37- 39.27)	36.03 (34.01- 38.17)

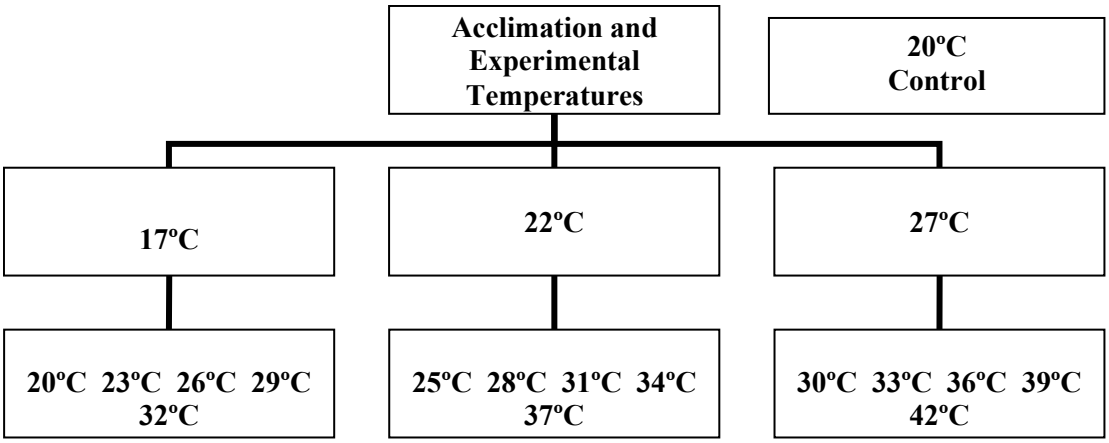

Figure 1. Experimental design showing acclimation and experimental temperature schemes for juvenile tests with freshwater mussels.

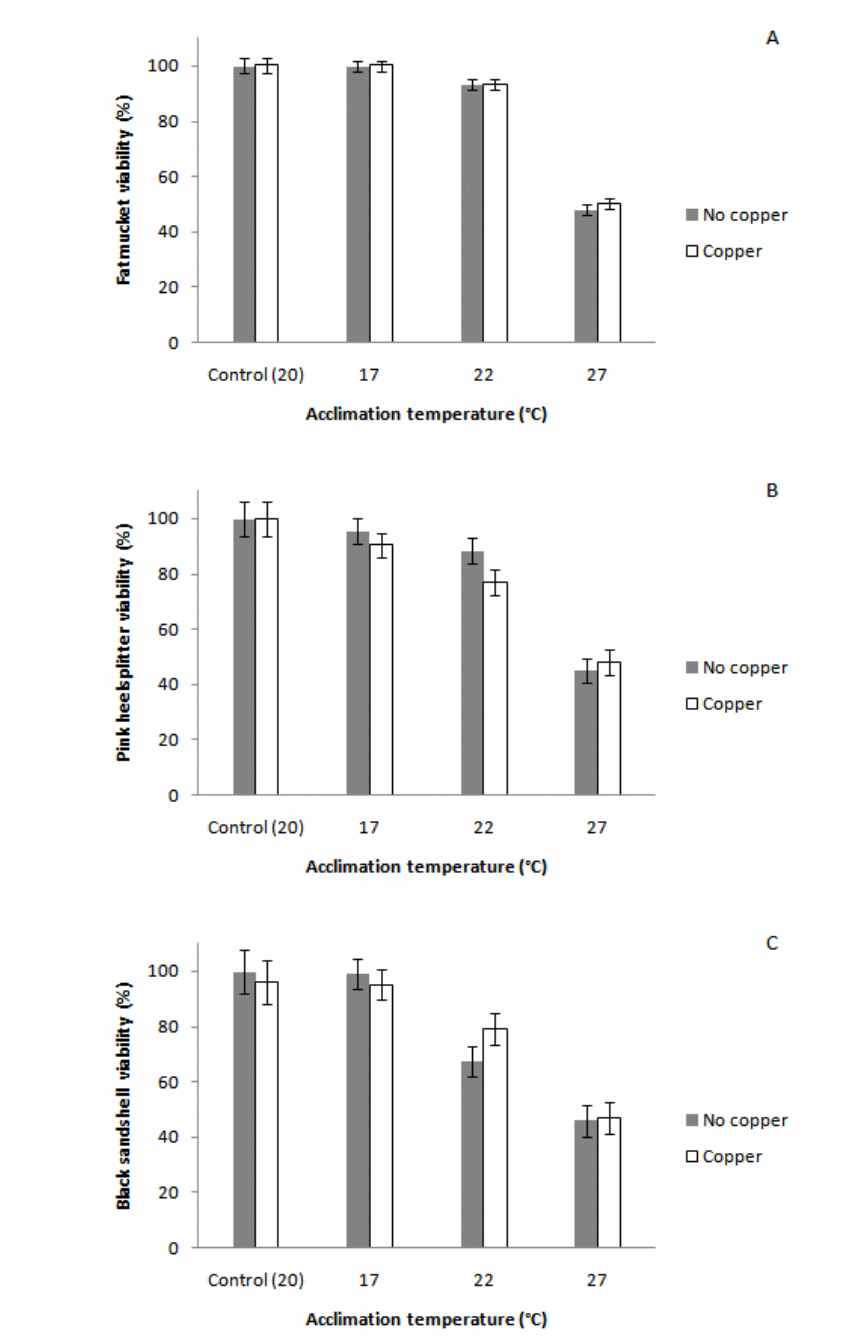

Figure 2. Mean viability of three species of juvenile freshwater mussels, fatmucket (A), pink heelsplitter (B), and black sandshell (C), at a range of experimental temperatures at three acclimation temperatures in the absence and presence of 10 µg/L of copper at 96 h. Error bars represent standard error. There were no significant differences at the α=0.01 level in overall experimental temperatures at any acclimation temperature caused by the addition of copper.

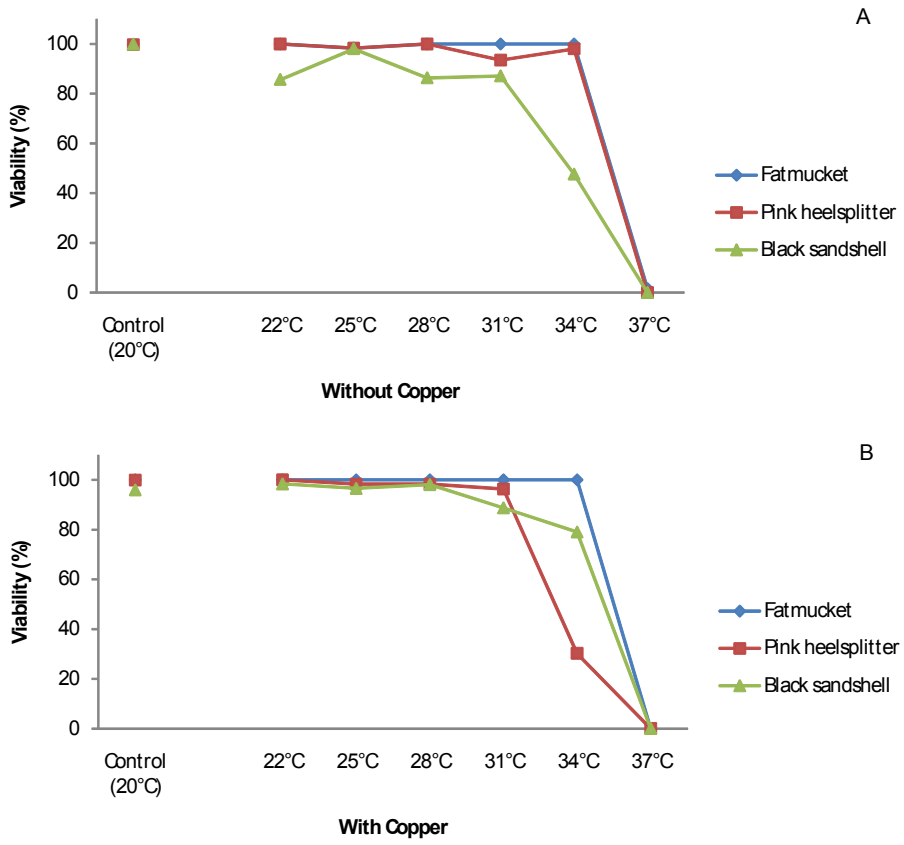

Figure 3. Viability trends over increasing experimental temperatures for three species of juvenile freshwater mussels at the 22ºC acclimation temperature in the absence (A) and presence (B) of 10 µg/L of copper at 96 h. Fatmucket follows pink heelsplitter trends when not visible.

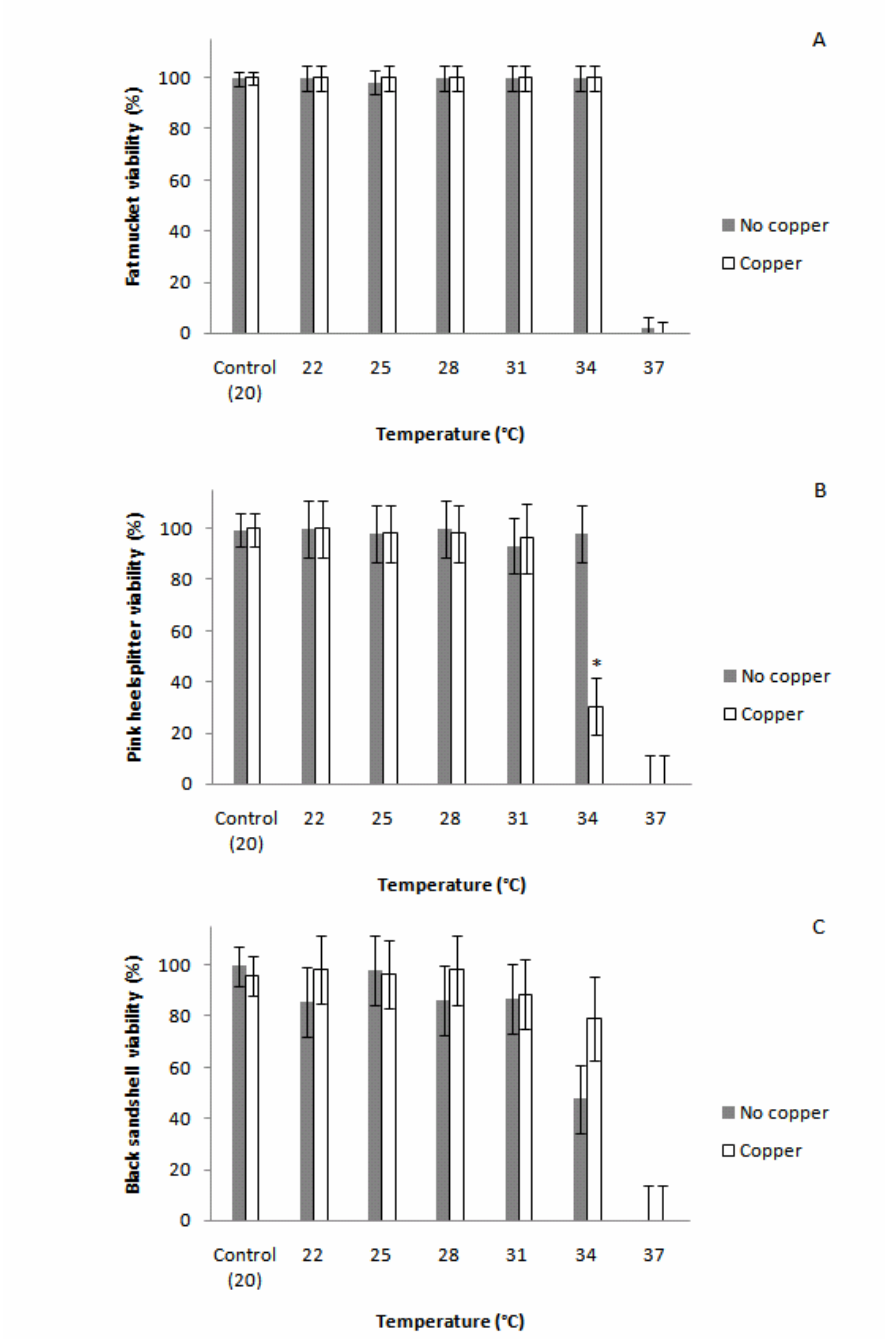

Figure 4. Viability of three species of juvenile freshwater mussels, fatmucket (A), pink heelsplitter (B), black sandshell (C), at the 22ºC acclimation temperature in the absence and presence of 10 µg/L of copper at 96 h. Error bars represent standard error.
* indicates significant difference at the α=0.01 level in a temperature treatment caused by the addition of copper.

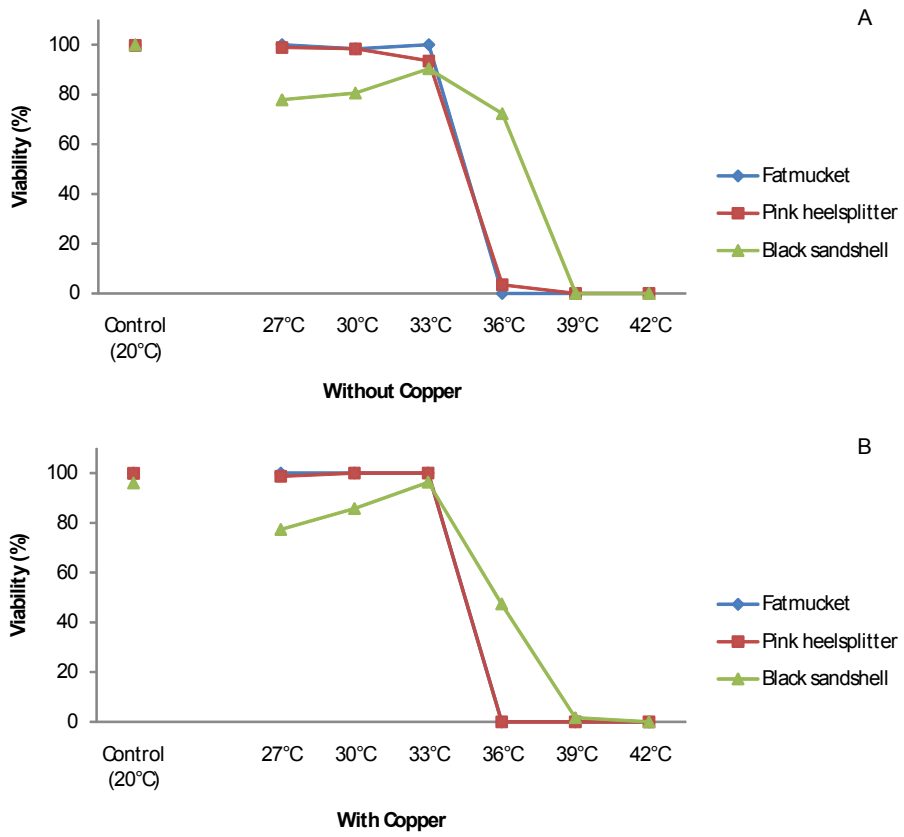

Figure 5. Viability trends over increasing experimental temperatures for three species of juvenile freshwater mussels at the 27ºC acclimation temperature in the absence (A) and presence (B) of 10 µg/L of copper at 96 h. Fatmucket follows pink heelsplitter trends when not visible.

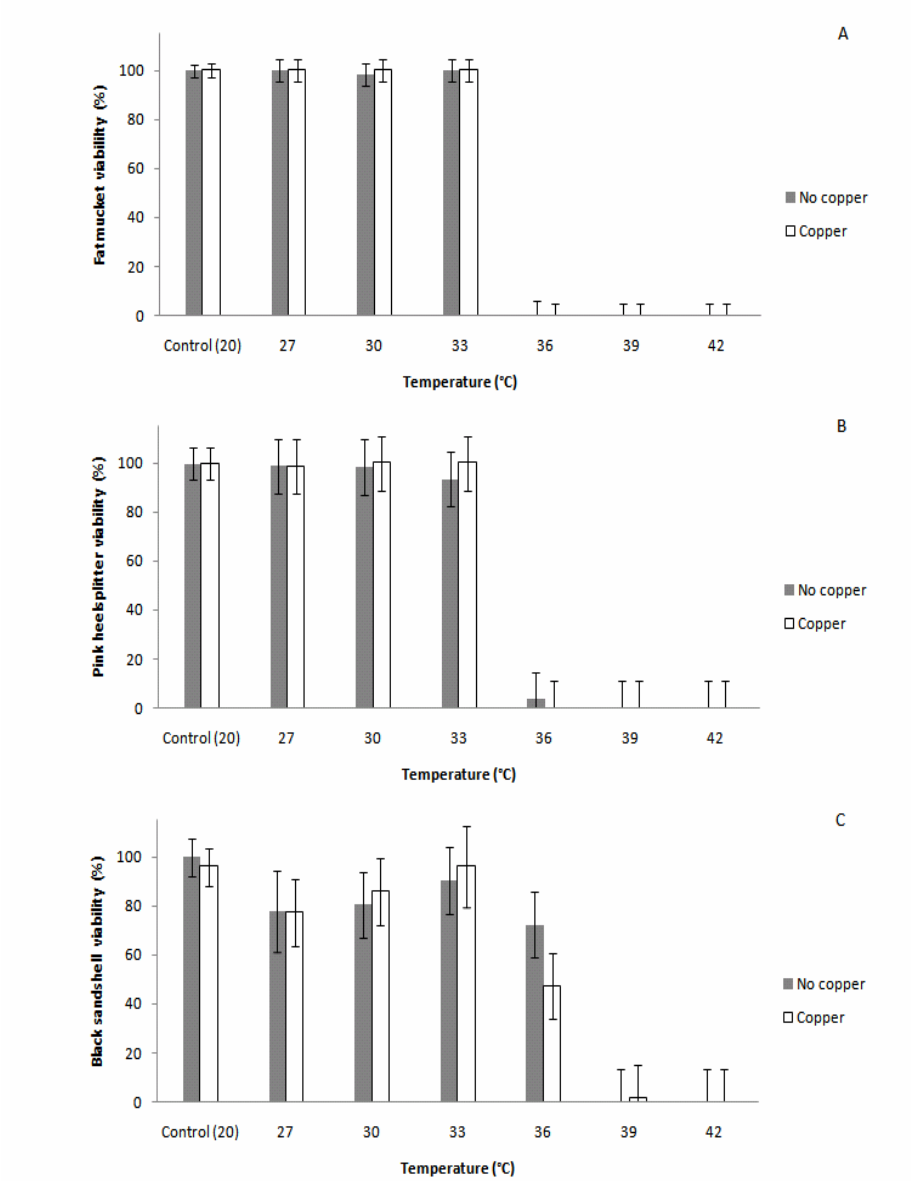

Figure 6. Viability of three species of juvenile freshwater mussels, fatmucket (A), pink heelsplitter (B), black sandshell (C), at the 27ºC acclimation temperature in the absence and presence of 10 µg/L of copper at 96 h. Error bars represent standard error. There were no significant differences at the α=0.01 level in any temperature treatment caused by the addition of copper.

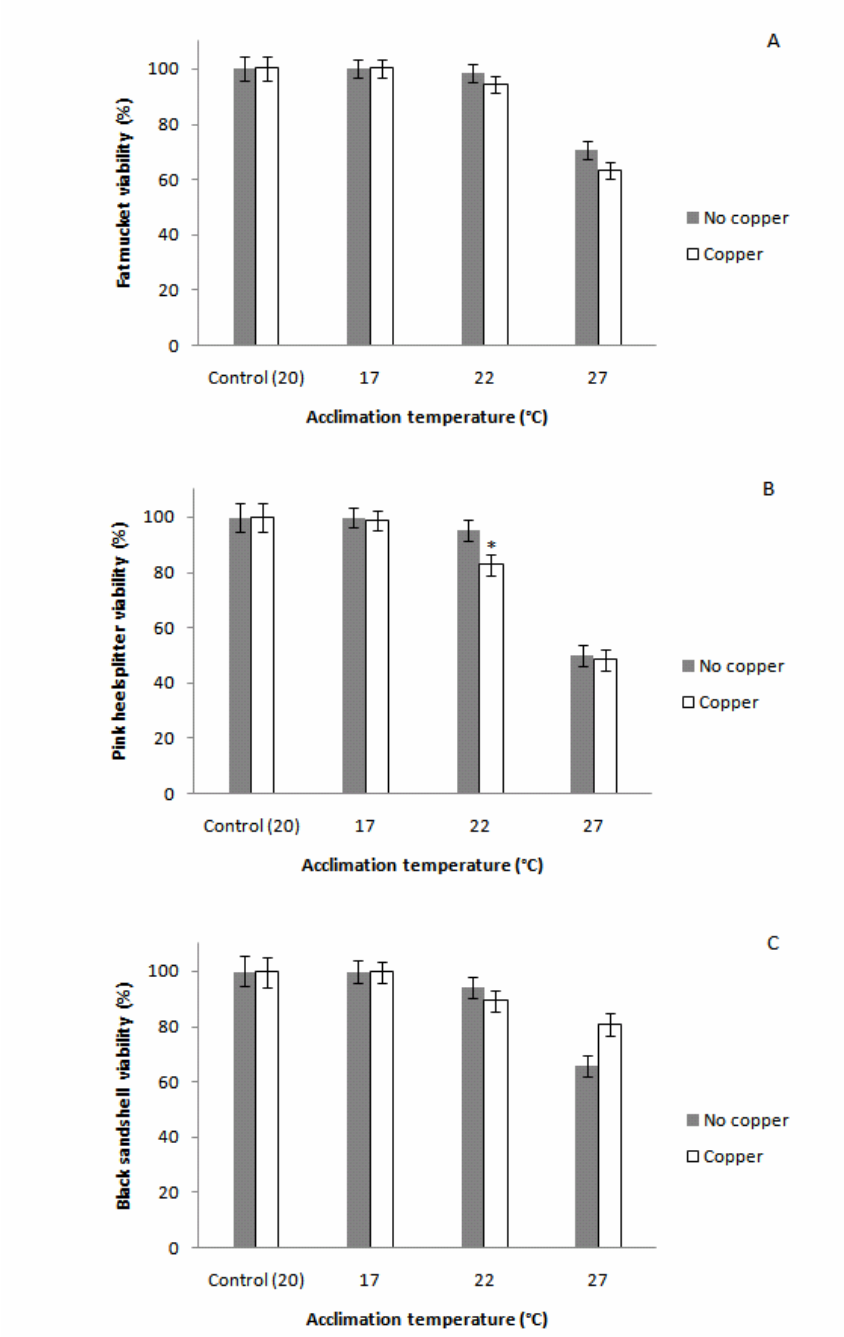

Figure 7. Mean viability of three species of juvenile freshwater mussels, fatmucket (A), pink heelsplitter (B), and black sandshell (C), at a range of experimental temperatures at three acclimation temperatures in the presence and absence of 10 µg/L copper at 48 h. Error bars represent standard error. * indicates significant difference at the α=0.01 level in overall experimental temperatures at an acclimation temperature caused by the addition of copper.

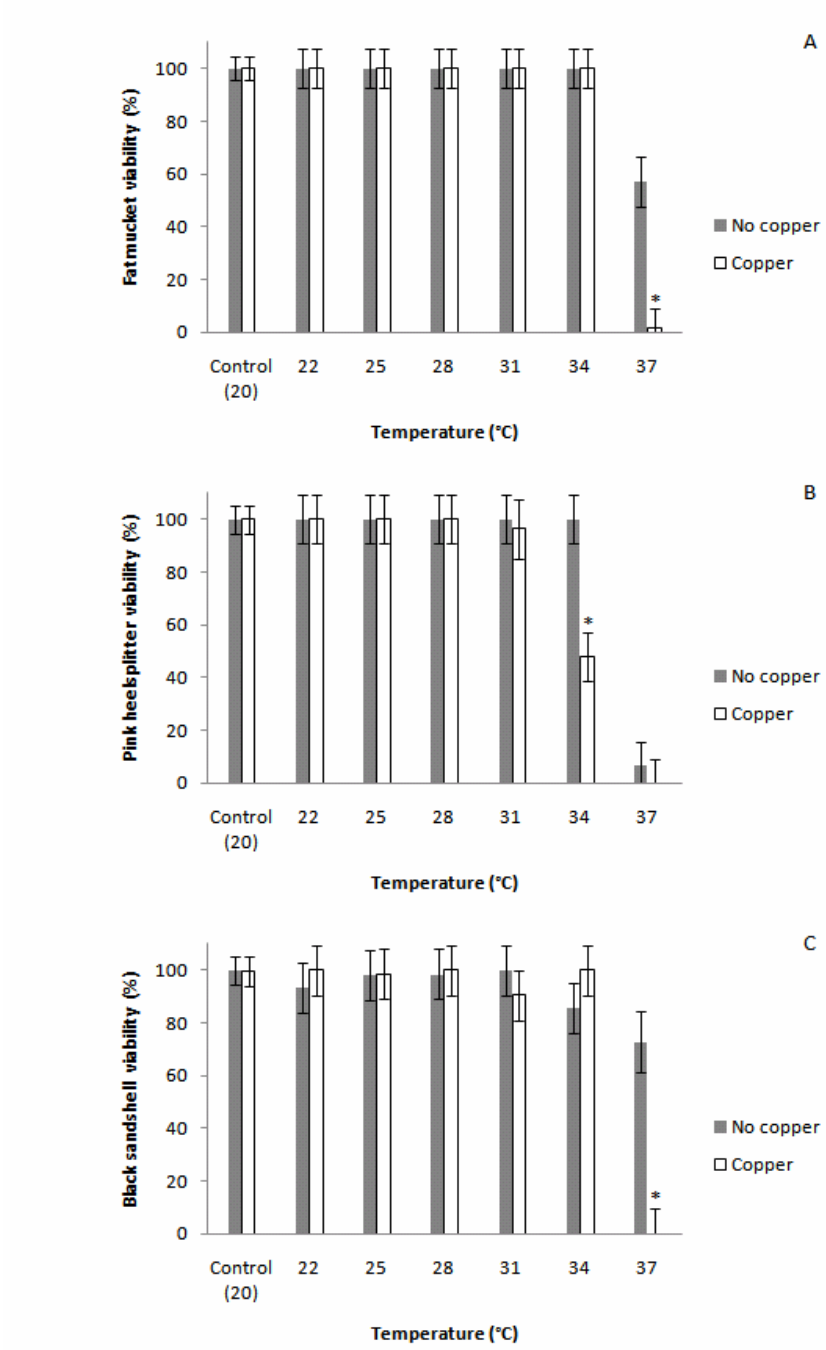

Figure 8. Viability of three species of juvenile freshwater mussels, fatmucket (A), pink heelsplitter (B), black sandshell (C), at the 22ºC acclimation temperature in the absence and presence of 10 µg/L of copper at 48 h. Error bars represent standard error.
* indicates significant difference at the α=0.01 level in a temperature treatment caused by the addition of copper.

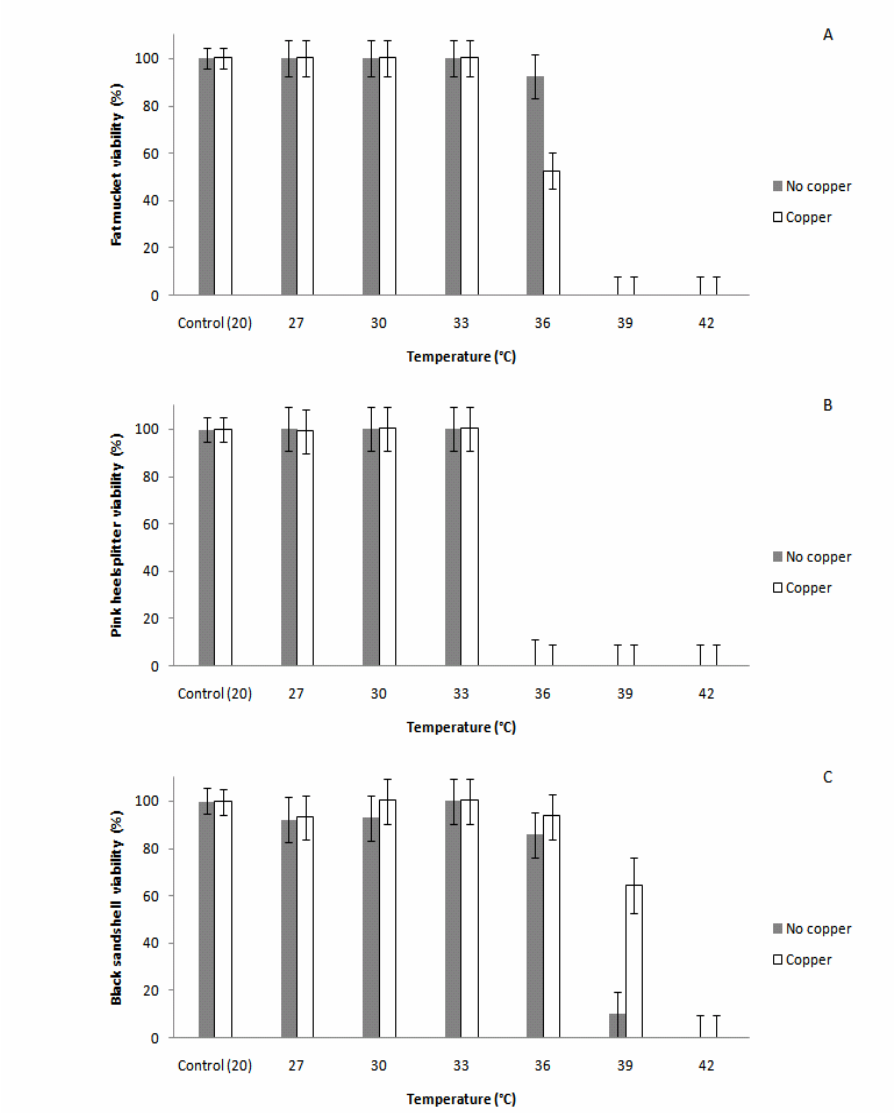

Figure 9. Viability of three species of juvenile freshwater mussels, fatmucket (A), pink heelsplitter (B), black sandshell (C), at the 27ºC acclimation temperature in the absence and presence of 10 µg/L of copper at 48 h. Error bars represent standard error. There were no significant differences at the α=0.01 level in any temperature treatment caused by the addition of copper.

Chapter 4. Are freshwater mussels a climate change aquatic canary? What we need to know about mussel, fish, and temperature interactions.

(Formatted for Ecology Letters)

Abstract

Rising environmental temperatures result from global climate change and can cause significant shifts in the composition and distribution of species within communities. In freshwater systems, the larval life stage, glochidia, of Unionoida mussel species develop as obligate parasites on host fish gills or fins before transforming into the juvenile life stage and dropping to the sediment to complete their life cycle. Because of the relationship between mussels and their often specific host fish species, freshwater mussels are not only potentially affected by their own variable thermal tolerance limits, but also by those of their host fish. The purpose of this paper was to compile data from available literature regarding sensitivities of eight species of freshwater mussels as well as their host fish, in order to determine whether the community structure of these systems is at risk from rising environmental temperatures. Relationships were complicated with mussels being both more and less thermally sensitive than certain host fish species, suggesting some mussels may be at risk due to the thermal tolerances of their host fish.

Keywords: Unionidae, temperature, freshwater mussel, thermal tolerance, host fish, climate change

Introduction

Rising atmospheric carbon dioxide concentrations from anthropogenic sources have caused global average air temperatures to rise by 0.6ºC since 1900, and more accelerated effects are anticipated in the current century (IPCC 2001). Rising environmental temperatures resulting from global climate change can cause significant shifts in the composition and distribution of species within communities (Smith *et al.* 2006). Aquatic systems are much more constrained than are terrestrial systems in the ways in which organisms can respond to warming, and therefore, effects of climate change may be more pronounced (Shuter & Post 1990). Because of this, and also because changes in temperature unrelated to climate change in stream ecosystems have been well documented (Feller 1981, Hewlett & Fortson 1982), aquatic systems are a valuable study system for climate change. Despite those advantages, the effect of climate change on aquatic communities has been understudied.

Freshwater mussels fulfill their considerable role in the aquatic community by converting particulate matter from the water column into a food source for other organisms (Vaughn *et al.* 2004, Howard & Cuffey 2006). Surprisingly, however, unionid mussels, which are among the most endangered group of aquatic organisms, have yet to be considered in the context of climate change. The unionids are suffering a high rate of extinction; in fact, nearly 70% of North America's nearly 300 species are extinct or vulnerable to extinction (Graf & Cummings 2007, Bogan 1993, Williams *et al.* 1993). The southeastern United States is one of two main areas of diversity and endemism for

these mussels. (Bogan 2008), and the decline of mussels has been well documented for this region (Bogan 1993, Lydeard *et al.* 2004). The most notable cause of decline in freshwater mussels is habitat degradation; other impacts include water withdrawal for industry, pollution, and urbanization (Bogan 2008). These factors not only affect freshwater mussels, but also the fish they rely on to complete their life cycle.

Freshwater mussels are a threatened group of organisms due in part to their complex life history strategies. The Unionoida order of freshwater mussels rely on host fish to complete their life cycle by allowing the larval life stage, glochidia, to infest the gills or fins of host fish as parasites before transforming into the juvenile life stage and dropping to the sediment to continue their development into benthic-dwelling adults (Wachtler *et al.* 2001). The complexities of their life cycle make freshwater mussels particularly susceptible to disruption by stressors, but the free living glochidial life stage is the most vulnerable (Bauer 2001).

Because of the relationship between mussels and their host fish species, freshwater mussels are not only potentially affected by their own variable thermal tolerance limits, but also by those of their host fish (Biro *et al.* 2007, Daufresne & Boet 2007, Schmutz *et al.* 2007, Steingraeber *et al.* 2007). Although it is often emphasized that species interactions could be important in the ability of species to respond to climate change, the possibility remains poorly explored. Because unionid mussels are host specific and have potentially different environmental requirements than their hosts, they represent an ideal case in which to explore the extent to which species interactions can

and will mediate responses to climate change. The freshwater mussel-host fish relationship is a fitting model to explore both climate change in an aquatic context and interspecies relationships in the context of global change.

To elucidate the links between climate change and freshwater mussel survival, representative thermal tolerance data were collected for eight species of mussels as well as their host fish. These data were used to compare the thermal tolerances of these two groups of organisms and discuss scenarios of population and functional changes related to rising environmental temperatures caused by global climate change.

Material and methods

Freshwater mussel thermal tolerance data were collected experimentally for eight species of glochidia and seven species of juvenile freshwater mussels. The species used represent three tribes from two subfamilies of the family Unionidae (Graf & Cummings 2007). From the Ambleminae subfamily, five species were from the Lampsilini tribe: fatmucket (*Lampsilis siliquoidea*, Barnes, 1823), pink heelsplitter (*Potamilus alatus*, Say, 1817), black sandshell (*Ligumia recta*, Lamarck, 1819), butterfly (*Ellipsaria lineolata*, Rafinesque, 1820), and eastern creekshell (*Villosa delumbis*, Conrad, 1834); while one species was from the Quadrulini tribe: washboard (*Megalonaias nervosa*, Rafinesque, 1820). From the Unioninae subfamily, two species belonged to the Anodontini tribe: white heelsplitter (*Lasmigona complanata*, Barnes, 1823), and brook floater (*Alasmidonta varicosa*, Lamarck, 1819).

These species were chosen because they encompass a variety of life history strategies and habitats, and because of their wide geographic distribution, particularly in the central and southeastern United States. The species represent three subregions of the Nearctic region (Graf & Cummings 2007): Interior Basin (fatmucket, white heelsplitter, butterfly, black sandshell, pink heelsplitter, and washboard), Gulf Coastal (white heelsplitter, butterfly, black sandshell, pink heelsplitter, and washboard), and the Atlantic Slope (brook floater and eastern creekshell). All test organisms came from propagation facilities at Missouri State University (Interior Basin and Gulf Coastal species) and North Carolina State University (Atlantic Slope species).

The methods used to determine the thermal tolerances of the mussel species are described in detail in a companion study (Pandolfo 2008). Briefly, each test was conducted at three acclimation temperatures: 17ºC, 22ºC, and 27ºC, and each acclimation temperature had five corresponding experimental temperatures in 3ºC increasing increments. A 20ºC reference control temperature was also assessed alongside each test (Figure 1).

Survival data from both glochidia and juvenile tests were used to generate median effective temperatures (ET50s) with the trimmed Spearman-Karber method using ToxCalc v.5.0.26 toxicity data analysis software (Tidepool Scientfic Software, McKinleyville, CA, USA). An ET50 is the temperature at which 50% of the exposed population exhibits some predefined effect; for glochidia this effect was loss of viability determined via shell closure response with the addition of saturated salt solution, and for

juveniles this effect was loss of viability determined by lack of foot movement within or outside of the shell, and/or lack of a heart beat (ASTM 2006).

Host fish were identified for the eight species of freshwater mussels used in this study through the Ohio State University Mussel/Host database (http://128.146.250.235/MusselHost/); only studies that observed juvenile metamorphosis in nature or in laboratory studies were included (Table 1). Host fish species that were used to transform the juveniles used in this study were provided by researchers at the propagation facilities. Thermal tolerance data for host fish species were collected from several sources. Lethal threshold temperatures (incipient lethal temperature; ILT) from the Environmental Protection Agency's Water Quality Criteria (1972) and Wismer and Christie (1987) were used when available, as these data coincided most clearly with the ET50 measure used for freshwater mussels. For species where no lethal threshold was available, critical thermal maximum temperatures (CTmax), using loss of equilibrium as an endpoint, were collected from Beitinger *et al*. (2000) and Wismer and Christie (1987). In addition, for species where ILT or CTmax were not available, upper thermal tolerance limit (UTTL) data were taken from Eaton *et al*. (1995).

Results

ET50s were generated for glochidia and juvenile freshwater mussels at two acclimation temperatures: 22°C and 27°C (Table 2). For both life stages, the overall ET50s ranged from 29.06°C to 37.51°C with a mean of 34.28°C. Glochidia ET50s

ranged from 29.06°C to 37.51°C with a mean of 33.80°C. Juvenile ET50s ranged from 32.90°C to 36.74°C with a mean of 34.76°C. Fish thermal tolerance values ranged from 25°C to 38.1°C with a mean of 33.09°C (Table 1). Fish thermal tolerance varied according to acclimation temperature, as well as the method used to determine the tolerance value.

Upper thermal tolerances for host fish were plotted with freshwater mussel ET50s against acclimation temperature for each freshwater mussel species (Figure 2). In most instances, fish thermal tolerance increased linearly with increasing acclimation temperature; because of this relationship, linear regressions for fish species with at least two thermal tolerance values can provide a good indication of the thermal threshold for that species (Figure 3). For mussel species with many hosts (i.e. fatmucket, black sandshell, washboard), fish species were included for linear regression only if they had three or more thermal tolerance data points. For mussel species with fewer hosts (i.e. white heelsplitter, brook floater, eastern creekshell), fish thermal tolerance data were included for regression if there were two or more data points. For fish species with only one thermal tolerance value reported, linear extrapolations were not possible. Linear regressions for pink heelsplitter and butterfly's host fish, freshwater drum (*Aplodinotus grunniens*, Rafinesque, 1819) were not possible, therefore they are not represented in Figure 3.

Mussel thermal tolerances were compared with fish thermal tolerances by determining on which side of the host fish thermal tolerance threshold they occurred. If

mussel tolerance occurred to the left of fish thermal tolerance, then the mussels were less thermally tolerant than the fish, whereas if mussel tolerance occurred to the right, then the mussels had a higher thermal tolerance. Fatmucket appeared more thermally tolerant than any of its host fish species. Pink heelsplitter and butterfly shared the same host fish, freshwater drum, which had only a limited amount of thermal tolerance data available. Both pink heelsplitter and butterfly had ET50s both higher and lower than freshwater drum's UTTL, though more thermal data is needed for freshwater drum to explore the relative thermal sensitivity of the freshwater mussels compared to this fish species. Black sandshell appeared more thermally tolerant than yellow perch (*Perca flavescens*, Mitchill, 1814) and bluegill (*Lepomis macrochirus*, Rafinesque, 1819), similar to largemouth bass (*Micropterus salmoides*, Lacapede, 1820), and more sensitive than pumpkinseed (*Lepomis gibbosus*, Linnaeus, 1758), green sunfish (*Lepomis cyanellus,* Rafinesque, 1819), longear sunfish (*Lepomis megalotis*, Rafinesque, 1820), and central stoneroller (*Campostoma anomalum*, Rafinesque, 1820). White heelsplitter was more thermally tolerant than all fish species except for green sunfish. Washboard was less thermally tolerant than green sunfish, longear sunfish, and central stoneroller but was more tolerant than brown bullhead (*Ameiurus nebulosus*, Lesueur, 1819), bluegill, and yellow perch. Brook floater was more thermally tolerant than slimy sculpin (*Cottus cognatus*, Richardson, 1836) and seagreen darter (*Etheostoma thalassinum*, Jordan and Brayton, 1878), but more sensitive than pumpkinseed. Eastern creekshell in this study was transformed by a hybrid bluegill-green sunfish (*Lepomis macrochirus cyanellus*),

therefore thermal tolerance data were used individually for these two species because data were not available for the hybrid. Eastern creekshell appeared to be more thermally tolerant than bluegill but less tolerant than green sunfish, thus it is unclear where the hybrid's thermal tolerance would occur.

Discussion

Research on terrestrial organisms has long suggested that the species most at risk for climate change are high latitude and high elevation species that will be pushed off the ends of their respective ranges as temperatures increase (Brown 1971); these shifts have been documented in a variety of taxa including plants, butterflies, invertebrates, zooplankton, fish, birds, and mammals (Hughes 2000, Walther *et al.* 2002). Freshwater systems can be easily paralleled to the latitudinal/altitudinal gradient studies of terrestrial systems by using stream order as the gradient measure. As stream order changes, the physical, chemical, and biological attributes of a stream also change (Vannote *et al.* 1980). Stream order size is directly related to the width, depth, area, volume, and speed of flow of streams, with low order streams being small headwaters and higher orders becoming larger rivers (Strahler 1964, Mackie 2001). The volume of water in a stream has a direct influence on the heating capacity of that stream, and it is generally observed that as stream order increases the mean daily water temperature also increases. If lower order streams are synonymous with higher elevations, species that reside in them may be

eliminated from the system by shifting species distributions caused by increases in environmental temperatures (Figure 4).

The bulk of thermal tolerance testing to date has been with fish (e.g. Beitinger *et al.* 2000). From these studies, we have learned of the effects of temperature on basic physiological processes. For instance, thermal stress can lead to acid-base disturbances (van Dijk *et al*. 1999), and effects on growth rate and capacity for metabolic performance (Widmer *et al*. 2006, Fontaine *et al*. 2007). Increases in environmental temperature have also been shown to adversely affect fish communities. One long term study found that an increase of 1.5ºC in the average water temperature in the Upper Rhone River caused southern fish species to displace northern fish species (Daufresne *et al.* 2003). The increase of southern warmwater fish into the range of the northern cooler water fish was consistent with predictions based on latitudinal/altitudinal/stream order gradient hypotheses.

Studies with mollusks have found, as in fish studies, that increases in temperature can affect various physiological functions, causing decreases in immune condition and increases in production of reactive oxygen species (Chen *et al*. 2007). Several studies have related increasing temperature with increases in filtration rate (Shulte 1975, Han *et al*. 2008), oxygen consumption (Newell *et al*. 1977, Han *et al*. 2008), excretion rates (Han *et al*. 2008), and growth (Han *et al*. 2008), while other studies have found a negative relationship (Petes *et al*. 2007). To a degree, increased energy input (e.g. through filtration) may be able to compensate for increased metabolic demands, but there

appears to be a thermal limit above which the positive relationship between temperature and physiological function plateaus or becomes negative due to increasing energetic costs (Schulte 1975, Newell *et al*. 1977). Rising temperatures have been associated with alterations in reproduction in the marine bivalve *Macoma balthica* (Linnaeus, 1758) (Philippart *et al*. 2003), and increased spawning in the marine *Perna canaliculus* (Gmelin, 1791) and *Mytilus galloprovincialis* (Lamarck, 1819) (Petes *et al*. 2007). In addition to the studies on sublethal effects of thermal stress, a number of studies have dealt with acute thermal limits (Ansell *et al*. 1980a, Ansell *et al*. 1980b, Iwanyzki & McCauley 1993, Urban 1994).

Laboratory tests with freshwater mussel glochidia have shown that viability of glochidia can vary widely even at a common temperature among species belonging to the same tribe (Cope *et al.* 2008). Laboratory tests also show that increasing temperatures cause a decrease in glochidial viability. Viability of glochidia of the species *Actinonaias pectorosa* (Conrad, 1834) and *Villosa iris* (I. Lea, 1829) decreased significantly at a temperature of 25ºC, when compared to 0 ºC or 10 ºC (Zimmerman & Neves 2002). Similar results were found for *Unio pictorum* (Linnaeus, 1758), *Unio crassus* (Retzius, 1788)*, Anodonta anatina* (Linnaeus, 1758)*, Anodonta cygnea* (Linnaeus, 1758)*, and Margaritifera margaritifera* (Linnaeus, 1758) (Jansen *et al.* 2001) and for *Margaritifera laevis* (Haas, 1910) glochidia (Akiyama & Iwakuma 2007). The density of *Oncorhynchus masou masou* (Brevoort, 1856), the host fish of *M. laevis* is also expected to decrease with increasing water temperature (Inoue *et al.* 1997).

The obligate parasite-host relationship between freshwater mussels and their host fish provides a valuable example for exploring how the loss of one species can have cascading effects for additional species. These cascades may lead to chains of extinction among any number of species that interact in a critical manner. In perhaps the clearest case of coextinction in the literature, severe reductions in populations of the eel grass *Zostera marina* (Linnaeus) caused the host-specific eelgrass limpet, *Lottia alveus* (Conrad, 1831) to become extinct (Carlton *et al.* 1991). Changes in environmental temperatures can also cause asynchrony in species interactions. Increased temperatures caused the bivalve *Macoma balthica* to adjust its reproductive schedule which caused asynchrony with the presence of phytoplankton and shrimp necessary for juvenile survival (Philippart *et al.* 2003). Synchrony between the oak tree's bud burst and winter moth egg hatching was disrupted by increases in spring temperature because the two species did not react to the changing temperature at the same speed (Visser & Holleman 2001). For freshwater mussels, asynchrony with the presence of host fish could lead to a collapse of mismatched populations.

As a response to global climate change, decreasing mussel survival may be a function of not only first order temperature effects, but also on scarcity of host fish due to fish thermal tolerances. Mussel population dynamics can also be impacted if increased water temperatures decrease the infestation success of glochidia on the host fish or if too few mussels are recruited to reproductive maturity. The mussels used in this comparative study have predominantly cool and warmwater assemblage species as their hosts (Stefan

et al. 1995), and therefore we can potentially classify these species based on the classification of their hosts. Though they have not been included in this study, there are mussel species that occupy source waters and therefore have coldwater fish as hosts. These mussels are the ones most likely to be adversely affected by global climate change, as proposed in Figure 4. It is also possible that mussels or fish that appear more heat tolerant may actually be more at risk from climate change because heat tolerant species may be living closer to their thermal limits (Tomanek & Somero 1999). There is evidence that some fish species are already encountering temperatures at their upper lethal limit in North America (Eaton *et al.* 1995, Caissie 2006).

There are a number of scenarios that that need to be considered in order to examine the interactions of freshwater mussels with their host fish in the context of climate change (Figure 5). The thermal tolerances of freshwater mussels can potentially be higher, lower, or similar to their host fish. Each of these possibilities leads to potentially very different outcomes, each with different implications for conservation and management of freshwater mussels and the waters they inhabit. If freshwater mussels and their host fish have similar thermal tolerances, then there are no expected species interaction effects to compound any adverse effects from climate change. This does not imply that climate change does not pose a risk to mussels or their hosts, but that they are expected to respond in similar manners and therefore their relationship can be conserved as long as their tolerances are not exceeded. However, even if the proper host fish remain

within range of freshwater mussels, glochidia may not transform successfully outside an optimal temperature range (Roberts & Barnhart 1999).

It must also be considered that freshwater mussels are more constrained in their movement than are their host fish. As temperatures increase, some species may change their distribution as a response, with warm water species moving into cooler habitats, or using the stream order model, species may relocate to lower order streams, therefore pushing out the species at the lowest orders. Because freshwater fish are able to detect differences in water temperature and to relocate to cooler water when available, the fish may more easily change their distribution and move outside of the range of the freshwater mussels that rely on them (Bardach & Bjorklund 1957, Kaya *et al.* 1977, Headrick & Carline 1993, Beitinger *et al.* 2000). This scenario could pose problems particularly if mussel thermal tolerances are higher than the tolerances of their host fish, because the fish may choose to leave the area for cooler waters. If fish relocate to another habitat, they can potentially bring glochidia with them, therefore dispersing the mussels to the cooler habitat that the fish prefer. Dispersal of mussels on small fishes such as darters and sculpins can be less than 100 m (McLain & Ross 2005), while larger fish with larger home ranges have a higher likelihood of allowing mussels to relocate to new habitats and differentiate genetically (Berg *et al.* 1998). Mussels may also be able to use other more tolerant fish as alternate hosts. Although most mussel species tend to specialize with one or a few species as hosts, specificity differs among species (Haag & Warren 2003), therefore some mussels may have limited transformation success using alternate fish

species. However, freshwater mussels may become locally adapted to their host fish and experience greater transformation success with fish within their native habitat than with fish from other areas (Rogers *et al.* 2001).

Another scenario is that if the host fish have thermal tolerances greater than the mussels, they will not need to relocate to cooler habitat. The possibility still remains in this scenario that through normal fish movement, the mussels may still be dispersed to cooler habitats where they will be more suited for survival. However, if this is not the case, the mussel populations may decline due to decreased glochidial infestation success or mortality of mussels of reproductive age despite the presence of their host fish. If mussel populations become too small and disconnected, sperm may not be able to reach females during the spawning season, and these populations will be unable to contribute genetically (Downing *et al.* 1993, Strayer *et al.* 2004, McLain & Ross 2005).

Organisms can adapt to environmental changes in two ways: changes within individuals (phenotypic plasticity) or changes between generations (evolution) (Berteaux *et al.* 2004). However, for freshwater mussels adaptation may be limited due to their extended life span, as species with long generation times do not respond as quickly to environmental changes (Berteaux *et al.* 2004, Rowe 2008). In addition, recruitment does not necessarily occur annually, a population study of the freshwater mussel *Fusconaia ebena* (I. Lea, 1831) found successful recruitment only once every 5 to 10 years (Payne & Miller 2000), thus, it can be difficult to assess population dynamics of freshwater

mussels because populations may be experiencing negative growth while long-lived individuals thrive (Strayer *et al.* 2004).

In addition to the dynamics of their interspecies relationships, freshwater mussels and their host fish will be exposed to a variety of impacts at the same time. Water withdrawal for industrial use and heated effluent discharges can further increase water temperatures and toxicity of contaminants in the water column can be exacerbated by increased temperatures (Langford 1990, Sokolova 2004, Caissie 2006). Species may also have to deal with the shifting distributions of more thermally tolerant non-indigenous species (Stachowicz *et al*. 2002, Carveth *et al*. 2006), and land-use changes can combine with climate change effects to the detriment of aquatic organisms (Peterson & Kwak 1999).

It is also important to consider that changes in environmental conditions associated with climate change are hard to predict because biological responses may not occur linearly, even if the underlying causes, such as air temperature, are linear. As a result, changes in environmental temperature may cause unexpected shifts in ecosystems as regime shifts occur (Hsieh *et al*. 2005), and the many factors involved in climate change may interact in a synergistic fashion (Portner *et al*. 2005). In fact, alterations in flow regimes as a result of changing precipitation patterns may be at least as threatening to aquatic species as increasing temperatures (Peterson & Kwak 1999).

There is still a great deal of research to do in the arena of climate change with the thermal tolerances of freshwater mussels and their host fish. More data must be collected

regarding the thermal tolerances of specific host fish-mussel pairs, as well as the presence and transformation success rate of alternate host fish before it is possible to determine which scenario is most likely for different freshwater mussel species, therefore the concepts developed in this paper will prove useful as more data is generated. Surveys of mussel community structure along temperature gradients would be a useful tool, as would laboratory studies regarding infestation success of glochidia on differing fish species in relation to changes in environmental temperature. In further studies, it will be important to consider the various scenarios and the potential outcomes of climate change on mussels and their hosts.

As it has already been noted, research on climate change can not be conducted for every species; therefore the focus must be on species with a disproportionately important role in their ecosystems (Bale *et al.* 2002). I further propose that freshwater mussels are a crucial fauna to study in the context of global change, not only because they are the most endangered aquatic faunal group in North America, but also because of their unique life history strategies. Unionids provide a means for measuring the importance of species interactions as a component of climate change using a sensitive model species in aquatic systems—if freshwater mussels will not be our aquatic climate change canary, which species will?

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Table 1. Freshwater mussel host fish thermal tolerance data compiled from literature. All temperatures are ºC, parentheses indicate acclimation temperature. ILT=incipient lethal temperature, CTmax=critical thermal maximum, UTTL=upper thermal tolerance limit. *fish used to transform juveniles in this study, **thermal data for *N. exilis* (slender madtom), ***thermal data for *E. blennioides* (greenside darter).

Freshwater mussel	Host fish	Fish species name	Thermal tolerance	Method	Source
Fatmucket	Largemouth bass*	*Micropterus salmoides*	32.5 (20)	ILT	EPA 1972
(*Lampsilis siliquoidea*)			31.5 (22)	ILT	EPA 1972
			34.5 (25)	ILT	EPA 1972
			36.4 (30)	ILT	EPA 1972
			36 (35)	ILT	Wismer and Christie 1987
	Bluegill	*Lepomis macrochirus*	31 (15)	ILT	Wismer and Christie 1987
			32 (20)	ILT	Wismer and Christie 1987
			33 (25)	ILT	Wismer and Christie 1987
			35.8 (27)	ILT	Wismer and Christie 1987
			34 (30)	ILT	Wismer and Christie 1987
			37 (33)	ILT	Wismer and Christie 1987
	Longear sunfish	*Lepomis megalotis*	31.1 (15.5)	ILT	Wismer and Christie 1987
			35.6 (25)	ILT	EPA 1972
			36.8 (30)	ILT	EPA 1972
			37.5 (35)	ILT	EPA 1972
	Striped shiner	*Luxilus chrysocephalis*	36.2 (26)	CTmax	Beitinger et al. 2000
	Smallmouth bass	*Micropterus dolomieu*	37 (35)	ILT	Wismer and Christie 1987
	White bass	*Morone chrysops*	33.5 (28)	UTTL	Eaton et al. 1995
	Yellow perch	*Perca flavescens*	27.7 (15)	ILT	EPA 1972
			29.7 (25)	ILT	EPA 1972
	Bluntnose minnow	*Pimephales notatus*	30.6 (15)	ILT	EPA 1972
			31.7 (20)	ILT	EPA 1972
			33.3 (25)	ILT	EPA 1972
	White crappie	*Pomoxis annularis*	32.8 (25.6)	UTTL	Eaton et al. 1995
	Sauger	*Stizostedion canadense*	28.7 (18.3)	ILT	Wismer and Christie 1987
			29.5 (19.9)	ILT	Wismer and Christie 1987
			29.9 (22)	ILT	Wismer and Christie 1987
			30.4 (23.9)	ILT	Wismer and Christie 1987
			30.4 (25.8)	ILT	Wismer and Christie 1987
	Walleye	*Stizostedion (Sander) vitreum*	30.5 (18.2)	ILT	Wismer and Christie 1987
			30.5 (22.1)	ILT	Wismer and Christie 1987
			31.5 (24)	ILT	Wismer and Christie 1987
			31.6 (25.8)	ILT	Wismer and Christie 1987
			34 (26)	ILT	Wismer and Christie 1987
Pink heelsplitter	Freshwater drum*	*Aplodinotus grunniens*	34 (21.2)	CTmax	Wismer and Christie 1987
(*Potamilus alatus*)			32.8 (29-35)	ILT	Wismer and Christie 1987
Black sandshell	Walleye*	*Stizostedion (Sander) vitreum*	30.5 (18.2)	ILT	Wismer and Christie 1987
(*Ligumia recta*)			30.5 (22.1)	ILT	Wismer and Christie 1987
			31.5 (24)	ILT	Wismer and Christie 1987
			31.6 (25.8)	ILT	Wismer and Christie 1987
			34 (26)	ILT	Wismer and Christie 1987
	Rock bass	*Ambloplites rupestris*	37.5 (23.9)	ILT	Wismer and Christie 1987
			35 (30)	ILT	Wismer and Christie 1987
	Central stoneroller	*Campostoma anomalum*	35.8 (23)	CTmax	Beitinger et al. 2000
			37.7 (24)	CTmax	Beitinger et al. 2000
			37.2 (26)	CTmax	Beitinger et al. 2000
	Banded killifish	*Fundulus diaphanus*	27.5 (15)	ILT	EPA 1972
			34.5 (25)	ILT	Wismer and Christie 1987
	Green sunfish	*Lepomis cyanellus*	35 (20)	ILT	Wismer and Christie 1987
			40 (30)	ILT	Wismer and Christie 1987
	Pumpkinseed	*Lepomis gibbosus*	28 (18)	ILT	Wismer and Christie 1987
			31.6 (20)	ILT	Wismer and Christie 1987
			30.2 (24)	ILT	Wismer and Christie 1987
			31.9 (28)	ILT	Wismer and Christie 1987
			33.5 (32)	ILT	Wismer and Christie 1987

Table 1. Continued.

Freshwater mussel	Host fish	Fish species name	Thermal tolerance	Method	Source
Black sandshell	Orangespotted sunfish	*Lepomis humilis*	36.4 (26)	CTmax	Beitinger et al. 2000
(*Ligumia recta*)	Bluegill	*Lepomis macrochirus*	31 (15)	ILT	Wismer and Christie 1987
continued			32 (20)	ILT	Wismer and Christie 1987
			33 (25)	ILT	Wismer and Christie 1987
			35.8 (27)	ILT	Wismer and Christie 1987
			34 (30)	ILT	Wismer and Christie 1987
			37 (33)	ILT	Wismer and Christie 1987
	Longear sunfish	*Lepomis megalotis*	31.1 (15.5)	ILT	Wismer and Christie 1987
			35.6 (25)	ILT	EPA 1972
			36.8 (30)	ILT	EPA 1972
			37.5 (35)	ILT	EPA 1972
	Largemouth bass	*Micropterus salmoides*	32.5 (20)	ILT	EPA 1972
			31.5 (22)	ILT	EPA 1972
			34.5 (25)	ILT	EPA 1972
			36.4 (30)	ILT	EPA 1972
			36 (35)	ILT	Wismer and Christie 1987
	White perch	*Morone americana*	34.6 (25-26)	ILT	Wismer and Christie 1987
			36.8 (26-27)	ILT	Wismer and Christie 1987
			36 (27)	ILT	Wismer and Christie 1987
	Yellow perch	*Perca flavescens*	27.7 (15)	ILT	EPA 1972
			29.7 (25)	ILT	EPA 1972
	White crappie	*Pomoxis annularis*	32.8 (25.6)	UTTL	Eaton et al. 1995
Butterfly	Freshwater drum*	*Aplodinotus grunniens*	34 (21.2)	CTmax	Wismer and Christie 1987
(*Ellipsaria lineolata*)			32.8 (29-35)	ILT	Wismer and Christie 1987
White heelsplitter	Golden shiner*	*Notemigonus crysoleucas*	30.5 (15)	ILT	EPA 1972
(*Lasmigona complanata*)			32 (20)	ILT	EPA 1972
			32.7 (22.8)	ILT	Wismer and Christie 1987
			33.5 (25)	ILT	EPA 1972
			34.5 (30)	ILT	EPA 1972
	Banded killifish	*Fundulus diaphanus*	27.5 (15)	ILT	EPA 1972
			34.5 (25)	ILT	Wismer and Christie 1987
	Green sunfish	*Lepomis cyanellus*	35 (20)	ILT	Wismer and Christie 1987
			40 (30)	ILT	Wismer and Christie 1987
	Orangespotted sunfish	*Lepomis humilis*	36.4 (26)	CTmax	Beitinger et al. 2000
	Largemouth bass	*Micropterus salmoides*	32.5 (20)	ILT	EPA 1972
			31.5 (22)	ILT	EPA 1972
			34.5 (25)	ILT	EPA 1972
			36.4 (30)	ILT	EPA 1972
			36 (35)	ILT	Wismer and Christie 1987
	White crappie	*Pomoxis annularis*	32.8 (25.6)	UTTL	Eaton et al. 1995
Washboard	Channel catfish*	*Ictalurus punctatus*	30.4 (15)	ILT	EPA 1972
(*Megalonaias nervosa*)			32.8 (20)	ILT	EPA 1972
			34.5 (25)	ILT	EPA 1972
			36.6 (26)	ILT	Wismer and Christie 1987
			37 (30)	ILT	EPA 1972
			38 (34)	ILT	Wismer and Christie 1987
	Black bullhead	*Ameiurus melas*	35 (23)	ILT	Wismer and Christie 1987
			38.1 (26)	CTmax	Beitinger et al. 2000
	Brown bullhead	*Ameiurus nebulosus*	31 (15)	ILT	EPA 1972
			32.5 (20)	ILT	EPA 1972
			33.8 (25)	ILT	EPA 1972
			34.8 (30)	ILT	EPA 1972
			41 (35)	ILT	Wismer and Christie 1987
	Freshwater drum	*Aplodinotus grunniens*	34 (21.2)	CTmax	Wismer and Christie 1987
			32.8 (29-35)	ILT	Wismer and Christie 1987
	Green sunfish	*Lepomis cyanellus*	35 (20)	ILT	Wismer and Christie 1987
			40 (30)	ILT	Wismer and Christie 1987
	Bluegill	*Lepomis macrochirus*	31 (15)	ILT	Wismer and Christie 1987
			32 (20)	ILT	Wismer and Christie 1987
			33 (25)	ILT	Wismer and Christie 1987
			35.8 (27)	ILT	Wismer and Christie 1987
			34 (30)	ILT	Wismer and Christie 1987
			37 (33)	ILT	Wismer and Christie 1987

Table 1. Continued.

Freshwater mussel	Host fish	Fish species name	Thermal tolerance	Method	Source
Washboard	Largemouth bass	*Micropterus salmoides*	32.5 (20)	ILT	EPA 1972
(*Megalonaias nervosa*)			31.5 (22)	ILT	EPA 1972
continued			34.5 (25)	ILT	EPA 1972
			36.4 (30)	ILT	EPA 1972
			36 (35)	ILT	Wismer and Christie 1987
	White bass	*Morone chrysops*	33.5 (25-31)	UTTL	Eaton et al. 1995
	White crappie	*Pomoxis annularis*	32.8 (25.6)	UTTL	Eaton et al. 1995
	Central stoneroller	*Campostoma anomalum*	35.8 (23)	CTmax	Beitinger et al. 2000
			37.7 (24)	CTmax	Beitinger et al. 2000
			37.2 (26)	CTmax	Beitinger et al. 2000
	Longear sunfish	*Lepomis megalotis*	31.1 (15.5)	ILT	Wismer and Christie 1987
			35.6 (25)	ILT	EPA 1972
			36.8 (30)	ILT	EPA 1972
			37.5 (35)	ILT	EPA 1972
	Yellow perch	*Perca flavescens*	27.7 (15)	ILT	EPA 1972
			29.7 (25)	ILT	EPA 1972
Brook floater	Margined madtom*	*Noturus insignis***	36.5 (26)	CTmax	Beitinger et al. 2000
(*Alasmidonta varicosa*)	Seagreen darter*	*Etheostoma thalassinum****	31.2 (10)	CTmax	Beitinger et al. 2000
			32.2 (15)	CTmax	Wismer and Christie 1987
			33.4 (20)	CTmax	Beitinger et al. 2000
	Slimy sculpin	*Cottus cognatus*	23.5 (15)	ILT	Wismer and Christie 1987
			29.4 (20)	CTmax	Wismer and Christie 1987
	Pumpkinseed	*Lepomis gibbosus*	28 (18)	ILT	Wismer and Christie 1987
			31.6 (20)	ILT	Wismer and Christie 1987
			30.2 (24)	ILT	Wismer and Christie 1987
			31.9 (28)	ILT	Wismer and Christie 1987
			33.5 (32)	ILT	Wismer and Christie 1987
	Blacknose dace	*Rhinicthys atratulus*	29.3 (15)	ILT	Wismer and Christie 1987
			29.3 (20)	ILT	EPA 1972
			29.3 (25)	ILT	EPA 1972
			29.3 (28)	ILT	EPA 1972
Eastern creekshell	Hybrid bluegill*	*Lepomis machrochirus cyanellus*			
(*Villosa delumbis*)	Bluegill	*Lepomis macrochirus*	31 (15)	ILT	Wismer and Christie 1987
			32 (20)	ILT	Wismer and Christie 1987
			33 (25)	ILT	Wismer and Christie 1987
			35.8 (27)	ILT	Wismer and Christie 1987
			34 (30)	ILT	Wismer and Christie 1987
			37 (33)	ILT	Wismer and Christie 1987
	Green sunfish	*Lepomis cyanellus*	35 (20)	ILT	Wismer and Christie 1987
			40 (30)	ILT	Wismer and Christie 1987

Table 2. Freshwater mussel thermal tolerance data. ET50s and 95% Confidence Intervals (in parentheses) for glochidia (24 h) and juvenile (96 h) mussels at 22ºC and 27ºC acclimation temperatures. ND indicates ET50s outside of tested temperature range, or unable to be determined, *no test run for white heelsplitter juveniles. All ET50s reported as ºC.

	22ºC Acclimation		27ºC Acclimation	
Species	Glochidia	Juveniles	Glochidia	Juveniles
Fatmucket	ND	35.54 (35.14- 35.96)	36.92 (35.29- 38.62)	34.31 (33.50- 35.14)
Pink heelsplitter	29.06 (25.55- 33.06)	34.79 (33.12- 36.54)	ND	34.60 (33.36- 35.90)
Black sandshell	ND	32.90 (29.58- 36.59)	33.89 (30.40- 37.79)	36.74 (34.37- 39.27)
Butterfly	33.65 (31.17- 36.32)	ND	30.64 (18.48- 50.79)	34.21 (33.20- 35.25)
White heelsplitter	35.99 (34.28- 37.79)	*	37.51 (36.94- 38.09)	*
Washboard	32.38 (29.58- 35.45)	34.16 (32.26- 36.18)	32.44 (29.23- 36.01)	34.98 (33.51- 36.52)
Brook floater	35.80 (34.58- 37.07)	35.05 (33.77- 36.39)	36.85 (35.28- 38.49)	35.29 (32.79- 37.99)
Eastern creekshell	32.87 (29.63- 36.47)	34.60 (32.75- 36.54)	31.43 (27.60- 35.79)	34.72 (33.19- 36.32)

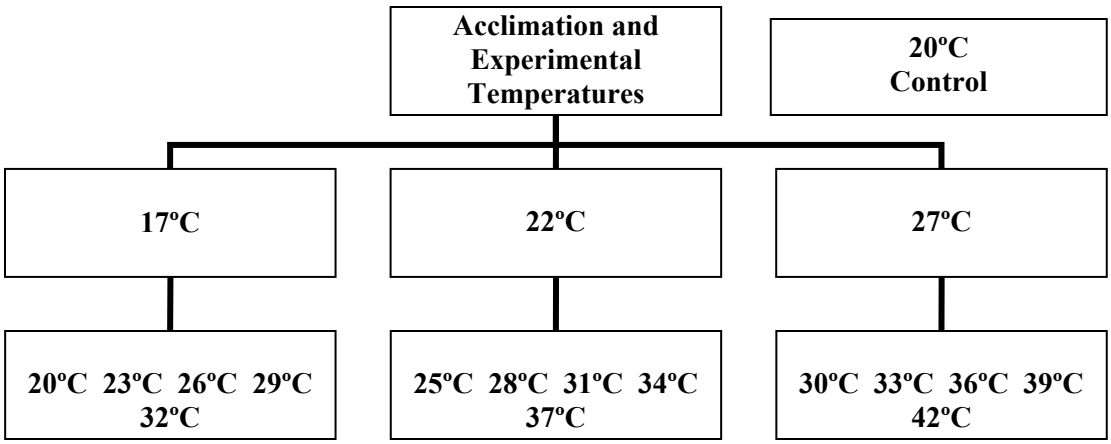

Figure 1. Experimental design showing acclimation and experimental temperature schemes for the early life stages tests with freshwater mussels.

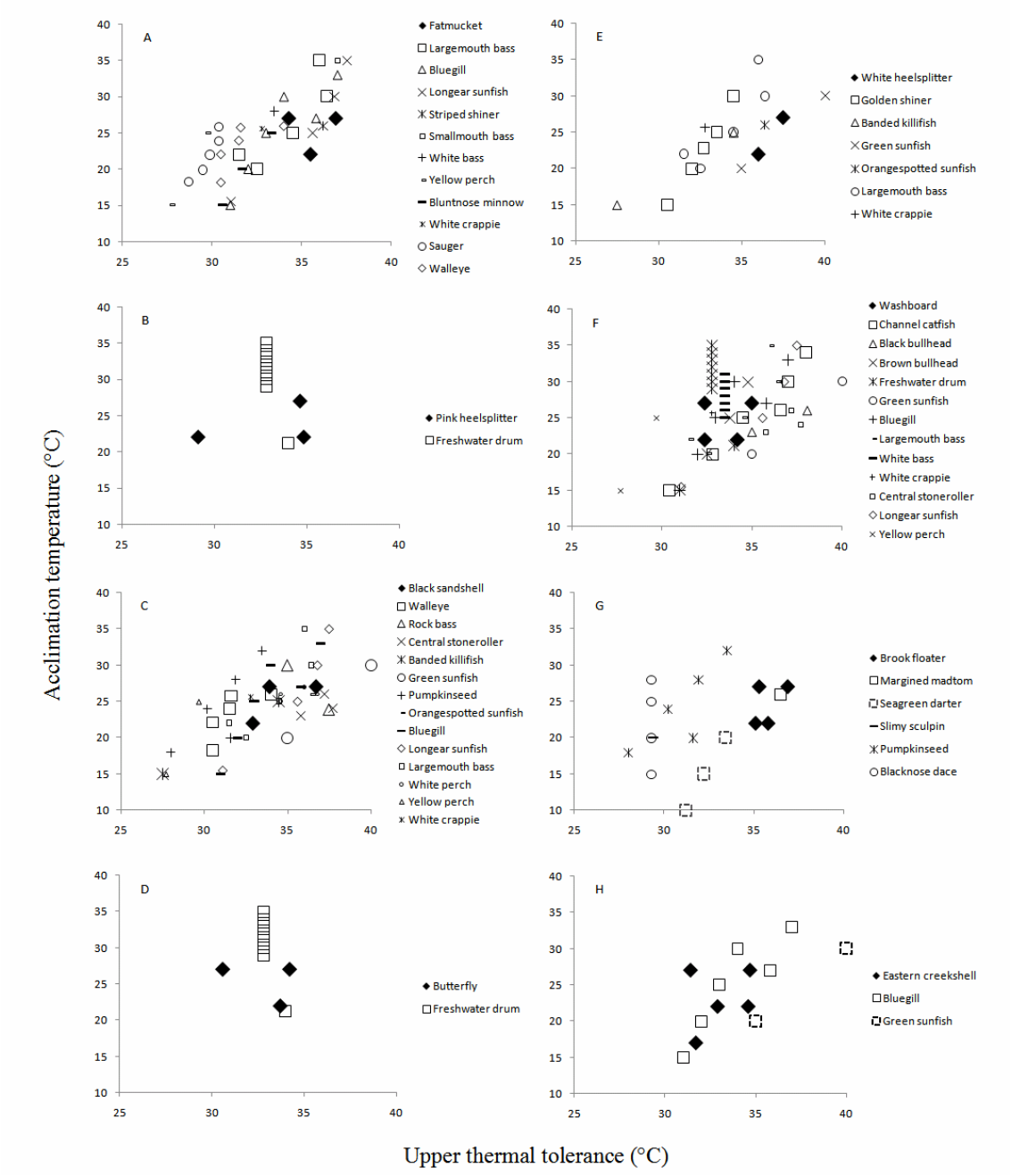

Figure 2. Upper thermal tolerances of eight species of freshwater mussels and their host fish. Each mussel species is graphed in a separate panel with its host fish: fatmucket (A), pink heelsplitter (B), black sandshell (C), butterfly (D), washboard (E), white heelsplitter (F), brook floater (G), and eastern creekshell (H). Freshwater mussels are denoted by the large diamond (◆), fish used to transform mussels used in this study are denoted by the large square (□).

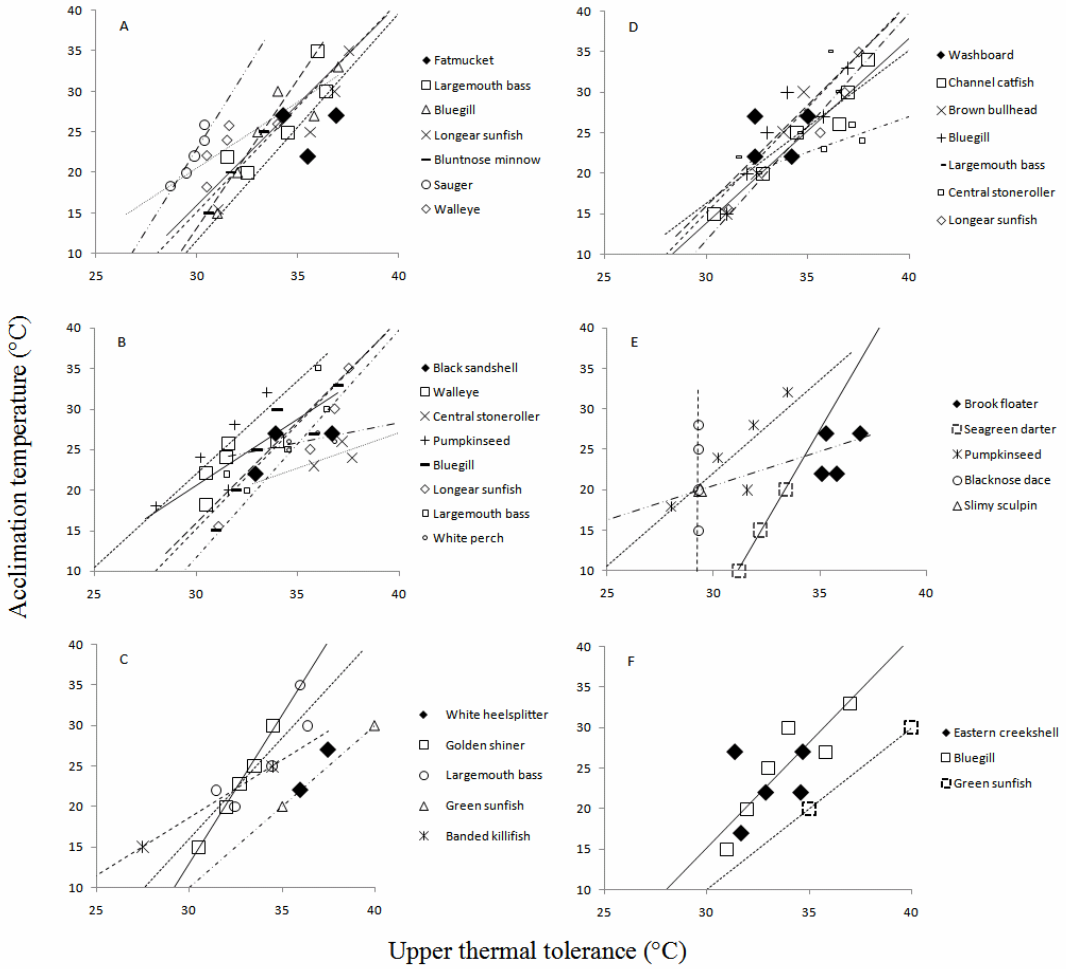

Figure 3. Linear regressions of selected host fish upper thermal tolerances plotted with the thermal tolerances of six species of freshwater mussels. Each mussel species is graphed in a separate panel with its host fish: fatmucket (A), black sandshell (B), white heelsplitter (C), washboard (D), brook floater (E), and eastern creekshell (F). Freshwater mussels are denoted by the large diamond (◆), fish used to transform mussels used in this study are denoted by the large square (□).

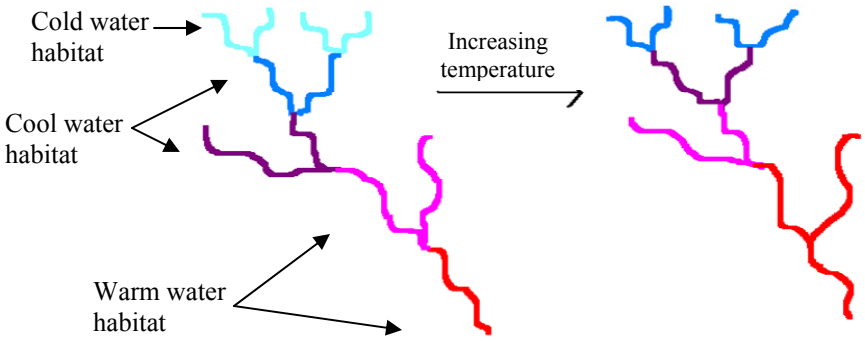

Figure 4. Rising environmental temperatures can cause shifts in the thermal regimes of streams and rivers. This example shows cold water habitat at the lowest stream orders being replaced with cool water habitat.

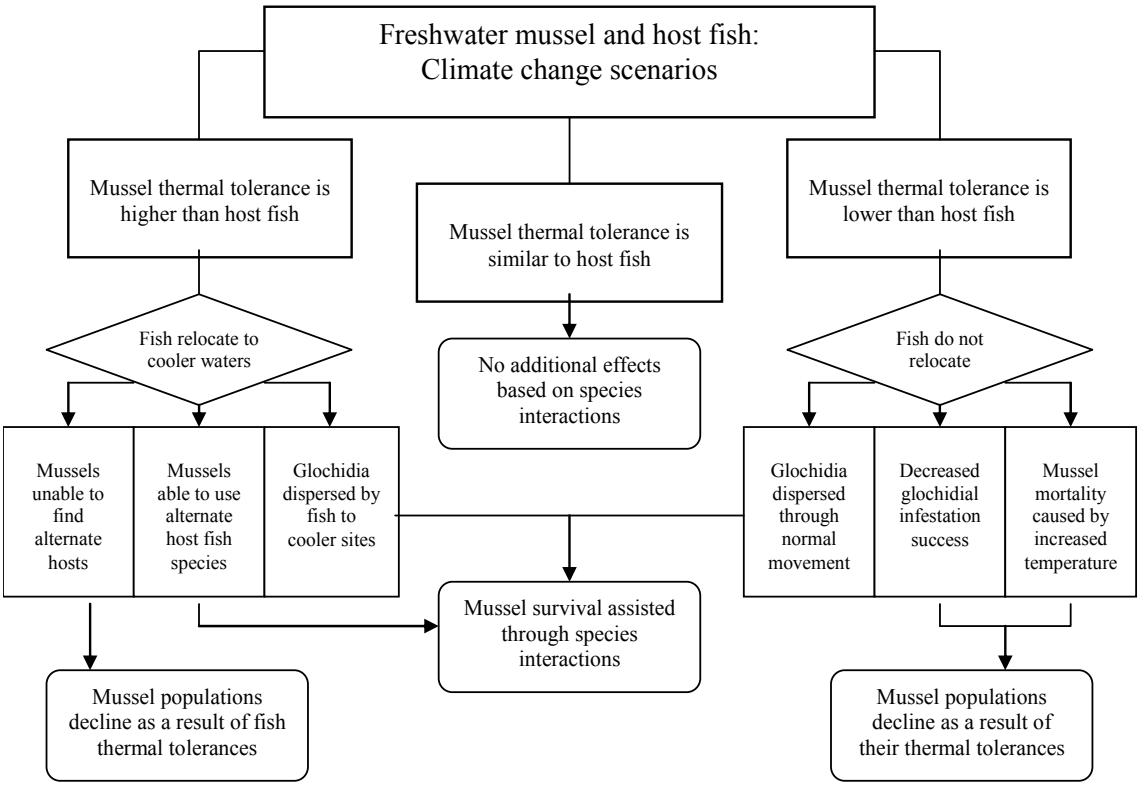

Figure 5. Freshwater mussel and host fish potential climate change scenarios.

APPENDIX

Table 1. Glochidia count duplicate verification (2007). Counts duplicated by time and person.

					Original count			Duplicate Count			Percent difference
Test	Time	Temp	Species	Rep	Mortality %	Avg Mort %	Counted by	Mortality %	Avg Mort %	Counted by	(Avg mort)
17 Acclimation	24	17	Fatmucket	1	16.30	16.87	S.M.	14.30	15.97	T.P.	5.48
17 Acclimation	24	17	Fatmucket	2	20.30			22.00			
17 Acclimation	24	17	Fatmucket	3	14.00			11.60			
17 Acclimation	24	17	Pink Heelsplitter	1	29.20	22.93	S.M.	28.12	21.82	E.T.	4.98
17 Acclimation	24	17	Pink Heelsplitter	2	24.60			21.82			
17 Acclimation	24	17	Pink Heelsplitter	3	15.00			15.52			
17 Acclimation	24	17	Black Sandshell	1	2.30	2.53	S.M.	2.27	2.04	E.T.	21.57 values <10
17 Acclimation	24	17	Black Sandshell	2	3.80			3.85			
17 Acclimation	24	17	Black Sandshell	3	1.50			0.00			
17 Acclimation	48	17	Fatmucket	1	26.20	27.40	S.M.	27.12	28.16	E.T.	2.72
17 Acclimation	48	17	Fatmucket	2	33.80			32.35			
17 Acclimation	48	17	Fatmucket	3	22.20			25.00			
17 Acclimation	48	17	Pink Heelsplitter	1	27.60	29.63	S.M.	25.45	26.30	E.T.	11.92
17 Acclimation	48	17	Pink Heelsplitter	2	30.40			25.45			
17 Acclimation	48	17	Pink Heelsplitter	3	30.90			28.00			
17 Acclimation	48	17	Black Sandshell	1	4.50	2.57	S.M.	4.44	2.00	E.T.	24.82 values <10
17 Acclimation	48	17	Black Sandshell	2	3.20			1.56			
17 Acclimation	48	17	Black Sandshell	3	0.00			0.00			
22 Acclimation	48	34	Black Sandshell	1	49.18	80.46	E.T.	52.54	81.32	T.P.	1.07
22 Acclimation	48	34	Black Sandshell	2	100.00			100.00			
22 Acclimation	48	34	Black Sandshell	3	92.19			91.43			
22 Acclimation	48	37	Black Sandshell	1	47.83	42.88	E.T.	47.92	43.56	T.P.	1.57
22 Acclimation	48	37	Black Sandshell	2	38.18			40.38			
22 Acclimation	48	37	Black Sandshell	3	42.62			42.37			
17 Acclimation	24	control	Pink Heelsplitter	1	17.02	17.41	E.T.	15.56	16.26	T.P.	6.85
17 Acclimation	24	control	Pink Heelsplitter	2	15.22			12.77			
17 Acclimation	24	control	Pink Heelsplitter	3	20.00			20.45			
17 Acclimation	48	32	Fatmucket	1	30.91	42.14	E.T.	33.33	45.41	T.P.	7.45
17 Acclimation	48	32	Fatmucket	2	49.37			53.66			
17 Acclimation	48	32	Fatmucket	3	46.15			49.23			
17 Acclimation	48	23	Black Sandshell	1	0.00	0.69	E.T.	3.39	1.81	T.P.	89.21 values <10
17 Acclimation	48	23	Black Sandshell	2	0.00			0.00			
17 Acclimation	48	23	Black Sandshell	3	2.08			2.04			
22 Acclimation	24	31-Cu	Fatmucket	1	100.00	100.00	E.T.	100.00	100.00	T.P.	0.00
22 Acclimation	24	31-Cu	Fatmucket	2	100.00			100.00			
22 Acclimation	24	31-Cu	Fatmucket	3	100.00			100.00			
22 Acclimation	24	25	Pink Heelsplitter	1	57.41	45.67	E.T.	69.49	50.64	T.P.	10.32
22 Acclimation	24	25	Pink Heelsplitter	2	35.85			37.50			
22 Acclimation	24	25	Pink Heelsplitter	3	43.75			44.93			

Table 1. Continued

					Original count			Duplicate Count			Percent difference	
Test	Time	Temp	Species	Rep	Mortality %	Avg Mort %	Counted by	Mortality %	Avg Mort %	Counted by	(Avg mort)	
22 Acclimation	24	control	Black Sandshell	1	6.67	6.48	E.T.	8.33	8.29	T.P.	24.56	values <10
22 Acclimation	24	control	Black Sandshell	2	3.33			3.33				
22 Acclimation	24	control	Black Sandshell	3	9.43			13.21				
22 Acclimation	24	37	Black Sandshell	1	32.20	28.99	E.T.	31.67	30.16	T.P.	3.97	
22 Acclimation	24	37	Black Sandshell	2	20.83			26.00				
22 Acclimation	24	37	Black Sandshell	3	33.93			32.81				
22 Acclimation	48	31	Fatmucket	1	29.31	34.12	E.T.	29.31	33.30	T.P.	2.42	
22 Acclimation	48	31	Fatmucket	2	41.38			40.91				
22 Acclimation	48	31	Fatmucket	3	31.67			29.69				
22 Acclimation	48	25-Cu	Pink Heelsplitter	1	100.00	95.24	E.T.	98.55	99.52	T.P.	4.40	
22 Acclimation	48	25-Cu	Pink Heelsplitter	2	85.71			100.00				
22 Acclimation	48	25-Cu	Pink Heelsplitter	3	100.00			100.00				
22 Acclimation	48	22	Black Sandshell	1	9.62	6.53	E.T.	9.62	7.52	T.P.	14.04	values <10
22 Acclimation	48	22	Black Sandshell	2	5.36			5.36				
22 Acclimation	48	22	Black Sandshell	3	4.62			7.58				
22 Acclimation	48	37-Cu	Black Sandshell	1	96.72	97.34	E.T.	98.41	98.97	T.P.	1.67	
22 Acclimation	48	37-Cu	Black Sandshell	2	98.46			100.00				
22 Acclimation	48	37-Cu	Black Sandshell	3	96.83			98.51				
27 Acclimation	24	36	Fatmucket	1	42.31	40.90	E.T.	40.38	41.69	T.P.	1.90	
27 Acclimation	24	36	Fatmucket	2	39.22			36.54				
27 Acclimation	24	36	Fatmucket	3	41.18			48.15				
27 Acclimation	24	30	Pink Heelsplitter	1	94.00	94.88	E.T.	94.44	98.15	T.P.	3.38	
27 Acclimation	24	30	Pink Heelsplitter	2	96.00			100.00				
27 Acclimation	24	30	Pink Heelsplitter	3	94.64			100.00				
27 Acclimation	24	control	Black Sandshell	1	1.69	5.31	E.T.	5.17	6.92	T.P.	26.22	values <10
27 Acclimation	24	control	Black Sandshell	2	8.00			8.00				
27 Acclimation	24	control	Black Sandshell	3	6.25			7.58				
27 Acclimation	24	39	Black Sandshell	1	100.00	100.00	E.T.	100.00	100.00	T.P.	0.00	
27 Acclimation	24	39	Black Sandshell	2	100.00			100.00				
27 Acclimation	24	39	Black Sandshell	3	100.00			100.00				
27 Acclimation	48	33	Fatmucket	1	31.48	46.47	E.T.	35.09	49.14	T.P.	5.59	
27 Acclimation	48	33	Fatmucket	2	47.27			51.67				
27 Acclimation	48	33	Fatmucket	3	60.66			60.66				
27 Acclimation	48	27	Pink Heelsplitter	1	100.00	98.89	E.T.	100.00	98.42	T.P.	0.48	
27 Acclimation	48	27	Pink Heelsplitter	2	100.00			96.72				
27 Acclimation	48	27	Pink Heelsplitter	3	96.67			98.53				
27 Acclimation	48	42-Cu	Pink Heelsplitter	1	92.73	93.55	E.T.	95.52	96.92	T.P.	3.54	
27 Acclimation	48	42-Cu	Pink Heelsplitter	2	89.66			95.24				
27 Acclimation	48	42-Cu	Pink Heelsplitter	3	98.25			100.00				
27 Acclimation	48	30	Black Sandshell	1	53.85	59.67	E.T.	57.41	61.44	T.P.	2.92	
27 Acclimation	48	30	Black Sandshell	2	65.52			67.24				
27 Acclimation	48	30	Black Sandshell	3	59.65			59.68				
27 Acclimation	48	39-Cu	Black Sandshell	1	100.00	100.00	E.T.	100.00	99.53	T.P.	0.47	
27 Acclimation	48	39-Cu	Black Sandshell	2	100.00			98.59				
27 Acclimation	48	39-Cu	Black Sandshell	3	100.00			100.00				

Max difference (%) for values > 10 = 11.90

Table 2. Glochidia count duplicate verification (2008). Counts duplicated in time only, all counts by same person (T.P.).

					Original count		Duplicate count		Percent Difference	
Test	Time	Temp	Species	Rep	Mortality %	Avg Mort %	Mortality %	Avg Mort %	(Avg mort)	
17 Acclimation	48	20	Butterfly	1	25.26	19.26	26.53	20.50	6.20	
17 Acclimation	48	20	Butterfly	2	15.25		16.67			
17 Acclimation	48	20	Butterfly	3	17.28		18.29			
17 Acclimation	24	29	White heelsplitter	1	0.00	0.52	0.00	1.52	97.87	values <10
17 Acclimation	24	29	White heelsplitter	2	0.00		2.99			
17 Acclimation	24	29	White heelsplitter	3	1.56		1.56			
17 Acclimation	24	17	Washboard	1	36.96	19.36	37.50	21.52	10.55	
17 Acclimation	24	17	Washboard	2	12.90		17.46			
17 Acclimation	24	17	Washboard	3	8.22		9.59			
17 Acclimation	48	26	Washboard	1	62.32	38.66	59.70	34.81	10.46	
17 Acclimation	48	26	Washboard	2	29.41		23.53			
17 Acclimation	48	26	Washboard	3	24.24		21.21			
22 Acclimation	24	37	Butterfly	1	89.71	88.99	88.41	89.37	0.43	
22 Acclimation	24	37	Butterfly	2	93.94		89.71			
22 Acclimation	24	37	Butterfly	3	83.33		90.00			
22 Acclimation	24	25	White heelsplitter	1	0.00	3.01	0.00	4.16	32.00	values <10
22 Acclimation	24	25	White heelsplitter	2	2.13		2.13			
22 Acclimation	24	25	White heelsplitter	3	6.90		10.34			
22 Acclimation	48	34	White heelsplitter	1	3.13	4.75	3.08	4.74	0.28	
22 Acclimation	48	34	White heelsplitter	2	5.00		5.13			
22 Acclimation	48	34	White heelsplitter	3	6.12		6.00			
22 Acclimation	48	28	Washboard	1	57.53	45.05	59.46	45.55	1.09	
22 Acclimation	48	28	Washboard	2	44.30		47.06			
22 Acclimation	48	28	Washboard	3	33.33		30.12			
27 Acclimation	24	42	Butterfly	1	100.00	99.48	100.00	97.42	2.09	
27 Acclimation	24	42	Butterfly	2	100.00		98.15			
27 Acclimation	24	42	Butterfly	3	98.44		94.12			
27 Acclimation	24	30	White Heelsplitter	1	1.92	2.07	3.77	2.68	25.40	values <10
27 Acclimation	24	30	White Heelsplitter	2	2.17		2.13			
27 Acclimation	24	30	White Heelsplitter	3	2.13		2.13			
27 Acclimation	48	30	White heelsplitter	1	1.64	1.05	1.64	1.05	0.00	
27 Acclimation	48	30	White heelsplitter	2	0.00		0.00			
27 Acclimation	48	30	White heelsplitter	3	1.52		1.52			

Table 2. Continued

Test	Time	Temp	Species	Rep	Original count Mortality %	Original count Avg Mort %	Duplicate count Mortality %	Duplicate count Avg Mort %	Percent Difference (Avg mort)	
27 Acclimation	48	36	White heelsplitter	1	28.99	21.48	26.76	20.61	4.13	
27 Acclimation	48	36	White heelsplitter	2	30.38		30.00			
27 Acclimation	48	36	White heelsplitter	3	5.08		5.08			
27 Acclimation	48	control	Washboard	1	15.85	29.61	14.81	28.61	3.41	
27 Acclimation	48	control	Washboard	2	41.94		42.62			
27 Acclimation	48	control	Washboard	3	31.03		28.41			
17 Acclimation	24	23	Brook floater	1	0.00	2.16	1.75	2.22	2.89	
17 Acclimation	24	23	Brook floater	2	1.64		1.69			
17 Acclimation	24	23	Brook floater	3	4.84		3.23			
17 Acclimation	48	32	Brook floater	1	7.81	5.91	12.50	10.79	58.52	values <10
17 Acclimation	48	32	Brook floater	2	4.55		11.11			
17 Acclimation	48	32	Brook floater	3	5.36		8.77			
22 Acclimation	48	25	Brook floater	1	3.17	4.08	4.76	6.53	46.13	values <10
22 Acclimation	48	25	Brook floater	2	6.25		6.25			
22 Acclimation	48	25	Brook floater	3	2.82		8.57			
27 Acclimation	24	39	Brook floater	1	98.31	97.85	100.00	98.17	0.32	
27 Acclimation	24	39	Brook floater	2	95.24		96.88			
27 Acclimation	24	39	Brook floater	3	100.00		97.62			
27 Acclimation	24	27	Eastern creekshell	1	52.38	58.77	54.69	61.38	4.35	
27 Acclimation	24	27	Eastern creekshell	2	61.43		65.67			
27 Acclimation	24	27	Eastern creekshell	3	62.50		63.79			
22 Acclimation	24	22	Eastern creekshell	1	47.78	38.62	43.62	38.05	1.48	
22 Acclimation	24	22	Eastern creekshell	2	44.55		44.14			
22 Acclimation	24	22	Eastern creekshell	3	23.53		26.40			
22 Acclimation	48	31	Eastern creekshell	1	76.47	72.06	76.40	71.42	0.89	
22 Acclimation	48	31	Eastern creekshell	2	63.38		64.18			
22 Acclimation	48	31	Eastern creekshell	3	76.32		73.68			
27 Acclimation	48	control	Eastern creekshell	1	43.40	57.13	44.23	58.65	2.62	
27 Acclimation	48	control	Eastern creekshell	2	52.38		53.66			
27 Acclimation	48	control	Eastern creekshell	3	75.61		78.05			

Max difference (%) for values > 10 = 10.55

Table 3. Glochidia freshwater mussel ET50s for 24 h and 48 h time points, with 95% confidence intervals and trim level. LL=lower limit, UL=upper limit, ND= ET50 unable to be determined, all ET50s are °C.

17°C Acclimation (24h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	ND			
Pink heelsplitter	ND			
Black sandshell	ND			
Butterfly	ND			
White heelsplitter	ND			
Washboard	ND			
Brook floater	ND			
Eastern creekshell	31.68	27.55	36.43	46.3

17°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	ND			
Pink heelsplitter	22.11	20.21	24.20	2.7
Black sandshell	ND			
Butterfly	ND			
White heelsplitter	ND			
Washboard	26.86	24.64	29.27	3.2
Brook floater	ND			
Eastern creekshell	29.66	27.58	31.89	8.4

22°C Acclimation (24h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	ND			
Pink heelsplitter	29.06	25.55	33.06	16.6
Black sandshell	ND			
Butterfly	33.65	31.17	36.32	14.6
White heelsplitter	35.99	34.28	37.79	26.2
Washboard	32.38	29.58	35.45	1.3
Brook floater	35.80	34.58	37.07	18.3
Eastern creekshell	32.87	29.63	36.47	13.5

22°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	35.51	33.82	37.28	19.9
Pink heelsplitter	ND			
Black sandshell	32.06	19.34	53.14	40.1
Butterfly	29.48	26.81	32.42	6.7
White heelsplitter	35.57	34.92	36.24	8.7
Washboard	28.45	26.53	30.50	2.2
Brook floater	35.47	34.77	36.18	1.0
Eastern creekshell	31.04	28.37	33.97	5.0

27°C Acclimation (24h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	36.92	35.29	38.62	2.0
Pink heelsplitter	ND			
Black sandshell	33.89	30.40	37.79	7.7
Butterfly	30.64	18.48	50.79	48.1
White heelsplitter	37.51	36.94	38.09	2.0
Washboard	32.44	29.23	36.01	24.9
Brook floater	36.85	35.28	38.49	2.6
Eastern creekshell	31.43	27.60	35.79	34.4

27°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	33.97	31.31	36.84	17.0
Pink heelsplitter	ND			
Black sandshell	27.37	15.04	49.80	48.9
Butterfly	ND			
White heelsplitter	36.67	34.82	38.62	0.0
Washboard	ND			
Brook floater	36.10	34.25	38.05	0.0
Eastern creekshell	ND			

Table 4. Juvenile freshwater mussel ET50s for 48 h and 96 h time points, with 95% confidence intervals and trim level. LL=lower limit, UL=upper limit, ND= ET50 unable to be determined, all ET50s are °C.

17°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	ND			
Pink heelsplitter	ND			
Black sandshell	ND			
Butterfly	ND			
Washboard	ND			
Eastern creekshell	ND			

17°C Acclimation (96h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	ND			
Pink heelsplitter	ND			
Black sandshell	ND			
Butterfly	ND			
Washboard	ND			
Eastern creekshell	ND			

22°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	ND			
Pink heelsplitter	35.63	35.01	36.26	9.5
Black sandshell	ND			
Butterfly	ND			
Washboard	34.88	33.50	36.30	0.0
Brook floater	35.22	34.25	36.21	0.0
Eastern creekshell	ND			

22°C Acclimation (96h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	35.54	35.14	35.96	4.8
Pink heelsplitter	34.79	33.12	36.54	0.0
Black sandshell	32.90	29.58	36.59	9.9
Butterfly	ND			
Washboard	34.16	32.26	36.18	0.0
Brook floater	35.05	33.77	36.39	0.0
Eastern creekshell	34.60	32.75	36.54	4.8

27°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	37.03	35.53	38.59	0.0
Pink heelsplitter	34.47	Graphical method		
Black sandshell	37.52	35.75	39.37	4.1
Butterfly	34.67	33.55	35.83	0.0
Washboard	37.14	35.82	38.51	0.0
Brook floater	37.47	Graphical method		
Eastern creekshell	37.41	36.96	37.86	4.0

27°C Acclimation (96h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	34.31	33.50	35.14	0.0
Pink heelsplitter	34.60	33.36	35.90	0.0
Black sandshell	36.74	34.37	39.27	16.0
Butterfly	34.21	33.20	35.25	0.0
Washboard	34.98	33.51	36.52	0.0
Brook floater	35.29	32.79	37.99	4.8
Eastern creekshell	34.72	33.19	36.32	9.7

Table 5. Glochidia freshwater mussel ET05s for 24 h and 48 h time points, with 95% confidence intervals. LL=lower limit, UL=upper limit, ND=unable to be determined, all ET05s are °C.

17°C Acclimation (24h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	ND		
Black sandshell	ND		
Butterfly	ND		
White heelsplitter	ND		
Washboard	ND		
Brook floater	ND		
Eastern creekshell	26.96	11.11	28.97

17°C Acclimation (48h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	20.84	ND	ND
Black sandshell	ND		
Butterfly	ND		
White heelsplitter	ND		
Washboard	ND		
Brook floater	ND		
Eastern creekshell	27.74	ND	ND

22°C Acclimation (24h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	21.69	12.41	24.79
Black sandshell	ND		
Butterfly	30.73	24.76	32.16
White heelsplitter	ND		
Washboard	28.18	19.97	30.38
Brook floater	ND		
Eastern creekshell	26.94	17.73	29.51

22°C Acclimation (48h)	ET05	95% LL	95% UL
Fatmucket	33.02	29.66	34.21
Pink heelsplitter	ND		
Black sandshell	23.83	4.23	28.03
Butterfly	27.62	ND	ND
White heelsplitter	34.46	31.49	35.35
Washboard	26.86	ND	ND
Brook floater	ND		
Eastern creekshell	26.51	20.27	28.48

27°C Acclimation (24h)	ET05	95% LL	95% UL
Fatmucket	35.28	33.99	35.79
Pink heelsplitter	ND		
Black sandshell	28.37	18.96	31.00
Butterfly	17.09	ND	ND
White heelsplitter	ND		
Washboard	27.60	21.07	29.61
Brook floater	35.26	32.94	35.76
Eastern creekshell	21.55	6.71	25.75

27°C Acclimation (48h)	ET05	95% LL	95% UL
Fatmucket	30.31	25.22	31.87
Pink heelsplitter	ND		
Black sandshell	21.40	5.57	24.79
Butterfly	ND		
White heelsplitter	ND		
Washboard	ND		
Brook floater	34.87	ND	ND
Eastern creekshell	ND		

Table 6. Juvenile freshwater mussel ET05s for 48 h and 96 h time points, with 95% confidence intervals. LL=lower limit, UL=upper limit, ND=unable to be determined, all ET05s are °C.

17°C Acclimation (48h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	ND		
Black sandshell	ND		
Butterfly	ND		
Washboard	ND		
Eastern creekshell	ND		

17°C Acclimation (96h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	ND		
Black sandshell	ND		
Butterfly	ND		
Washboard	ND		
Eastern creekshell	ND		

22°C Acclimation (48h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	ND		
Black sandshell	ND		
Butterfly	ND		
Washboard	ND		
Brook floater	ND		
Eastern creekshell	ND		

22°C Acclimation (96h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	ND		
Black sandshell	29.13	23.22	30.92
Butterfly	ND		
Washboard	33.18	ND	ND
Brook floater	ND		
Eastern creekshell	32.52	27.58	33.35

27°C Acclimation (48h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	ND		
Black sandshell	35.45	33.93	36.23
Butterfly	33.52	27.55	34.56
Washboard	ND		
Brook floater	ND		
Eastern creekshell	ND		

27°C Acclimation (96h)	ET05	95% LL	95% UL
Fatmucket	ND		
Pink heelsplitter	33.21	31.61	33.96
Black sandshell	35.37	ND	ND
Butterfly	ND		
Washboard	33.55	ND	ND
Brook floater	32.33	27.17	33.74
Eastern creekshell	32.56	30.57	33.46

Table 7. Juvenile freshwater mussel duplicate heart rate counts (2007/2008). Heart beats assessed by different counters on same mussel within <1 min of each other.

			Original count Heart Beats/15 s						Duplicate count Heart Beat/15 s						Percent difference
Species	**Temp**	**Rep**	**1**	**2**	**3**	**4**	**Mean/Rep**	**Counted by**	**1**	**2**	**3**	**4**	**Mean/Rep**	**Counted by**	**(Mean/rep)**
Black sandshell	20	1	13	23	20	22	19.5	T.P.	13	22	23	21	19.8	S.M.	1.3
		2	19	14	20		17.7		18	13	22		17.7		0.0
		3	19	14	16		16.3		20	15	15		16.7		2.0
Pink heelsplitter	20	1	27	20	18		21.7	T.P.	25	20	17		20.7	S.M.	4.7
		2	25	17	20		20.7		27	15	19		20.3		1.6
		3	16	27	26		23.0		16	26	27		23.0		0.0
Pink heelsplitter	28	1	none found					T.P.	none found					S.M.	N/A
		2	24	16			20.0		24	14			19.0		5.1
		3	none found						none found						N/A
Black sandshell	28	1	13	12			12.5	T.P.	12	11			11.5	S.M.	8.3
		2	none found						none found						N/A
		3	none found						none found						N/A
Washboard	20	1	22	14			18.0	T.P.	20	15			17.5	S.M.	2.8
		2	none found						none found						N/A
		3	none found						none found						N/A

Table 8. Juvenile freshwater mussel ET50s for 48 h and 96 h time points with the addition of 10 µg/L copper, with 95% confidence intervals and trim level. LL=lower limit, UL=upper limit, ND=unable to be determined, all ET50s are °C.

17°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	ND			
Pink heelsplitter	ND			
Black sandshell	ND			

17°C Acclimation (96h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	ND			
Pink heelsplitter	ND			
Black sandshell	ND			

22°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	35.54	35.14	35.95	4.8
Pink heelsplitter	33.63	31.60	35.78	0.0
Black sandshell	35.05	33.73	36.41	0.0

22°C Acclimation (96h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	35.47	Graphical method		
Pink heelsplitter	32.99	30.67	35.47	0.0
Black sandshell	34.73	32.91	36.66	0.0

27°C Acclimation (48h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	36.01	34.32	37.79	0.0
Pink heelsplitter	34.45	34.27	34.64	1.1
Black sandshell	39.23	37.07	41.53	0.1

27°C Acclimation (96h)	ET50	95% LL	95% UL	Trim Level (%)
Fatmucket	34.47	Graphical method		
Pink heelsplitter	34.45	34.25	34.65	1.2
Black sandshell	36.03	34.01	38.17	5.6