Abstract

ARCHAMBAULT, JENNIFER MICHELE. Thermal Sensitivity of Freshwater Mussels: Incorporating Benthic Ecology into Laboratory Mesocosm Experiments. (Under the direction of W. Gregory Cope and Thomas J. Kwak).

The consequences of global climate change on aquatic ecosystems are predicted to result from altered intensity, variability, and distribution of precipitation, and more frequent flooding and droughts. In freshwater systems, these changes may result in degradation or loss of habitat due to dry stream beds or low flows, and increased water temperatures, pollution, and erosion. Freshwater mussels (Order Unionida) are especially vulnerable to disturbance because they are incapable of escaping detrimental changes at any practical temporal scale. Quantitative information on lethal temperatures (LT) to native freshwater mussels is currently limited to fewer than 10 species, and these few studies have been restricted to the water-only standard method for toxicity testing. The results of these prior studies indicate that some species may be living near their upper thermal tolerances; however, evaluation of the thermal sensitivity of these benthic organisms has never been conducted in sediment. Thus, I sought to increase the complexity and ecological realism of laboratory exposures of freshwater mussels to acute thermal stress by including factors that affect mussels in natural systems, including sediment, flow regime, and a vertical thermal gradient. I developed a method for assessing thermal sensitivity of freshwater mussels in sediment, and, using these testing protocols, I evaluated the relative sensitivities of juveniles of four species (*Amblema plicata, Lampsilis abrupta, Lampsilis cariosa,* and *Lampsilis siliquoidea*) and adults of one species (*Lampsilis fasciola*) to a range of temperatures common during summer in streams with low flow and drought conditions, using two temperature acclimation (22 and 27°C) and surrogate flow regimes (low water and dewatered

treatments). I then added a vertical sediment temperature gradient and evaluated the thermal sensitivities of two species (*Lampsilis abrupta* and *Lampsilis radiata*). Endpoints were survival, burrowing behavior, byssus production (in juveniles), and biomarkers of thermal stress (in adults). Acute (96-h) median lethal temperatures (LT50s) ranged from 29.9 to 37.2°C, with a grand mean of 34.8°C, indicating a narrow range of upper thermal sensitivity, regardless of test type, species, life stage, or conservation status. LT50s from sediment tests generally did not differ from water-only tests, suggesting that any stream thermal refuge would emanate from other ecological or physical habitat interactions. Increasing temperature significantly reduced burrowing and byssus production in the species tested. Elevated concentrations of stress biomarkers were detected in some experiments. My findings suggest that rising stream temperatures and altered hydrologic flows from climate change and other anthropogenic factors may directly impact freshwater mussel diversity by causing mortality, and may have indirect sublethal effects.

Thermal Sensitivity of Freshwater Mussels: Incorporating Benthic Ecology into Laboratory Mesocosm Experiments

by
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Dedication

To Dr. Gregory Forbes, my first advisor at Grand Rapids Community College, Grand Rapids, Michigan – the first of several extraordinary advisors that was instrumental in my path to becoming a fisheries and wildlife ecologist. As a professor, he was the first to introduce me to important ecological concepts and to teach me wildlife identification and management skills. As my advisor, he was genuinely interested, and helped to guide my educational decisions toward the career I desired, even without my knowing all the right questions to ask. Perhaps most importantly, he teaches with contagious enthusiasm and humor, vital tools for keeping the attention of, and creating memories for, sleepy college students – for I will never forget the day of the dogfish shark dissection, when Dr. Forbes screamed as he pulled a Barbie doll's leg from the "man-eater's" stomach!

Biography

Before joining North Carolina State University as a Graduate Research Assistant in 2010, I worked as a Wildlife Biologist and Project Manager at an environmental consulting firm in the Sandhills Region of North Carolina. I specialized in liaising with clients and regulatory agencies for development projects; floral and faunal surveys for endangered and threatened species; stream classification; wetland and stream permitting, mitigation monitoring, restoration, and delineation; preparation of reports required by regulatory agencies; GPS and GIS; and monitoring and management of federally-endangered red-cockaded woodpeckers, including banding and demographic monitoring of color-banded birds. Before securing employment as a professional biologist, I earned a B.S. in Fisheries and Wildlife Sciences at North Carolina State University, an A.S. Emphasis in Biological Sciences at Grand Rapids Community College, made a mean Long Island iced tea as I worked through college, and proudly served as one of the fewest of the few in the United States Marine Corps. Semper fidelis.

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Chapter 1. Influence of Sediment Presence on Freshwater Mussel Thermal Tolerance

Abstract

Global climate is warming, and available lethal temperature (LT) data on early life stages of freshwater mussels suggest they may be living near their upper thermal tolerances in some systems. Thus, we expanded mussel LT research to include ecological factors that affect mussels in natural systems, such as sediment and flow regime. We developed a method for assessing thermal sensitivity of juvenile freshwater mussels in sediment, thus incorporating their benthic ecology into tolerance tests. Using these testing protocols, we evaluated the relative sensitivity of four species of mussels (juvenile life stage) to a range of temperatures common during summer in streams with low flow and drought conditions, using two temperature acclimation and exposure regimes. We also conducted water-only LT tests with mussel larvae (glochidia) and juveniles of four previously untested mussel species. The median lethal temperatures (LT50s) for all water-only and sediment tests ranged from 33.3 to 37.2°C, indicating a narrow range of upper thermal sensitivity, regardless of test type, mussel species, life stage, or conservation status. Moreover, LT50s from sediment tests generally did not differ from water-only tests, suggesting that any stream thermal refuge would emanate from other ecological or physical habitat interactions.

Keywords: acute thermal sensitivity, benthic stream ecology, climate change, LT50, sediment, Unionidae

Introduction

In its latest State of the Climate assessment, the National Oceanic and Atmospheric Administration (NOAA) reported that all 11 years in the 21st century (2001 – 2011) rank among the 13 warmest in the history of the instrumental record of global surface temperature (NOAA 2012a). In the United States (US), temperatures were warmer than average in much of the country, and July 2012 was the all-time hottest month ever recorded (NOAA 2012a,b). Hydrology in the US during 2011 was characterized as a year of extremes; areas that were extremely wet combined with other extremely dry areas to account for a record high combination at 58% of the US land area (NOAA 2012a). These climate statistics and many climate records set in 2011 and 2012 exemplify climate trends of warming and extreme weather that have dominated the past few decades. Significant perturbations are expected to continue as a result of global climate change, and include increasing annual mean global temperatures; sea level rise; changes in precipitation trends; reduction of snow cover, mountain glaciers, and polar ice; and increased frequency of severe weather events such as floods, drought, and hurricanes (IPCC 2007a).

The consequences of global climate change to aquatic ecosystems will likely result from altered intensity, variability, and distribution of precipitation, and more frequent flooding and droughts (Bates 2008). In freshwater systems, these changes may result in degradation or loss of habitat due to dry stream beds or excessively low flows, and increased water temperatures, runoff, pollution, sedimentation, and erosion. For instance, an average global temperature increase of just 1°C above 1990 levels could result in a loss of 8% of North American freshwater fish habitat, and an increase of 3°C above 1990 levels could lead

to a 24% loss (IPCC 2007b). Concurrent changes in land use may also have deleterious consequences to aquatic habitats by contributing to additional heated point- and non-point-source effluents (Hester and Doyle 2011), thus exacerbating thermal stress to aquatic organisms. Further, stream temperature in urbanized areas is greatly affected by reduction of riparian vegetation, modified flow regimes, and alteration of stream geomorphology (LeBlanc et al. 1997).

Freshwater mussels are uniquely vulnerable to disturbance in the aquatic environment because they are incapable of escaping detrimental changes at any practical temporal scale. These largely sedentary, benthic invertebrates are already extremely imperiled (Lydeard et al. 2004). Freshwater mussel populations have experienced steep declines worldwide, and in North America, only 70 (24%) of the nearly 300 species are considered stable (Williams et al. 1993). Declining trends have continued, and the faunal status is presumably worse than that reported by Williams et al. (1993) nearly 2 decades ago. Modern causes implicated in the freshwater mussel decline include pollution and water quality degradation, habitat alteration and destruction, or a combination of large-scale, chronic stressors (Strayer et al. 2004, Cope et al. 2008). In a literature review of recent mussel decline (Strayer et al. 2004), most articles attributed population declines to more than one causal factor. When multiple stressors affect a population, determining the cumulative and chronic effects can be problematic and difficult to remedy. Given the multitude of challenges facing freshwater mussel survival, further alteration to aquatic systems associated with climate change (e.g., warmer water, dry or intermittent stream beds, or altered flow regimes) could have

synergistic effects on freshwater mussel decline, leading to further imperilment (Galbraith et al. 2010).

Few investigators have evaluated the effects of temperature stress on native freshwater mussels of the US. Recent research suggests that mussels can be classified into 2 thermal functional guilds – tolerant and sensitive – based on physiological condition and performance at 35°C (Spooner and Vaughn 2008). To date, quantitative information on lethal temperatures to freshwater mussels is limited to fewer than 10 species (Dimock and Wright 1993, Pandolfo et al. 2009, 2010a,b). These few studies have been restricted to the water-only standard method for toxicity testing (ASTM 2006a). Existing research indicates that some species of native freshwater mussels may already be living near their upper thermal tolerance limit, based on water-only temperature exposures (Pandolfo et al. 2010b); however, evaluation of the thermal sensitivity of these benthic organisms has never been conducted in sediment. In the wild, benthic habitats have many components not represented in water-only exposures; two such components are sediment and flow regime. In this study, we sought to (1) increase the complexity and ecological realism of laboratory exposures of freshwater mussels to acute thermal stress by including factors that affect mussels in natural systems, such as sediment and flow regime, and (2) expand the temperature sensitivity database of the early life stages of freshwater mussels by conducting standardized water-only thermal exposures (ASTM 2006a) on previously untested glochidia and juveniles of freshwater mussel species.

Methods

We developed a standardized method for conducting thermal exposures to juvenile freshwater mussels in sediment, with 2 experimental water treatments that served as proxies for different flow regimes. We controlled ambient temperature, allowing evaluation of the presence of sediment on mussel thermal tolerance by directly comparing our results to those from water-only tests that we conducted and from previous research (Pandolfo et al. 2010b). Currently, a standard protocol for conducting toxicity tests with freshwater mussels in sediment does not exist. To allow for comparability across test types and to ensure quality assurance, sediment tests were conducted following the same standards (ASTM 2006a) as water-only exposures to the extent practical.

Test Chambers

We employed a novel dual-chamber static-renewal design for thermal exposure of juvenile mussels in sediment (Figure 1). The dual-chamber design allowed the use of a sufficient water volume and sediment depth to test for sediment effects on thermal sensitivity, while reducing the total amount of sediment to be searched upon test termination for small juvenile mussels, thus allowing for efficient recovery of test organisms. The outer chamber, a 1-L glass beaker, was filled with 400 mL of silica sand to achieve a sediment depth of 5 cm. The inner chamber was constructed of a 5-cm length of 5-cm diameter PVC pipe joined to a 5-cm-by-3.8-cm PVC adapter coupling, with a layer of 400-µm Nitex® mesh fitted between the pipe section and adapter coupling, allowing mussels to burrow to a depth of 2.5 cm. We drilled 18 holes into the PVC chambers and tested them to ensure a lack of thermal insulation.

Two proxies for flow regime were simulated by controlling the amount of reconstituted hard water (ASTM 2006b) added to the test chambers. A low water scenario, intended to simulate low-flow stream conditions (e.g., a reach with a patchy distribution of water or slack-water), included 400 mL of overlying water (Figure 1). The total water volume added to the low water treatments averaged 482 mL (± 15 mL, SD). A dewatered scenario served to simulate extensive drought conditions and included just enough water to wet the sand, and a maximum 50 mL overlying water to mitigate evaporative loss during the exposure (Figure 1). The total water volume added to dewatered treatments averaged 186 mL (± 11 mL). Water-only tests were conducted according to the ASTM guideline for conducting laboratory toxicity tests with freshwater mussels, including no sediment in a 250-mL dish filled with 200 mL of water (ASTM 2006a).

Sediment.— Commercially available, contaminant-free filter sand (Southern Products and Silica Co., Inc. Hoffman, North Carolina) served as the sediment for the exposures. This silica sand is widely used in applications, such as drinking water filtration, meets or exceeds the current American Water Works Association Standard for Filter Material (Southern Products and Silica Co., Inc. 2011), and was suited for use in this application (i.e., the materials did not introduce any confounding influences, e.g., parasites, pathogens, or chemical toxicants).

Test Organisms

We tested 5 species representing 2 tribes in the Unionidae family, including *Amblema plicata* (Say) (Amblemini tribe); and *Lampsilis abrupta* (Say), *L. cariosa* (Say), *L. fasciola* (Rafinesque), and *L. siliquoidea* (Barnes) (all Lampsilini tribe). All juveniles were

propagated via host-fish infection in facilities at the Alabama Aquatic Biodiversity Center (Marion, Alabama), Missouri State University (Springfield, Missouri), or North Carolina State University, College of Veterinary Medicine (Raleigh, North Carolina), using standard propagation and culture methods (Barnhart 2006).

Test species were chosen based on native range, conservation status, and availability. We selected a suite of species native to the southeastern and central US that represented distribution in the Atlantic Slope and Interior basins and that represented a range of conservation statuses, from secure to federally endangered. *Amblema plicata* and *L. siliquoidea* are both widely-distributed species that are ubiquitous to the Interior Basin. *Lampsilis fasciola* is considered globally secure, but it is classified as critically imperiled in Canadian provinces and several states, including Georgia and North Carolina in the southeastern US (NatureServe 2011, NC Wildlife Resources Commission 2011). *Lampsilis cariosa* is vulnerable or imperiled across much of its range and is further listed under some state wildlife protection programs, including North Carolina (state endangered; NC Wildlife Resources Commission 2011). *Lampsilis abrupta* is federally-listed as endangered (US Fish and Wildlife Service 1985).

Test Conditions

We conducted acute (96 h) thermal exposures that were similar in design among test types (low-water sediment, dewatered sediment, and water-only) and consisted of 7 test treatments as follows: a control held at 20°C (ASTM 2006a), an acclimation temperature (22 or 27°C), and 5 treatment temperatures, 4 of which were similar between the 2 acclimation groups (Figure 2). To elicit a likely response from the test organisms, optimal acclimation

and test temperatures were informed by results of Pandolfo et al. (2010b). Test temperatures in the 22°C acclimation exposures ranged from 27 to 37°C, and test temperatures in the 27°C acclimation exposures ranged from 31 to 39°C (Figure 2). Similar temperature treatments between the 2 acclimation regimes facilitated the identification and analysis of any acclimation-related effect.

Juveniles of 4 mussel species were used to test thermal sensitivity. Because of limited availability, *L. fasciola* was omitted from juvenile testing, and *A. plicata* was not tested at the 22°C acclimation temperature in low water or dewatered sediment tests. Because *L. siliquoidea* juvenile acute thermal tolerance was previously evaluated in water-only tests by Pandolfo et al. (2010b), we tested this species only in sediment. Individuals used in sediment tests ranged in age from 3 to 5 months. Average shell lengths were 4.08 mm (± 0.95 mm, SD) for *A. plicata*, 4.93 mm (± 0.85 mm) for *L. abrupta*, 3.09 mm (± 0.78 mm) for *L. cariosa*, and 4.00 mm (± 0.61 mm) for *L. siliquoidea*. Individuals within a species for a given test type differed in age by 1 week at most. Average shell lengths in water-only tests were 1.60 mm (± 0.32 mm) for *A. plicata*, 594 µm (±87 µm) for *L. cariosa*, and 231 µm (±17 µm) for *L. abrupta*. *Amblema plicata* and *L. cariosa* ranged in age from 4 to 6 weeks, and *L. abrupta* were younger than 1 week.

Juveniles were acclimated to the test acclimation temperature by adjusting their shipping temperature upon arrival by 2.5°C/d, with a minimum 24-h acclimation period once the target temperature was attained (ASTM 2006a, Pandolfo et al. 2010b). Shipping temperatures averaged 23°C (±1°C, SD) from May through August, and 18°C (±1°C) from October through February. Experiments were nonaerated static-renewal tests with 90%

reconstituted hard water renewed at 48 h (ASTM 2006a,b). Survival was assessed visually with an Olympus SZ61 microscope (Olympus America, Center Valley, Pennsylvania) to detect foot movement outside of the shell, foot movement inside the shell, or the presence of a heartbeat for the 7 mussels in each of 3 replicates per treatment. Controls included 10 mussels per replicate.

Glochidia of 3 species (*A. plicata*, *L. abrupta*, and *L. fasciola*) were tested. Glochidia were less than 24 h old at the initiation of each test. Glochidia were acclimated by adjusting their shipping temperature on arrival by 1°C/h, with a 2-h acclimation period once the target temperature was reached. Tests were 24-h nonaerated static experiments done in reconstituted hard water in accordance with the ASTM guideline for glochidia (ASTM 2006a,b). Survival was assessed at 24 h for a subsample of approximately 50 of the 150 glochidia in each of 3 replicates per treatment. A saturated NaCl solution was used to stimulate a shell-closure response that was observed with either an Olympus SZ61 microscope and QCapture Pro 5.1 digital photographic software (Quantitative Imaging Corporation, Burnaby, British Columbia, Canada) or a Leica EZ4 D stereo microscope with integral digital camera and Leica Application Suite EZ digital photographic software (Leica Microsystems, Ltd., Switzerland).

Quality assurance and control were ensured by conducting all tests according to the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM 2006a). Tests were conducted in light- and temperature-controlled environmental chambers (Precision Model 818, Thermo Fisher Scientific, Marietta, Ohio, and Isotemp Model 146E, Fisher Scientific, Dubuque, Iowa). Thermometers used for daily temperature monitoring

were certified for accuracy by the National Institute of Standards and Technology (NIST). Target test temperatures were ± 1°C (n = 1,206) for 97.1% of trials and ± 2°C for 99.4% of trials, with a maximum departure of 3.5°C. Sediment temperatures were monitored with partial-immersion thermometers (Fisherbrand® Red-Spirit®, Fisher Scientific, Pittsburgh, Pennsylvania) that met NIST tolerances for accuracy. Mean sediment temperatures differed from target incubator temperatures by ≤ 1.1°C in low water exposures (n = 394) and by ≤ 0.9°C in dewatered exposures (n = 392). Mean water quality conditions among all tests were 108.2 mg $CaCO_3$/L alkalinity, 143.3 mg $CaCO_3$/L hardness, 534.9 µS/cm conductivity, 8.13 pH, and 7.52 mg/L dissolved oxygen (n = 21 for alkalinity and hardness, n = 167 for all other variables).

Statistical Analysis

The effects of temperature treatments on mussels were analyzed with Comprehensive Environmental Toxicity Information Software (CETIS)™ (v1.8.0.12, Tidepool Scientific, LLC, McKinleyville, California). The median lethal temperature (LT50) was defined as the temperature that caused mortality in 50% of the individuals in the exposed sample, and the LT05 was the temperature that caused mortality in 5% of those in the sample. Survival data were used to generate LT50s and LT05s with the Trimmed Spearman-Karber method. LT50s and their 95% confidence intervals (CI) were compared between acclimation exposures, test types (water-only vs. low water, water-only vs. dewatered, and low water vs. dewatered), species, and life stages for a given species, to detect significant differences. LT50 values were considered statistically different when the 95% CIs did not overlap.

Results

Tests were conducted on 5 species and 2 life stages (glochidia of 3 species and juveniles of 4 species; Tables 1 – 4). Fourteen sediment tests on juveniles of 4 species were conducted; sediment tests at the 22°C acclimation temperature were not possible for *A. plicata* due to limited availability (Tables 1 and 2). Six water-only tests on glochidia of 3 species and 6 water-only tests on juveniles of 3 species were completed; *A. plicata* glochidia and *L. fasciola* juveniles were unavailable (Tables 3 and 4). LT50s for glochidia (24 h) and juveniles (96 h) for all exposures within 22°C and 27°C acclimation groups ranged from 33.3 to 37.2°C, with a grand mean of 35.6°C (Tables 1 and 3), and LT05s ranged from 22.2 to 36.7°C, with a grand mean of 31.1°C (Tables 2 and 4). In general, results were similar between acclimation groups, between low water and dewatered sediment tests, and among juvenile water-only and sediment tests (Tables 1 – 4); results were mixed in comparisons between life stages (i.e., glochidia and juvenile water-only tests; Tables 3 and 4).

Sediment Exposures

We achieved 99.3% (n = 4,205) total recovery of juvenile mussels from the sediment chambers among all sediment tests. Recovery in 11 of 14 sediment tests was ≥ 99%, with a minimum recovery rate of 96.8% for any 1 test. Recovery was 100% in 7 tests.

LT50s for sediment tests ranged from 33.3 to 37.2°C, with a mean of 35.9°C for the low water treatment, 35.2°C for the dewatered treatment, and an overall mean of 35.6°C. Results were similar among most tests. No significant differences were detected among species within a given acclimation temperature and proxy flow regime treatment combination (e.g., 22°C acclimation, low water test). Within a species, no significant effects of

acclimation temperature or flow regime treatment were detected on *L. abrupta* or *L. cariosa.* In the 27°C acclimation temperature, *A. plicata* exhibited significantly greater thermal sensitivity in the dewatered treatment compared to the low water treatment. Within the low water treatment, *L. siliquoidea* exhibited greater thermal sensitivity at the 22°C acclimation temperature compared to the 27°C acclimation temperature (Table 1).

Overall, LT05s in sediment tests ranged from 26.7 to 34.8°C, with a mean of 31.1°C. Low water flow regime treatments yielded LT05s ranging from 28.7 to 34.3°C, with a mean of 31.4°C, and dewatered flow regime treatments yielded LT05s ranging from 26.7 to 34.8°C, with a mean of 30.9°C (Table 2). Of the 14 sediment tests, LT05s could not be determined from survival data in 6 cases, due lack of partial mortality responses, and 95% CIs could not be estimated in 2 cases. No significant differences were detected among all tests within or between species for a given acclimation temperature and flow regime (Table 2).

Water-Only Exposures

LT50s for glochidia and juvenile water-only exposures ranged from 33.3 to 37.2°C, with a mean of 35.7°C. Juvenile LT50s ranged from 34.8 to 36.8°C, with a mean of 35.8°C. No differences in thermal sensitivity were associated with acclimation temperature for a given species, and only one difference was detected in comparisons among species. At the 27°C acclimation temperature, *L. abrupta* was significantly more thermally sensitive than *A. plicata* (Table 3).

Glochidial LT50s ranged from 33.3 to 37.2°C, with a mean of 35.6°C. Significant differences in thermal sensitivity associated with acclimation temperature were detected for

L. cariosa and *L. fasciola.* Both species were more thermally sensitive at the 27°C acclimation temperature than at the 22°C acclimation (Table 3). Comparisons among species revealed differences at the 27°C acclimation temperature. *Lampsilis fasciola* was more thermally sensitive than *L. abrupta*, and *L. cariosa* was more thermally sensitive than both *L. fasciola* and *L. abrupta.* No differences in thermal sensitivity between species were detected at the 22°C acclimation temperature (Table 3). Comparisons among life stages within species indicate that *L. abrupta* glochidia exposed to the 27°C acclimation temperature were more thermally tolerant than juvenile conspecifics exposed in water-only at either acclimation temperature, and *L. cariosa* glochidia exposed to the 27°C acclimation temperature were more thermally sensitive than conspecific glochidia exposed at the 22°C acclimation temperature and juveniles exposed at either acclimation (Table 3).

Overall, LT05s in water-only exposures ranged from 22.2 to 36.7°C, with a mean of 31.2°C. Juvenile LT05s ranged from 22.2 to 35.0°C, with a mean of 30.5°C. Of the 6 juvenile water-only tests conducted, LT05s could not be determined from survival data in 3 cases due to lack of partial mortality responses, and 95% CIs could not be estimated for the other 3 tests (Table 4). Therefore, comparisons within or among species were not possible.

Glochidial LT05s ranged from 23.8 to 36.7°C, with a mean of 31.7°C. Of the 6 glochidia tests conducted, LT05s could not be determined from survival data in 2 cases due to lack of partial mortality, and 95% confidence intervals could not be estimated in 2 cases. *Lampsilis cariosa* glochidial LT05s did not differ between acclimation temperatures (Table 4).

Discussion

To our knowledge, this is the first report of the acute lethal thermal sensitivities for juvenile freshwater mussels held in sediment in laboratory tests. Our standard water-only tests served both as a comparison to the sediment exposure results and to expand the knowledge-base on temperature sensitivity for the early life stages of freshwater mussels. Overall, we found that LT50s generated in sediment exposures conducted in controlled-temperature incubators were similar to those generated from water-only tests. In a comparison of LT50s and their 95% CIs, those generated in both the low water and dewatered sediment treatments for a given species were not significantly different from water-only test results, except in one case. At the 27°C acclimation temperature, *L. abrupta* juveniles were more thermally tolerant in the low water sediment treatment than in water-only (Tables 1 and 3). In a comparison with results from Pandolfo et al. (2010b), the LT50s they reported in *L. siliquoidea* juvenile water-only tests were not significantly different from our LT50s in the low water or dewatered sediment treatments. Further, in the only previous studies on acute lethal temperatures for early life stages of freshwater mussels, Pandolfo et al. (2010b) reported 35.8°C (32.5 – 38.8°C) as the mean and range of LT50s for juveniles, and Dimock and Wright (1993) reported LT50s for juvenile *Utterbackia imbecillis* (Say) and *Pyganodon cataracta* (Say) as 31.5 and 33.0°C, respectively. The results of those studies are similar to the mean (35.6°C) and range (33.3 – 37.2°C) of LT50s for all juvenile experiments in our study, both in sediment and water-only exposures.

Effects of acclimation temperature were largely absent, but results for a given species and test type differed between acclimation temperatures in 3 cases. *Lampsilis siliquoidea*

was more thermally sensitive in the low water sediment treatment at the 22°C acclimation temperature, whereas glochidia of both *L. cariosa* and *L. fasciola* were more thermally sensitive at the 27°C acclimation temperature. No consistent pattern of acclimation effect was observed. While our acclimation duration was longer and the rate of change was more conservative (i.e., slower) than the 3°C/h recommended by the ASTM (2006a) guidelines, it still may have been too brief to allow the mussels to establish true acclimation or, conversely, may be unimportant in acute exposures, especially when the two acclimation temperatures are relatively high and proximate in range (22°C and 27°C). In a review of temperature tolerance for 50 aquatic organisms, including 16 mollusks, deVries et al. (1998) reported that acclimation periods were typically longer than 96 h, but Hicks and McMahon (2002) reported that acclimation temperature did not affect the upper lethal thermal limits in acute exposures with the marine brown mussel (*Perna perna*). It may be impractical to hold early life stages of mussels for longer periods while following the ASTM (2006a) guidelines for acute tests (e.g., no feeding of test organisms); however, recent thermal research with adult mussels (not following the ASTM mussel early life stage acute guidelines) detected differences in temperature sensitivity between divergent cool and warm (15°C and 25°C) acclimation temperatures (Galbraith et al. 2012) when mussels were fed and held for 7 d prior to testing. Similar future research with lethal temperatures and juvenile freshwater mussels may benefit from a longer acclimation period to determine any pattern of effect.

Guidelines for conducting chronic exposures with the early life stages of freshwater mussels are needed, but despite their absence, researchers are advancing knowledge of the effects of thermal exposures of longer duration on mussels. For example, Ganser (2012)

determined LT50s of 4 species of 2-month-old juvenile freshwater mussels with test lengths of 7 to 28 d. The LT50 values after 7 d for *L. abrupta* [mean of 33.6°C, (95% CI 32.5 – 34.6°C)] and *L. siliquoidea* [mean of 32.5°C, (95% CI 31.5 – 33.5°C)] are substantially less than results from our 96-h tests with sediment on those species (Table 1), and the LT50 values after 28 d are significantly less, based on comparisons of 95% CIs [mean 27.2°C, (95% CI 26.3 – 28.2°C) and 25.3°C, (95% CI 24.1 – 26.7°C), respectively] (Ganser 2012). These findings substantiate the potential increase in thermal sensitivity of freshwater mussels with longer duration exposures and demonstrate the need for better understanding of long-term thermal exposures. The 7-d LT50s determined by Ganser (2012) are qualitatively similar to our 96-h LT05s, and the 28-d LT50s are lower than our LT05s for both species, anecdotally suggesting that prediction of chronic median lethal temperatures may be possible with acute test results, but further investigation is needed to develop that relationship. Nonlethal chronic effects of elevated temperatures have rarely been studied directly; however, in a study of the stream ecosystem roles of 8 species of unionid mussels, Spooner and Vaughn (2008) showed thermally sensitive species had decreased clearance and oxygen consumption rates and increased catabolism at 35°C, compared to 25°C, whereas thermally tolerant species had highest respiration, oxygen consumption and anabolism at 35°C. Their research indicates that thermally sensitive species may be negatively affected by warming summer trends, via less resource acquisition and assimilation, and potential degradation of body condition due to increased catabolism, which in turn may alter ecosystem services provided and have an overall impact on stream ecosystem integrity. Further research on

nonlethal chronic effects of temperature on additional freshwater mussel species would help elucidate impacts on mussel populations and, in turn, subsequent impacts on stream ecology.

Our results are relevant in the context of global change, because surface water temperatures are regularly impacted by anthropogenic activities (LeBlanc et al. 1997, Hester and Doyle 2011), and because climate change is expected to alter stream temperatures as a result of increased air temperatures and changes in patterns of precipitation [i.e., greater frequency of extreme rainfall events (e.g., hurricanes), and prolonged weather patterns (e.g., droughts)] (Bates 2008). A recent severe drought in 2000 caused mussel density to decline as much as 83% in some southeastern US streams, and led to a reduction in species richness in these systems, primarily through the loss of rare species (Haag and Warren 2008). Some unionids may be living near their upper thermal tolerance limits (Pandolfo et al. 2010b); Hester and Doyle (2011) suggested that that most organisms are more sensitive to increases in water temperature than to decreases, and that human impacts tend to increase the temperature of surface waters more often than decrease it. Many anthropogenic activities affecting river temperatures could have adverse population-level effects, because the resulting temperature increases approach or exceed limits that cause a 50% reduction in organismal performance (e.g., growth, reproduction, or survival) (Hester and Doyle 2011). Further, fish may be more sensitive than invertebrates, suggesting that adverse consequences to obligate mussel/host-fish relationships could result (e.g., Pandolfo et al. 2012). While the Hester and Doyle (2011) review did not include mollusks due to lack of species performance curve data in the literature, Burlakova et al. (2011) suggested that climate, land use, and human population density influence freshwater mussel species diversity, and specifically,

that human population density was negatively correlated with species diversity and the proportion of rare species. They also concluded that the most important environmental factors influencing freshwater mussel diversity included climatic parameters (i.e., precipitation and evaporation) and hydraulic variables (i.e., river relief and discharge; Burlakova et al. 2011) – proximate factors that are affected by climate change.

Conclusions

We developed and applied a new method for conducting thermal toxicity tests in sediment with juvenile freshwater mussels. Construction of the treatment chambers was simple and low-cost, contaminant-free substrate was commercially available and inexpensive, and we achieved nearly 100% recovery of test organisms. Overall, this sediment testing method was simple, efficient, and reproducible. Our findings may assist the ASTM or others in development of guidelines amended for toxicity testing with freshwater mussels that include our sediment testing methods and those recently employed by other researchers (Newton and Bartsch 2007, Maio et al. 2010).

We expanded the acute temperature sensitivity database for the early life stages of North American freshwater mussels to include 5 additional species of the Interior and Atlantic Slope Basins, representing a range of conservation statuses. These data not only increase the understanding of the effects of thermal stress on freshwater mussels, but begin to elucidate patterns among taxa and life stages and can be used in other applications to predict the response of mussels to changes in temperature, as related to flow and climate change scenarios. These data may also be directly relevant to the establishment of modern thermal

water quality criteria that would be protective of mussels. Water quality criteria for temperature are currently species-specific, based solely on fish species (US Environmental Protection Agency 1986), and – at more than 25 years old – would benefit from review and augmentation with recent findings.

Water-only tests appear to reflect the acute thermal tolerance of juvenile mussels in sediment exposures when performed in temperature-controlled incubators; however, because the lethal temperatures that we observed in the laboratory are regularly exceeded in surface waters of the southeastern and central US, we suggest that more complex interactions are involved in determining thermal sensitivity in natural systems and, ultimately, in mitigating survival during periods of excessive heat. For example, the maximum temperatures occurring at 5 and 15 cm below the sediment/water interface in streams in the North Carolina Piedmont were an average of 1.9 and 2.9°C cooler, respectively, than the surface water temperature from July to October 2011 (T. J. Pandolfo, North Carolina State University, personal communication). Freshwater mussels regularly burrow to 10 cm, and as deep as 20 cm depth (Schwalb and Pusch 2007), suggesting that they may experience thermal buffering when burrowed in stream sediments. Physiological factors that may influence thermal sensitivity of freshwater mussels, such as induced thermotolerance (Jackson et al. 2011) and interactive effects of temperature with dissolved oxygen concentration, which have been shown to affect other bivalves (Polhill and Dimock 1996, Pörtner et al. 2006, 2007, Peck et al. 2007), also remain poorly understood. Topics warranting future research on the lethal thermal sensitivities of freshwater mussels include studies of physiological parameters that may alter thermal tolerance, and additional ecological and physical habitat variables, such as

a vertical temperature gradient often present in stream substrates and pore water, daily temperature flux of surface waters, chronic exposures to thermal stress, and thermal stress coupled with other stressors, such as aquatic contaminants.

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Table 1. Median lethal temperatures (LT50) causing 50% mortality (with 95% confidence intervals) in juvenile mussels at 22°C and 27°C acclimation temperatures in low water and dewatered sediment exposures (96 h). LT50 values with the same letter for a given species are not significantly different. LT50 values between species for a given acclimation temperature and proxy flow regime treatment did not differ in any case. ND = value could not be determined, * = no test run for *Amblema plicata* juveniles at the 22°C acclimation temperature.

	22°C Acclimation LT50		27°C Acclimation LT50	
Species	Low Water	Dewatered	Low Water	Dewatered
Amblema plicata	*	*	37.2 A (36.7 – 37.7)	35.3 B (34.6 – 36.1)
Lampsilis abrupta	ND	35.1 A (34.4 – 35.9)	36.5 A (35.8 – 37.2)	35.0 A (34.2 – 35.9)
Lampsilis cariosa	ND	35.1 A (33.8- 36.3)	36.5 A (36.0 – 36.9)	35.5 A (34.8 – 36.2)
Lampsilis siliquoidea	33.3 A (32.4 – 34.2)	35.4 AB (34.1 – 36.7)	36.0 B (35.4 – 36.5)	35.3 B (34.9 – 35.7)

Table 2. Protection-level lethal temperatures (LT05) causing 5% mortality (with 95% confidence intervals) in juvenile mussels at 22°C and 27°C acclimation temperatures in low water and dewatered sediment exposures (96 h). LT05 values with the same letter for a given species are not significantly different. LT05 values between species for a given acclimation temperature and proxy flow regime treatment did not differ in any case that could be compared. ND = value could not be determined, * = no test run for *Amblema plicata* juveniles at the 22°C acclimation temperature.

	22°C Acclimation LT05		27°C Acclimation LT05	
Species	Low Water	Dewatered	Low Water	Dewatered
Amblema plicata	*	*	ND	34.8 (30.1 – 35.6)
Lampsilis abrupta	34.3 (ND)	30.0 (ND)	31.2 A (6.4 – 33.9)	30.9 A (0.2 – 33.7)
Lampsilis cariosa	ND	26.7 (0.2- 30.4)	ND	ND
Lampsilis siliquoidea	28.7 A (17.0 – 31.1)	32.2 A (23.9 – 33.8)	ND	ND

Table 3. Median lethal temperatures (LT50) causing 50% mortality (with 95% confidence intervals) in glochidia (24-h exposure) and juvenile mussels (96-h exposure) at 22°C and 27°C acclimation temperatures in water-only exposures. LT50 values with the same letter for a given species are not significantly different. LT50 values between species for a given life stage and acclimation temperature with the same symbols (#, &, ^) are not significantly different. ND = value could not be determined, * = no test run for *Amblema plicata* glochidia or *Lampsilis fasciola* juveniles.

	22°C Acclimation LT50		27°C Acclimation LT50	
Species	Glochidia	Juvenile	Glochidia	Juvenile
Amblema plicata	*	36.4 A# (35.9 – 36.9)	*	36.4 A# (35.7 – 37.1)
Lampsilis abrupta	ND	34.8 A# (33.6 – 36.0)	37.2 B# (37.0 – 37.4)	34.9 A§ (34.3 – 35.5)
Lampsilis cariosa	35.8 A# (35.3 – 36.2)	36.8 A# (35.6- 37.9)	33.3 B§ (32.7 – 33.8)	35.5 A#§ (35.0 – 36.0)
Lampsilis fasciola	36.3 A# (36.2 – 36.4)	*	35.5 A^ (35.3 – 35.7)	*

Table 4. Protection-level lethal temperatures (LT05) causing 5% mortality (with 95% confidence intervals) in glochidia (24-h exposure) and juvenile mussels (96-h exposure) at 22°C and 27°C acclimation temperatures in water-only exposures. LT05 values with the same letter for a given species are not significantly different. Comparisons between species were not made. Few comparisons were made due to lack of generated LT05s from the survival data. ND = value could not be determined, * = no test run for *Amblema plicata* glochidia or *Lampsilis fasciola* juveniles.

	22°C Acclimation LT05		27°C Acclimation LT05	
Species	Glochidia	Juvenile	Glochidia	Juvenile
Amblema plicata	*	ND	*	ND
Lampsilis abrupta	ND	34.6 (ND)	36.7 (ND)	34.7 (ND)
Lampsilis cariosa	31.1 A (25.1 – 32.8)	22.2 (ND)	23.8 A (13.4 – 27.8)	ND
Lampsilis fasciola	ND	*	35.0 (ND)	*

Figure 1. The dual-chamber design used thermal exposures of juvenile freshwater mussels in sediment consisted of a PVC chamber inside a 1-L beaker (left – low water treatment; right – dewatered treatment).

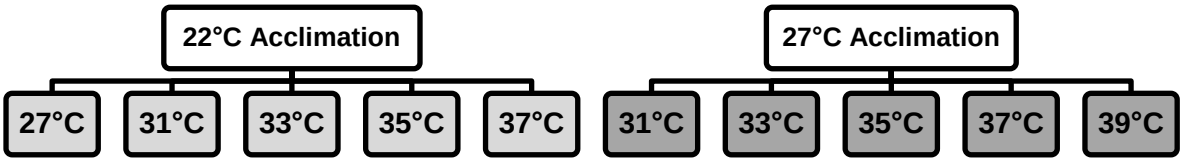

Figure 2. Schematic diagram of experimental design showing acclimation temperatures (22 and 27°C) and experimental temperature treatments for all glochidia and juvenile mussel exposures. All experiments employed a control temperature of 20°C.

Chapter 2. Burrowing, byssus, and biomarkers: Behavioral and physiological indicators of sublethal thermal stress in freshwater mussels

Abstract

Recent research has elucidated the acute lethal effects of elevated water temperatures to glochidia (larvae), juvenile, and adult life stages of freshwater mussels (Order Unionida), but few studies have focused on sublethal effects of thermal stress. We evaluated the sublethal effects of elevated temperatures on burrowing behavior and byssus production in juveniles, and on enzymatic biomarkers of stress in adults in acute (96 h) laboratory experiments in sediment, with two acclimation temperatures and two experimental water levels as proxies for flow regime. Increasing temperature significantly reduced burrowing in all 5 species tested, and the dewatered treatment (a proxy for drought conditions) reduced burrowing in all but *Amblema plicata*. Production of byssal threads was affected most drastically by flow regime, with the probability of byssus presence reduced by 93 – 99% in the dewatered treatment, compared to the low water treatment (a proxy for low flow conditions); increasing temperature reduced byssus production by 18 – 35%. Alanine aminotransferase and aspartate aminotransferase, biomarkers of tissue damage , were significantly affected by treatment temperature in the 27°C acclimation, low water test (p = 0.04 and 0.02, respectively). Our results are important in the context of climate change, because stream temperature and flow are expected to change with increasing air temperature and altered precipitation patterns.

Key words: climate change; benthic; stream flow; Unionidae; LT50; endangered species

Introduction

Aquatic fauna and flora are potentially affected by a myriad of stressors in the aquatic environment. Freshwater mussels belonging to the Order Unionida are especially vulnerable to disturbance because they are incapable of escaping detrimental changes at any practical temporal scale. In North America, where approximately half of the worldwide unionid diversity exists, 213 (71.7%) of the nearly 300 species are endangered, threatened, or of special concern (Williams et al. 1993). Declining trends have continued, and the faunal status has presumably worsened since the Williams et al. (1993) assessment nearly two decades ago. Increasing temperature and altered hydrology (i.e., precipitation and discharge; Burlakova et al. 2011) due to rapid climate change may exacerbate current trends in species decline or may be a factor in future decline of both common and imperiled species.

The consequences of global climate change on aquatic ecosystems will likely result from altered intensity, variability, and distribution of precipitation, and more frequent flooding and droughts (Bates 2008). Hydrology in the United States (US) during 2011 was characterized by the National Oceanic and Atmospheric Administration (NOAA) as a year of extremes — areas that were extremely wet combined with other extremely dry areas that accounted for a record high combination of 58% of the US land area affected (NOAA 2012a). The agency also reported that all 11 years in the 21st century (2001 – 2011) ranked among the 13 warmest in the history of the instrumental record of global surface temperature, and July 2012 was ranked as the warmest month ever recorded in US history (NOAA 2012a,

2012b). These climate statistics and other climate records broken elsewhere in recent years exemplify climate trends of warming and extreme weather. Concurrent changes in land use may also have deleterious consequences to aquatic habitats by contributing to additional heated point- and non-point-source effluents (Hester and Doyle 2011), thus exacerbating thermal stress to aquatic organisms. Further, stream temperature in urbanized areas is greatly affected by reduction of riparian vegetation, modified flow regimes, and alteration of stream geomorphology (LeBlanc et al. 1997).

Though thermal inputs to aquatic systems are common, and despite extensive thermal research associated with fisheries and fish populations, research on thermal stress to freshwater mussels has gained momentum only in the past decade. Recent research has revealed the acute lethal effects of elevated water temperature to glochidia (larvae), juvenile, and adult life stages of freshwater mussels (Dimock and Wright 1993; Pandolfo et al. 2010; Archambault 2012), but only a few studies have focused on sublethal effects of thermal stress [e.g., Pandolfo et al. 2009 (heart rate); Galbraith et al. 2012 (gaping)].

Mussel Burrowing

Research on freshwater mussel burrowing has centered mainly on the ecology of burrowing, such as horizontal and vertical movements (Yeager et al. 1994, Schwalb and Pusch 2007; Allen and Vaughn 2009; Negishi et al. 2011), effects of particle size on burrowing (Lewis and Riebel 1984; Troia and Ford 2010), and ecosystem services provided by bioturbation (Vaughn and Hakenkamp 2001). Most burrowing studies have concentrated on adult freshwater mussels (but see Yeager et al. 1994; Rogers 1999, Schwalb and Pusch 2007; Negishi et al. 2011), and few have addressed the effects of stressors on burrowing

(e.g., Nichols and Wilcox 1997). Waller et al. (1999) considered the effects of common stream temperatures on righting and burrowing behaviors, but to our knowledge, few studies have quantified the effects of extreme temperatures on burrowing behavior (e.g., Bartsch et al. 2000), and no one has done so with juvenile mussels.

Byssus Production

Research on stressors to byssus production has concentrated primarily on efforts to control the nonnative zebra mussel (*Dreissena polymorpha*) (Clark and McMahon 1996; Cope et al. 1997). Native freshwater mussels apparently use byssus chiefly for attachment to the substrate and for drift, typically as juveniles (Bradley 2011). Although some investigators have attempted to elucidate the significance of byssus, few have explored potential stressors to byssus production. Clark and McMahon (1996) found that invasive zebra mussels produced more byssus at higher temperatures, with the greatest rate at 30°C, which is, interestingly, near their upper lethal limit. We investigated the effects of thermal stress on byssus production in juvenile native unionid freshwater mussels.

Biomarkers

While the literature provides extensive examples of non-lethal techniques for assessing stress in marine and freshwater bivalves, the vast majority of studies have focused on stress due to chemical contaminants (Gagne and Blaise 2003; Boutet et al. 2005) or other stress events such as hypoxia (Lee et al. 2008), starvation (Patterson et al. 1999), and relocation (Naimo and Monroe 1999). Few others have attempted to use biomarkers as a non-lethal means of evaluating thermal stress in freshwater bivalves (e.g., Greseth et al. 2003), but there have been advances in this area for marine mollusks (Corporeau and Auffret

2003; Liu et al. 2004; Chen et al. 2007; An and Choi 2010). Researchers at the University of Georgia (USA) have recently examined the use of biomarkers to evaluate stress related to climate change (i.e., high temperatures, low flow) in freshwater bivalves (2012 email from A. Fritts to JMA, unreferenced, see 'Acknowledgements'). The objectives of our study were to assess the sublethal effects of elevated temperatures on burrowing behavior and byssus production in juvenile freshwater mussels, and on enzymatic biomarkers of stress in adult mussels.

Methods

We developed a standardized method for conducting thermal exposures to freshwater mussels in sediment, with two acclimation temperatures (22 and 27°C), five temperature treatments, and two experimental water treatments (low water and dewatered) that served as surrogates for different stream flow regimes. Though a standard protocol for conducting toxicity tests with freshwater mussels in sediment does not currently exist, exposures in sediment were conducted following the same standards (ASTM 2006a) as for water-only exposures, to the extent practical, to ensure data quality and comparability. We evaluated the sublethal effects of temperature, proxy flow regime, and acclimation on the endpoints of burrowing behavior, byssus production, and enzyme biomarker levels.

Test Chambers

We employed a novel dual-chamber static-renewal design for thermal exposure of juvenile mussels in sediment. The dual-chamber design allowed the use of a sufficient water volume and sediment depth to test for sediment effects on thermal sensitivity, while reducing

the total amount of sediment to be searched upon test termination for small juvenile mussels, thus allowing for efficient recovery of test organisms. The outer chamber, a 1-L glass beaker, was filled with 400 mL of silica sand to achieve a sediment depth of 5 cm. The inner chamber was constructed of a 5-cm length of 5-cm diameter PVC pipe joined to a 5-cm-by-3.8-cm PVC adapter coupling, with a layer of 400-µm Nitex® mesh fitted between the pipe section and adapter coupling, allowing mussels to burrow to a depth of 2.5 cm. Eighteen holes (0.6-cm diameter) were drilled into the inner PVC chambers to ensure uniformity of temperature between the outer and inner chambers.

Two proxy flow regimes (hereafter called flow regime) were simulated by controlling the amount of reconstituted hard water (ASTM 2006b) added to the test chambers. A low water treatment, intended to simulate low-flow stream conditions (e.g., a reach with a patchy distribution of water or slack-water), included 400 mL of overlying water. The total water volume added to the low water treatments averaged 508 (± 38, SD) mL. A dewatered treatment served to simulate extensive drought conditions and included just enough water to wet the sand, and a maximum of 50 mL overlying water to mitigate evaporative loss during the experiment. The total water volume added to dewatered treatments averaged 200 (± 20) mL.

Sediment.— Commercially available, contaminant-free filter sand (Southern Products and Silica Co., Inc. Hoffman, North Carolina) served as the substrate for the experiments. This silica sand is widely used in applications, such as drinking water filtration, meets or exceeds the current American Water Works Association Standard for Filter Material (Southern Products 2011), and was suited for use in this application (i.e., the materials did

not introduce any confounding influences of organic matter, parasites, pathogens, or chemical toxicants). Before use, the sand was dry sieved to a more uniform size range of 500 – 850 µm and heated to 200°C in a drying oven to ensure the lack of organisms and low starting moisture content.

Test Organisms

We tested five species of mussels representing two tribes in the Unionidae family, including *Amblema plicata* (Say) (Amblemini tribe); and *Lampsilis abrupta* (Say), *Lampsilis cariosa* (Say), *Lampsilis fasciola* (Rafinesque), and *Lampsilis siliquoidea* (Barnes) (all Lampsilini tribe). All juveniles were propagated via host-fish infection in facilities at the Alabama Aquatic Biodiversity Center (Marion, Alabama), Missouri State University (Springfield, Missouri), or North Carolina State University, College of Veterinary Medicine (Raleigh, North Carolina) using standard propagation and culture methods (Barnhart 2006).

Test species were chosen based on native range, conservation status, and availability. We selected a suite of species native to the southeastern and central US that represented distribution in the Atlantic Slope and Interior Basin and that represented a range of conservation statuses, from secure to federally endangered. *Amblema plicata* and *L. siliquoidea* are both widely-distributed species that are ubiquitous to the Interior Basin. *Lampsilis fasciola* is considered globally secure, but it is classified as critically imperiled in Canadian provinces and several states, including Georgia and North Carolina in the southeastern US (NatureServe 2011, North Carolina Wildlife Resources Commission 2011). *Lampsilis cariosa* is vulnerable or imperiled across much of its range and is further listed under some state wildlife protection programs, including North Carolina (state endangered;

North Carolina Wildlife Resources Commission 2011). *Lampsilis abrupta* is federally-listed as endangered (US Fish and Wildlife Service 1985).

Test Conditions

We conducted acute (96 h) thermal exposures in the low water and dewatered sediment treatments that consisted of seven temperature treatments as follows: a control held at 20°C (ASTM 2006a), an acclimation temperature (22 or 27°C), and five experimental temperatures, four of which were similar between the two acclimation groups (Figure 1). To elicit a likely response from the test organisms, optimal acclimation and test temperatures were informed by results of Pandolfo et al. (2010). Test temperatures in the 22°C acclimation exposures ranged from 27 to 37°C, and test temperatures in the 27°C acclimation exposures ranged from 31 to 39°C (Figure 1). Similar temperature treatments between the two acclimation regimes facilitated the identification and analysis of any acclimation-related effects.

Juveniles of four mussel species were used to test thermal sensitivity. Because of limited availability, *L. fasciola* was omitted from juvenile testing, and *A. plicata* was not tested at the 22°C acclimation temperature in low water or dewatered sediment tests. Mussels used in the juvenile tests ranged in age from three to five months. Average shell lengths were 4.08 mm (± 0.95 mm, SD) for *A. plicata*, 4.93 mm (± 0.85 mm) for *L. abrupta*, 3.09 mm (± 0.78 mm) for *L. cariosa*, and 4.00 mm (± 0.61 mm) for *L. siliquoidea*. Mussels within a species for a given test type differed in age by one week at most.

Juveniles were acclimated to the test acclimation temperature by adjusting their shipping temperature upon arrival by 2.5°C/d, with a minimum 24-h acclimation period once

the target temperature was attained (ASTM 2006a, Pandolfo et al. 2010). Shipping temperatures averaged 23°C (±1°C, SD) from May through August, and 18°C (±1°C) from October through February. Experiments were nonaerated static-renewal tests with reconstituted hard water renewed (90% volume) at 48 h (ASTM 2006a, 2006b). Seven mussels were in each of three replicates per treatment and 10 mussels per replicate in controls.

Adult *L. fasciola* used in the biomarker studies were 22-23 months old and reproductively mature. They were acclimated from their ambient temperature in laboratory holding tanks to the test acclimation temperature following the same procedure as for juveniles. Ambient temperatures averaged 21.4°C (±2°C). Acute (96 h) experiments with these mussels followed the same procedure as those with juveniles. Survival of adults was assessed visually by checking for foot retraction or valve closure in response to a blunt probe in mussels with open shells, and by checking for resistance to opening in mussels with closed shells. In these experiments, three mussels were in each of four replicates per treatment.

Quality assurance and control were ensured by conducting all tests according to the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM 2006a). Tests were conducted in light- and temperature-controlled environmental chambers (Precision Model 818, Thermo Fisher Scientific, Marietta, Ohio, and Isotemp Model 146E, Fisher Scientific, Dubuque, Iowa). Thermometers used for daily temperature monitoring were certified for accuracy by the National Institute of Standards and Technology (NIST). Target test temperatures were ± 1°C (n = 1,206) for 97.1% of trials and ± 2°C for 99.4% of trials, with a maximum departure of 3.5°C. Sediment temperatures were monitored with

partial-immersion thermometers (Fisherbrand® Red-Spirit®, Fisher Scientific, Pittsburgh, Pennsylvania) that met NIST tolerances for accuracy. Mean sediment temperatures differed from target incubator temperatures by ≤ 1.1°C in low water exposures (n = 494) and by ≤ 0.9°C in dewatered exposures (n = 500). Mean water quality conditions among all juvenile tests were 108.2 mg $CaCO_3$/L alkalinity, 143.3 mg $CaCO_3$/L hardness, 534.9 µS/cm conductivity, 8.13 pH, and 7.52 mg/L dissolved oxygen (n = 21 for alkalinity and hardness, n = 167 for all other variables). Mean water quality conditions among the adult tests were 105.2 mg $CaCO_3$/L alkalinity (n = 2), 149.0 mg $CaCO_3$/L hardness (n = 2), 568.27 µS/cm conductivity (n = 15), 7.72 pH (n = 15), and 4.63 mg/L dissolved oxygen (n = 21).

Data Collection and Statistical Analysis

Burrowing data were recorded upon completion of 96-h thermal exposures in all tests. The number of mussels visible on the sediment surface in each chamber was recorded. Mussels were considered not burrowed if they were lying flat or relatively flat on the sediment surface and no attempt at burrowing was apparent. Mussels were considered burrowed if they were visibly upright and in position for siphoning at the sediment-water interface, as indicated by the observation of mantle tissue or the anterior edge of the shell or were not visible beneath the sediment-water interface. The presence of byssal threads on juvenile mussels in each chamber at the end of tests was assessed visually using a magnifying lamp and was recorded using the dichotomous dependent variable index, with 1 representing “byssus detected” and 0 representing “byssus not detected.” The effects of temperature, flow regime and acclimation treatment on burrowing and byssus production were analyzed with logistic regression (PROC LOGISTIC; SAS version 9.2; SAS Institute,

Inc., Cary, North Carolina). The best-fit statistical models for burrowing and byssus production for each species were selected from all possible models using Akaike's Information Criterion adjusted for low sample sizes (AIC_C; Burnham and Anderson 2002). Because of the nature of the byssus data (i.e., one datum per replicate), analysis of interactive effects was not possible, and only main effects on byssus production were interpreted.

Hemolymph was collected from the anterior adductor muscle of each adult *L. fasciola* surviving the 96-h thermal exposures. Hemolymph taken from mussels in each replicate was composited to create one sample per replicate, then immediately stored at -80°C until analysis. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), calcium, and bicarbonate in each hemolymph sample were analyzed by standard methods in the Clinical Pathology Laboratory at the North Carolina State University, Veterinary Teaching Hospital (Raleigh, North Carolina). The effects of temperature treatment on each hemolymph parameter were analyzed by Analysis of Variance (ANOVA) with JMP® Pro (version 9.0; SAS Institute, Inc.). Significant temperature treatment effects ($p < 0.05$) were further analyzed through a pairwise comparison of differences among the samples from the 20°C unacclimated control and experimental temperatures for a given acclimation temperature and flow regime treatment combination using a Dunnett's post-hoc test.

The effects of temperature treatment on survival of adult mussels were analyzed with Comprehensive Environmental Toxicity Information Software (CETIS)™ (v1.8.0.12, Tidepool Scientific, LLC, McKinleyville, California). The median lethal temperature (LT50) was defined as the temperature that caused mortality in 50% of the individuals in the exposed

sample, and the LT05 was the temperature that caused mortality in 5% of those in the sample. Survival data were used to generate LT50s and LT05s with the Trimmed Spearman-Karber method. LT50s and their 95% confidence intervals (CI) were compared between acclimation temperatures and test types (i.e., low water vs. dewatered) to detect significant differences. LT50 values were considered statistically different when the 95% CIs did not overlap (i.e., $\alpha = 0.05$).

Results

We found that elevated water and sediment temperatures generally reduced burrowing and byssus production, and that the dewatered flow regime simulation treatment also depressed these behaviors, compared to the low water treatment. The effects of acclimation temperature on burrowing and byssus were mixed, affecting some species negatively, some positively, and some were unaffected (Tables 1 and 2, Figure 2). LT50s for adult *L. fasciola* (96 h) averaged 34.1°C (Table 3) and were similar to acute lethal temperatures of the juveniles used in this study (Archambault 2012) and to those in other thermal studies (Dimock and Wright 1993; Pandolfo et al. 2010; Ganser 2012). Results of the biomarker analyses in adult *L. fasciola* were mixed; increased levels of ALT and AST were observed in both the low water and dewatered flow regime treatments in the 27°C acclimation test, but were statistically significant only in the low water test; biomarkers were apparently unaffected in the 22°C acclimation tests, even in the elevated treatment temperatures (Figure 3).

Burrowing behavior

Treatment temperatures affected burrowing behavior in all five species observed (Figure 2). In all tests except those with *A. plicata*, treatment temperature had interactive effects with either acclimation temperature, flow regime treatment, or both; however, regardless of interactions, increasing treatment temperatures always reduced mussel burrowing ability (Table 1). The burrowing behavior of four species was affected by flow regime treatment; *A. plicata* was not significantly affected. In three species (*L. cariosa, L. fasciola,* and *L. siliquoidea*), flow regime interacted with either treatment temperature, acclimation temperature, or both, to partially mitigate the negative effects of increasing treatment temperatures; however, the mitigative effect of the interactions was not strong enough to overcome the overall negative main effects of temperature or proxy flow in any case. The effects of acclimation temperature varied among species. Acclimation temperature did not significantly affect burrowing in *L. fasciola*. In *L. abrupta* and *L. siliquoidea*, the interactions partially mitigated the negative effects of treatment temperature, but in *L. cariosa*, acclimation temperature interactions generally exacerbated the negative effect of increasing treatment temperature on burrowing. Effects of acclimation were not analyzed for *A. plicata* because it was only tested at the 27°C acclimation due to lack of availability.

The most parsimonious logistic regression models explaining burrowing differed among species (Table 1), but the directional effects of temperature and flow regime were similar. Increasing temperature significantly reduced burrowing in *A. plicata* ($p < 0.0001$). Every degree increase in temperature decreased the odds of burrowing by a factor of 0.722

(95% confidence interval, 0.645 – 0.807), or approximately 28%. Flow regime treatment did not significantly affect the burrowing behavior of *A. plicata* (Table 1, Figure 2).

The burrowing behavior of *L. fasciola* was affected by treatment temperature and flow regime. While these effects were strongly interactive ($p = 0.0098$), increasing temperature reduced burrowing in both the low water and dewatered flow regime treatments (Table 1, Figure 2). The negative effect of temperature on burrowing was somewhat mitigated in the dewatered treatment compared to low water. However, while the interactive effect of flow on temperature seems to be mitigative, the main effect of flow had a much stronger overall negative impact on burrowing behavior.

In *L. abrupta*, burrowing was affected by treatment temperature, flow regime, and acclimation temperature. For a given acclimation and treatment temperature, the odds of burrowing was reduced in the dewatered flow regime ($p < 0.0001$), compared to low water, by a factor of 0.332 (0.196 – 0.562), or approximately 67%. The effects of temperature and acclimation were strongly interactive ($p = 0.0094$) (Table 1). Acclimation mitigated the negative effect of treatment temperature on burrowing; however, increasing temperature reduced burrowing in both the 22 and 27°C acclimation tests [i.e., regardless of whether the acclimation term in the logistic model equation was entered as 22 or 27, the slope of the coefficient for temperature (β_1) remained negative] (Figure 2). The 27°C acclimation temperature had a greater mitigative effect ($\beta_1 = -0.2563$) than did the 22°C acclimation ($\beta_1 = -0.5742$).

In the burrowing behavior of *L. cariosa*, treatment temperature had interactive effects with the flow regime ($p = 0.0004$) and acclimation temperature ($p = 0.0362$); however, for

any given acclimation and flow regime treatment combination, increasing treatment temperature always reduced burrowing [i.e., when values for acclimation temperature (22 or 27) and flow regime (0 or 1) were entered into the model, β_1 remained negative, regardless of the flow/acclimation treatment combination] (Table 1, Figure 2). The interactive effects of temperature and flow regime were mitigative, as was shown in *L. fasciola*; however, like in *L. fasciola*, the main effect of flow was much stronger on the burrowing behavior of *L. cariosa*. In this species, the interaction of temperature and acclimation exacerbated the negative effect on burrowing behavior, but the interaction had a much smaller impact on burrowing than the main effects of treatment and acclimation temperatures.

The most parsimonious model explaining burrowing in *L. siliquoidea* was the full model, containing a second-order interaction among acclimation temperature, flow regime, and treatment temperature ($p = 0.0046$), in addition to first-order interactions among the combinations of treatments (Table 1). The three treatments were strongly interactive, but increasing treatment temperature reduced burrowing in *L. siliquoidea* for any given combination of acclimation temperature and flow regime treatments (Figure 2). The negative effect on burrowing was greatest in the 27°C acclimation dewatered test.

Siphoning.— Siphoning data were analyzed for juveniles of three species (*L. abrupta*, *L. cariosa*, and *L. siliquoidea*) and on adult *L. fasciola*. Of the mussels that were burrowed at the end of the 96-h acute tests, we observed a substantial percentage burrowed and in upright position for siphoning. Among burrowed adult *L. fasciola* mussels, 71.4% were in siphoning position. In juveniles, 68.4% of burrowed *L. abrupta* were siphoning at the surface, as were 32.2% of burrowed *L. cariosa* and 62.0% of burrowed *A. plicata*. The grand mean for

percentage of burrowed mussels that were siphoning in juveniles of all species was 53.0%. Because siphoning was not a pre-determined sublethal endpoint, the specific effects of acclimation, treatment temperature, or proxy flow regime to siphoning were not analyzed.

Byssus production

Byssus production data were analyzed for three species (*L. abrupta*, *L. cariosa*, and *L. siliquoidea*; Table 2); no byssus was observed in the adult *L. fasciola*, and *A. plicata* produced byssus in only one treatment among all experiments. The most parsimonious logistic regression models explaining the effects of treatment temperature, flow regime, and acclimation temperature on byssus production were assumed to be additive for each species. In summary, production of byssal threads was affected most drastically by flow regime in all three species, with the probability of byssus presence reduced by 93 – 99% in the dewatered treatment when compared to the low water treatment. Increasing temperatures reduced byssus production in all species analyzed by 18 – 35%. Acclimation temperature reduced byssus production only in *L. abrupta*; *L. cariosa* was unaffected by acclimation temperature (Table 2).

Treatment temperature and flow regime effects on byssus production in *L. cariosa* were highly significant (p = 0.0002 and 0.0005, respectively; Table 2). For a given flow regime, each degree increase in temperature reduced the odds of byssus production by a factor of 0.651, or approximately 35%. After controlling for temperature, the dewatered flow regime reduced the odds of byssus production by a factor of 0.006, or more than 99%, compared to the low water treatment (Table 2).

Byssus production in *L. siliquoidea* was also significantly affected by treatment temperature and flow regime (Table 2). For a given flow regime, each degree increase in temperature reduced the odds of byssus production ($p = 0.0032$) by a factor of 0.723, or approximately 28%, and after controlling for temperature, the dewatered flow regime reduced the odds of byssus, compared to low water ($p = 0.0071$), by a factor of 0.025, or approximately 97% (Table 2).

Byssus production in *L. abrupta* was negatively affected by all three experimental factors (Table 2). After controlling for the other factors, increasing acclimation temperature reduced the odds of byssus production ($p = 0.0210$) by a factor of 0.758, or approximately 24%; increasing treatment temperature reduced the odds of byssus ($p = 0.0004$) by a factor of 0.818, or approximately 18%; and the dewatered flow regime decreased the odds, compared to low water ($p < 0.0001$), by a factor of 0.068, or about 93% (Table 2).

Adult survival and biomarkers of thermal stress

LT50s for *L. fasciola* ranged from 33.7 to 34.7°C, with a mean of 34.6°C for the low water treatments, 33.7°C for the dewatered treatments, and a grand mean of 34.1°C. No significant differences in survival were detected between acclimation temperatures or proxy flow regime treatments (Table 3). LT05s ranged from 26.6 to 28.4°C, with a mean of 27.5°C. LT05s could not be determined from survival data for the low water treatments in either the 22 or the 27°C acclimation test due to lack of partial mortality responses.

The effects of treatment temperature on hemolymph biomarkers varied. The only notable effects were on ALT and AST (Figure 3). ALT and AST concentrations were significantly increased by treatment temperature in the 27°C acclimation, low water test ($p =$

0.04 and 0.02, respectively). Concentrations of ALT in the control samples averaged 9.6 IU/L (± 1.7 IU/L, SE), and AST averaged 15.2 IU/L (± 2.4 IU/L). Mean ALT concentrations in the temperature treatments ranged 2.0 – 16.5 IU/L, and mean AST concentrations ranged 4.0 – 27.5 IU/L. A Dunnett's post-hoc test indicated that ALT and AST concentrations in individual treatment temperatures did not differ significantly from the controls, but the AST in the 33°C treatment (27.5 IU/L) was tending toward significance ($p = 0.07$) (Figure 3C).

Concentrations of ALT and AST showed a qualitatively similar response to temperature in the dewatered treatment of the 27°C acclimation test (Figure 3D); however, ANOVA results were not statistically significant ($p > 0.05$). Concentrations of ALT in the control samples averaged 7.5 IU/L (± 1.4 IU/L, SE), and AST averaged 12.5 IU/L (± 4.2 IU/L). Mean ALT concentrations in the temperature treatments ranged 3.0 – 10.0 IU/L, and mean AST concentrations ranged 6.0 – 25.8 IU/L.

Neither ALT nor AST were significantly affected by temperature in the 22°C acclimation tests (Figure 3A, B). Mean ALT concentrations in the low water and dewatered treatments ranged 7.5 – 11.2 IU/L, and 3.8 – 7.0 IU/L, respectively, and mean AST concentrations ranged 12.6 – 17.0 IU/L, and 6.0 – 13.4 IU/L, respectively.

Discussion

Overall, we found that increasing temperatures negatively affected burrowing in all five mussel species tested, and that the dewatered treatment, our proxy for drought conditions, negatively affected burrowing, compared to the low water treatment, in all species except for *A. plicata*. In the three species for which the effects of experimental

treatments on byssal thread production were evaluated (*L. abrupta*, *L. cariosa*, and *L. siliquoidea*), we found that increasing temperature had a negative effect on production. Proxy flow was by far the most influential factor affecting byssus production, with the dewatered treatment causing a reduction in byssus production of ≥ 93% for all 3 species. In our analyses of biomarkers in hemolymph, we found no effects of elevated temperatures in the 22°C acclimation tests and mixed results for ALT and AST in the 27°C acclimation tests.

Increasing temperature reduced burrowing in the five species we studied, and the effects were exacerbated in the dewatered treatment for all but *A. plicata*. These findings support previous research by Bartsch et al. (2000), who found that adult unionids took longer to upright in sediment and had lower survival after emersion for up to 60 minutes in high (45°C) air temperatures. The mitigative effects of some of the treatment interactions complicate interpretation of the logistic regression models explaining burrowing behavior; however, the magnitude of the interactive effects was typically very small, and seemingly much less consequential, compared to main effects. Mussels may burrow to escape high velocity currents (e.g., flash floods) (Schwalb and Pusch 2007), to remove zebra mussel infestations (Nichols and Wilcox 1997), or to feed (Rogers 1999), and while adult mussels have evolved impressive adaptations for surviving emersion during low flow events, survival time depends on temperature, humidity, and duration of the event (Byrne and McMahon 1994; Bartsch et al. 2000). The same adaptations may not be as effective for very small juveniles (Ricciardi et al. 1994) or for adults of smaller, thin-shelled species (Waller et al. 1995). Our findings suggest that hotter stream temperatures and extreme low flow events may decrease fitness in freshwater mussels by diminishing their ability to burrow into the

substrate and escape predation, detrimentally fast currents (e.g., washing them to less suitable habitats downstream), or fouling organisms. Poor survival of juvenile mussels could result in population level effects that may go undetected until relict adults begin to phase out of the system. Moreover, because mussels exhibit seasonal patterns in vertical movement associated with temperature, day length (Schwalb and Pusch 2007), and reproductive timing (Amyot and Downing 1997; Negishi et al. 2011), stream temperature regimes altered by climate change, point-source, and non-point source thermal inputs, have potential to disrupt the phenology of important seasonal cues, and could potentially decouple spatial/temporal relationships with host fishes, and, ultimately, reduce recruitment. Decoupling of mussel-host fish relationships and diminished recruitment over time could have disastrous effects, further endangering species' survival and imperiling species now considered secure (Pandolfo et al. 2012). Temperature and flow are likely not the only factors limiting burrowing. Peck et al. (2007) showed that the effects of temperature on the burrowing of the marine Antarctic clam (*Laternula elliptica*) were exacerbated in hypoxic conditions and ameliorated under hyperoxic conditions. They also found an interactive effect of temperature and body size on burrowing capacity. Freshwater bivalves may respond similarly, and further investigation into additional variables that may affect burrowing is warranted.

Several studies suggest that juvenile unionids remain largely burrowed in the sediment for the first 2 – 4 years of their life, garnering their nutrients primarily from sediment pore water by employing a pedal feeding strategy (Yeager et al. 1994; Balfour and Smock 1995; Strayer et al. 2004; Schwalb and Pusch 2007). However, we regularly observed juvenile mussels siphoning at the sediment/water interface. A majority of *A.*

plicata and *L. abrupta* were observed siphoning (62 and 68.4% of those burrowed, respectively), and both species exhibited a maximum of 100% siphoning for a given treatment. Even *L. cariosa*, which siphoned the least on average overall (32.2% of those burrowed), exhibited a maximum of 71% siphoning for a given treatment. Our results suggest that research on diet, contaminant exposure, or other parameters with juvenile mussels should not rule out surface water as a potential source.

Because the majority of native freshwater mussels known to produce byssus are Lampsilines (Bradley 2011), the three juvenile *Lampsilis* species in our study were ideal for observing effects of temperature on byssus production. Like burrowing, byssal thread production was negatively affected by elevated temperatures, but flow regime had a greater effect than temperature. The dewatered flow regime reduced byssus by 93 – 99% among species, compared to low water — an intuitive finding, because water is used in the production of byssus (Waite 1983; Cope et al. 1997). Increasing temperature reduced byssus by 18 – 35% per degree Celsius. If byssus is used for both drift and attachment as suspected (Bradley 2011), hampered byssus production by high stream temperatures and low flows may reduce the ability to disperse, or conversely, to retain position within a stream bed. A situation common to southeastern streams during summer is the combination of very low flows due to seasonal drought, followed by flashy stream conditions caused by strong thunderstorms; our findings suggest that this combination may reduce byssus production and ability to attach, and then sweep juveniles downstream, resulting in dispersal to potentially unsuitable habitats.

Increasing temperature significantly affected ALT and AST enzymes in the 27°C acclimation low water experiment. Though differences between each treatment compared to the control were not statistically significant ($\alpha = 0.05$), the spike in enzyme activity, especially in AST, in the 33°C treatment (Figure 3C) may be biologically meaningful. The LT50 for the 27°C acclimation low water experiment was 34.7°C (33.9 – 35.7°C, 95% confidence interval (CI)). Spikes in ALT and AST concentrations in the 33°C treatment suggest that mussels may become detrimentally stressed several degrees less than the median lethal outcome. In the 27°C dewatered treatment, despite the lack of statistical significance, the qualitative evidence of a spike in AST at 31°C (Figure 3D) compared to the LT50 of 33.7°C (32.5 – 34.9°C), suggests not only that the mussels became stressed before a lethal outcome, but that physiological stress is likely exacerbated in extreme low flow or drought situations (i.e., the spike occurred at a lower temperature than was observed in the low water treatment). A recent severe drought in 2000 caused mussel density to decline as much as 83% in some southeastern US streams, and led to a reduction in species richness in these systems, primarily through the loss of rare species (Haag and Warren 2008).

The enzymes ALT and AST were responsive only in the 27°C acclimation tests. One possibility for the difference between acclimation tests is that the acute (96 h) test duration was not long enough to elicit a response in the candidate biomarkers. The mussels in the 22°C acclimation tests were already acclimated before the acclimation period began because the ambient temperature in their holding tanks was approximately 22°C for a relatively long duration (2 weeks). Those test mussels experienced thermal ramping and high temperatures only for the duration of the 96-h acute exposure. Conversely, ambient temperatures in the

holding tanks in the weeks before the 27°C acclimation and testing period were approximately 19°C. The mussels were then ramped to the test acclimation temperature following the ASTM (2006a) guideline. As no guidance for acclimation of adults currently exists, the protocols for juveniles (ASTM 2006a) were used, to maintain consistency with our other thermal experiments and to maintain protocol. However, it is possible that the acclimation period was too brief and the rate of thermal ramping was too fast for the adult *L. fasciola* to achieve true acclimation to 27°C. In a review of temperature tolerance for 50 aquatic organisms, including 16 mollusks, deVries et al. (1998) reported that acclimation periods were typically longer than 96 h. If the mussels used in the 27°C acclimation tests were not truly acclimated, then they may have elicited elevated levels of enzyme biomarkers of thermal stress in response to a longer duration of thermal ramping and high temperatures, including approximately 72 h of acclimation time in addition to the 96-h acute thermal exposure. Further, the disparity in the results of the 22 and 27°C tests may be an indicator that ALT and AST would be more suitable for longer duration (i.e., chronic) thermal exposures. Boutet et al. (2005) observed changes in AST mRNA expression after more than seven days of exposure to stress in the Pacific oyster (*Crassostrea gigas*), and found that enzyme protein and mRNA levels were not always paired. These and other physiological parameters may vary seasonally (Monroe and Newton 2001), or with reproductive status or body size (Gustafson et al. 2005). Moreover, An and Choi (2010) found that ALT and AST responded to high temperature (30°C) stress in a time-dependent manner, peaking at the end of a 48-h experiment, in the marine ark shell (*Scapharca broughtonii*). While there are no other studies with *L. fasciola* for comparison, the concentrations of AST we report fall within

reference ranges reported by Gustafson et al. (2005) for another freshwater mussel species (*Elliptio complanata*). More research is needed to determine if these biomarkers are good indicators of thermal stress, and further, to determine concentrations indicative of a detrimental stress event, as opposed to natural variation and reactions to common natural stressors, like acute duration spikes in seasonal temperatures. Based on our results, future studies of biomarkers of thermal stress would be enhanced with a non-lethal endpoint and long enough duration to elucidate reaction time (Liu et al. 2004), hemolymph samples from individual mussels rather than composite samples, and additional methods of stress detection, including biomarker gene expression (Corporeau and Auffret 2003; Boutet et al. 2005).

The acclimation effects on sublethal endpoints in this study are unrelated to those of the acute median lethal temperatures for the same test organisms. In an analysis of lethal endpoints (LT50s), effects of acclimation temperature were largely absent (Archambault 2012). In this study, we observed an effect of acclimation temperature on burrowing in three species, affecting *L. abrupta* and *L. siliquoidea* negatively overall, with a positive main effect on burrowing in *L. cariosa* (Table 2). In each species, the effects of acclimation were interactive with treatment temperature, although not to a great enough extent to mitigate the main effects of either treatment. In testing for acclimation effects to byssus production, we observed that *L. abrupta* was negatively affected and *L. cariosa* was unaffected (Table 3). In a related study of lethal endpoints with the same organisms (Archambault 2012), acclimation duration was longer and the rate of change was more conservative (i.e., slower) than the 3°C/h recommended by the ASTM (2006a) guidelines, but that still may have been too brief to allow the mussels to establish true acclimation or, conversely, may be unimportant in acute

exposures, especially when the two acclimation temperatures are relatively high and proximate in range (22°C and 27°C). While it may be impractical to hold early life stages of mussels for longer periods while following the ASTM (2006a) guidelines for acute tests (e.g., no feeding of test organisms), it may be worthwhile to consider effects of acclimation to sublethal endpoints, even when a short-duration acclimation period like that recommended by the ASTM (2006a) is used. No consistent pattern of acclimation effect was observed in our study of sublethal measures of environmental stress, and our results suggest that the topic warrants further investigation.

The results of this study are important in the context of climate change because global warming is expected to alter stream temperatures as a result of increased air temperature and change in patterns of precipitation [i.e., greater frequency of extreme rainfall events (e.g., tropical storms), and prolonged weather patterns (e.g., droughts)] (Bates 2008), and because surface water temperatures are regularly impacted by anthropogenic activities (LeBlanc et al. 1997; Hester and Doyle 2011). Urban stormwater runoff and wastewater effluents are important anthropogenic contributors to elevated stream temperatures (Kinouchi et al. 2007; Thompson et al. 2008). Many organisms are more sensitive to increases in water temperature than to decreases, and human impacts tend to increase the temperature of surface waters more often than decrease it (Chen et al. 2007; Hester and Doyle 2011). Additionally, the global prevalence of large-scale impoundments along rivers regularly alters downstream discharge, water temperatures, and sediment transport (Poff et al. 2007), and may be detrimental to freshwater mussel biodiversity, particularly in the southeastern US, where dams are plentiful and global mussel biodiversity is greatest (Bogan et al. 2008). Galbraith

and Vaughn (2011) found that unionids downstream of dam releases that were unnatural (e.g., peaking flows for hydropower generation) had lower body condition, higher hermaphroditism and parasite loads, and occurred in lower densities than mussels that were downstream of dam releases that more closely mimicked natural flow regimes. Burlakova et al. (2011) suggested that climate, land use, and human population density influence freshwater mussel diversity, and specifically, that human population density was negatively correlated with species diversity and the proportion of rare species. They also concluded that the most important environmental factors influencing freshwater mussel diversity included climatic parameters (i.e., precipitation and evaporation) and hydraulic variables (i.e., river relief and discharge; Burlakova et al. 2011) — proximate factors that are affected by climate change.

Conclusions

We quantified effects of increasing temperature, proxy flow regime, and acclimation temperature on behavioral and physiological measures of stress in five species of freshwater mussels. We showed that increasing temperature and proxy drought flow negatively affected burrowing behavior. Mussels acclimated to warmer temperatures may experience mitigation or exacerbation of those negative effects, and the response may be species specific. Because some freshwater mussel species exhibit seasonal vertical movements (Amyot and Downing 1997; Negishi et al. 2011), water temperature may act as an environmental cue on the molecular clock of mussels, signaling that it is time to surface. Stream discharge may also be a driving factor in burrowing behavior (Schwalb and Pusch 2007). While our use of a

surrogate for flow regime was informative and significant, future endeavors exploring burrowing behavior may benefit from a flow-through experimental design.

We showed that decreased proxy flow greatly diminished or abolished the ability of unionids to produce byssus, and that increasing temperature reduced byssus production by as much as 35% per degree rise. Summer weather patterns of seasonal droughts coupled with heavy rainfall in the southeastern US where worldwide unionid diversity is greatest (Bogan 2008) may regularly induce these negative effects to byssus production, especially in streams more susceptible to running dry (e.g., headwater streams). Additional research on stressors to byssus production may benefit from more replication and more intensive observations to help define potential interactions among multiple factors.

We found that ALT and AST enzymes in *L. fasciola* were significantly affected by temperature in the 27°C acclimation low water experiment, and showed a qualitatively similar response in the 27°C acclimation dewatered experiment. The results of the 27°C acclimation tests indicate that thermal stress occurred below LT50s and that thermal stress can occur at relatively moderate temperatures. Stream temperatures greater than 30°C (> 35°C is not uncommon) coupled with below-normal discharge regularly occur in streams in the eastern US, especially in the southeastern region (USGS 2012). Our findings suggest that freshwater mussels subjected to seasonally common moderately warm or hot conditions may experience thermal stress, and such stress may be intensified during droughts. The lack of agreement among all analyses of ALT and AST suggests that they may take longer than 96 h to respond to environmental stress, and may serve as physiological cues of stress in chronic tests or field monitoring of native populations of mussels. While warm temperatures may

provide some benefit to unionid life histories (e.g., affecting seasonal diet, increased growth, or cueing reproductive timing), we suggest that above-average stream temperatures and changes in the seasonal phenology of stream temperature profiles and flows may have detrimental behavioral and physiological effects to this already imperiled faunal group.

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Table 1. Burrowing behavior in 5 species of freshwater mussels tested in thermal exposure experiments, as explained by the most parsimonious logistic regression models, selected using Akaike's Information Criterion, corrected for small samples (AIC_C). Let *t* = treatment temperature, *f* = flow regime, and *a* = acclimation temperature. Then let $x_1 = t$; $x_2 = f$; $x_3 = a$; $x_4 = t * f$; $x_5 = t * a$; $x_6 = f * a$; and $x_7 = t * f * a$. Models are listed in order of increasing model complexity, coefficients for flow regime are for dewatered with respect to low water (reference level), and all models = logit (burrowed/exposed), or the log odds of burrowing.

		***p*-values for partial slopes**						
Species	**Model**	**x_1 (temp)**	**x_2 (flow)**	**x_3 (acc)**	**x_4 (temp* flow)**	**x_5 (temp* acc)**	**x_6 (flow* acc)**	**x_7 (temp* flow* acc)**
Amblema plicata	$y = 12.2702 - 0.3263x_1$	< 0.0001		not tested				
Lampsilis fasciola	$y = 19.0125 - 0.5455x_1 - 10.4234x_2 + 0.2710x_4$	< 0.0001	0.0037		0.0098			
Lampsilis abrupta	$y = 71.6998 - 1.9735x_1 - 1.1019x_2 - 2.2637x_3 + 0.0636x_5$	0.0023	< 0.0001	0.0086		0.0094		
Lampsilis cariosa	$y = 19.6913 - 0.1715x_1 - 33.5030x_2 + 0.9455x_3 + 0.8963x_4 - 0.0390x_5$	0.7580	0.0003	0.1398	0.0004	0.0362		
Lampsilis siliquoidea	$y = 73.9041 - 2.1036x_1 - 83.6619x_2 - 2.3302x_3 + 2.4625x_4 + 0.0659x_5 + 2.9265x_6 - 0.0862x_7$	0.0055	0.0020	0.0187	0.0019	0.0222	0.0048	0.0046

Table 2. Byssus production behavior in 3 species of freshwater mussels in thermal exposures, as explained by the most parsimonious logistic regression models, selected using Akaike's Information Criterion, corrected for small samples (AIC_C). Let x_1 = treatment temperature, x_2 = flow regime, and x_3 = acclimation temperature. Models are listed in order of increasing model complexity; coefficients for flow regime are for dewatered with respect to low water (reference level); all models = logit (byssus), or the log odds of byssus occurring; and odds ratios are point estimates, with 95% confidence intervals in parentheses.

		***p*-values for Partial Slopes**			**Odds Ratio Estimates**		
Species	**Model**	**x_1 (temp)**	**x_2 (flow)**	**x_3 (acc)**	**x_1 (temp)**	**x_2 (flow)**	**x_3 (acc)**
Lampsilis siliquoidea	$y = 10.9634 - 0.3241x_1 - 3.66801x_2$	0.0032	0.0071	not tested	0.723 (0.583 – 0.897)	0.025 (0.002 – 0.367)	
Lampsilis cariosa	$y = 13.6241 - 0.4285x_1 - 5.1814x_2$	0.0002	0.0005		0.651 (0.520 – 0.817)	0.006 (<0.001 – 0.102)	
Lampsilis abrupta	$y = 14.1435 - 0.2013x_1 - 2.6914x_2 - 0.2776x_3$	0.0004	< 0.0001	0.0210	0.818 (0.732 – 0.913)	0.068 (0.018 – 0.256)	0.758 (0.599 – 0.959)

Table 3. Median lethal temperatures (LT50) causing 50% mortality and protection-level lethal temperatures (LT05) causing 5% mortality (with 95% confidence intervals) in adult *Lampsilis fasciola* mussels at 22°C and 27°C acclimation temperatures in low water and dewatered sediment exposures (96 h). LT50 values among acclimation and proxy flow regime treatment combinations did not differ in any case. LT05 values between acclimation temperatures did not differ in the dewatered treatment. ND = value could not be determined.

	LT50		LT05	
Lampsilis fasciola	Low Water	Dewatered	Low Water	Dewatered
22°C Acclimation	34.4 (33.5 – 35.4)	33.7 (32.1 – 35.4)	ND	26.6 (20.6 – 29.0)
27°C Acclimation	34.7 (33.9 – 35.7)	33.7 (32.5 – 34.9)	ND	28.4 (19.0 – 31.0)

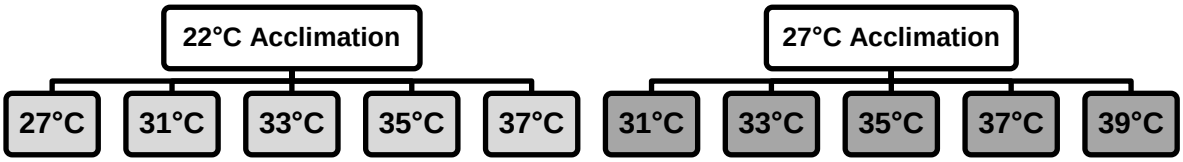

Figure 1. Schematic diagram of experimental design showing acclimation temperatures (22 and 27°C) and experimental temperature treatments for all juvenile and adult mussel exposures. All experiments employed a control temperature of 20°C.

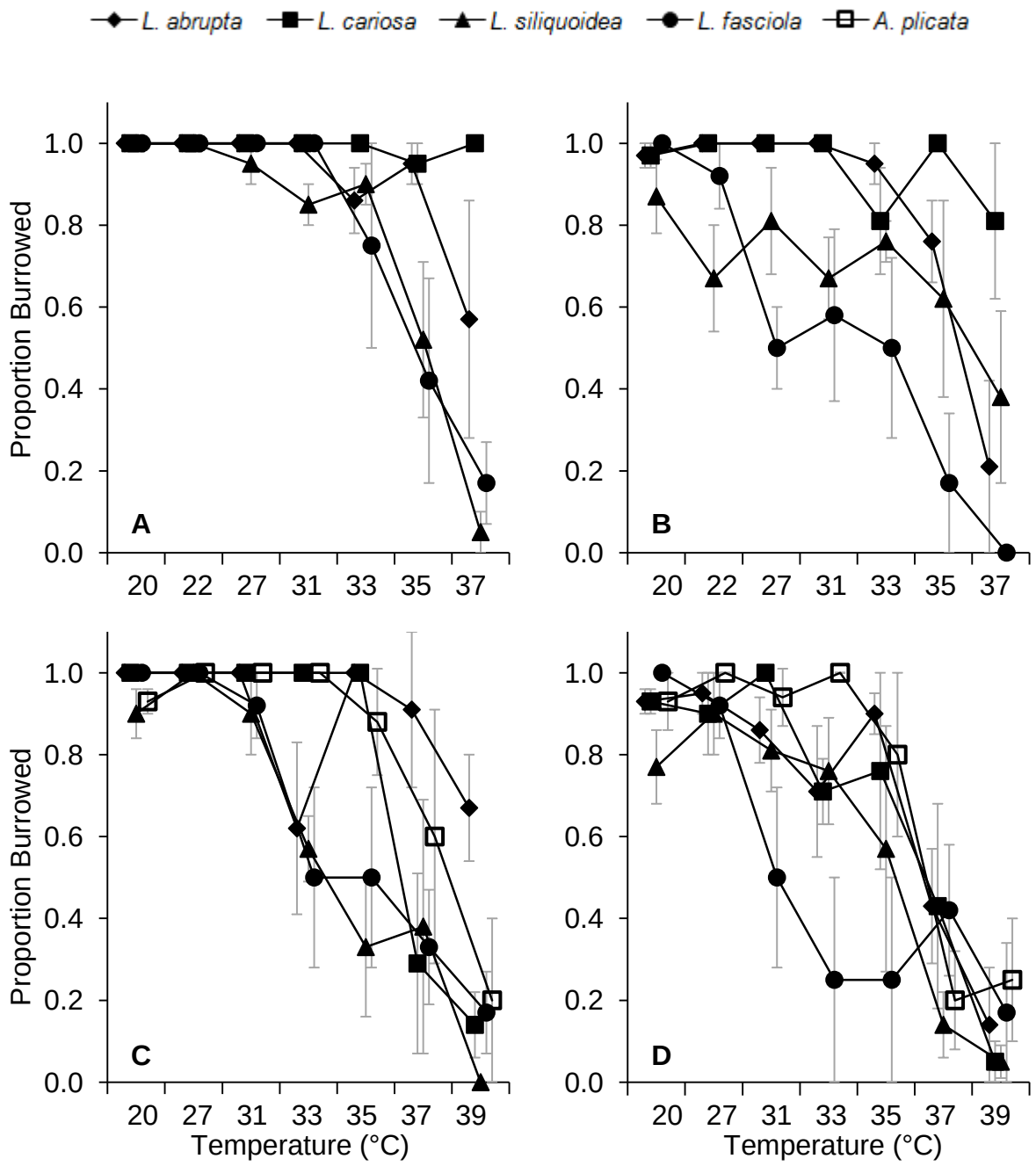

Figure 2. The mean (± SE) proportion of mussels that were burrowed at the end of the acute (96 h) exposures in the (A) 22°C acclimation, low water; (B) 22°C acclimation, dewatered; (C) 27°C acclimation, low water; and (D) 27°C acclimation, dewatered experiments.

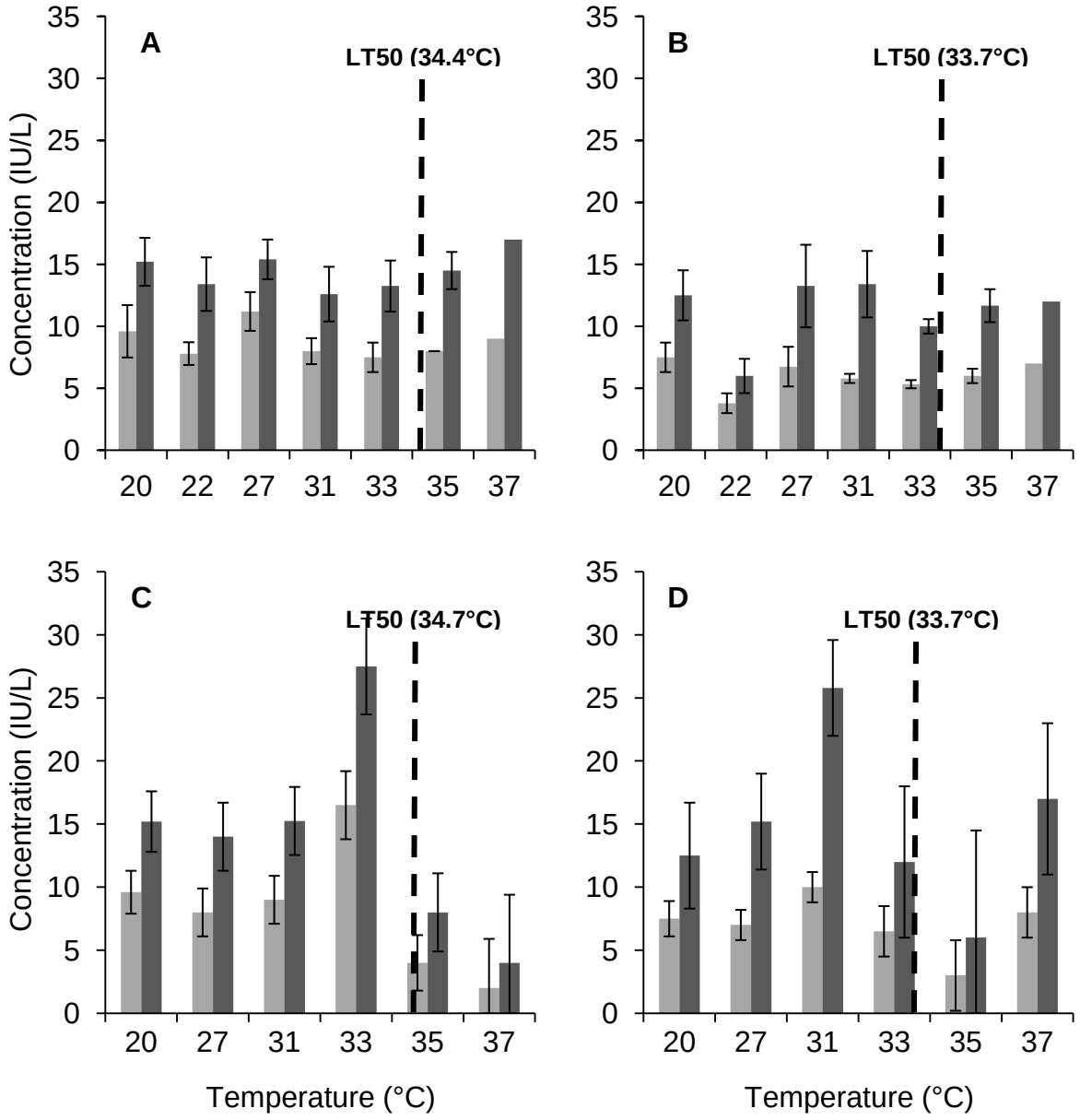

Figure 3. The mean concentrations of alanine aminotransferase (ALT; light grey bars) and aspartate aminotransferase (AST; dark grey bars) (± SE) in composite samples of hemolymph extracted from surviving adult *Lampsilis fasciola* at the end of acute (96 h) exposures in the (A) 22°C acclimation, low water; (B) 22°C acclimation, dewatered; (C) 27°C acclimation, low water; and (D) 27°C acclimation, dewatered experiments. The vertical dashed line indicates the acute (96-h) median lethal temperature (LT50).

Chapter 3. Toward ecological relevance in laboratory thermal tests: incorporating multiple components of the benthic environment

Abstract

Mesocosm experiments are a popular tool for increasing environmental complexity while maintaining experimental control; however, relatively few mesocosm studies have been used to investigate threats to freshwater mussel (Unionidae) survival and biodiversity, despite their status as a highly imperiled group. One such critical threat is thermal sensitivity, because global climate change and other anthropogenic activities contribute to increasing stream temperatures and altered hydrology and flows that may be detrimental to freshwater mussels. We incorporated four benthic environmental components – temperature, sediment, a surrogate for flow, and a vertical thermal gradient in the sediment column – in laboratory experiments with two species of juvenile freshwater mussels (*Lampsilis abrupta* and *Lampsilis radiata*) and tested their effects on survival, burrowing behavior, and byssus production. Increasing temperature significantly diminished burrowing behavior in both species ($p < 0.0001$), and the dewatered flow regime significantly reduced burrowing in *L. radiata*, compared to that in low water. Increasing temperature also significantly reduced byssus production in both species (*L. abrupta* $p = 0.0080$, *L. radiata* $p < 0.0001$). Median lethal temperature (LT50) ranged from 29.9 to 35.6°C, a relatively narrow range of thermal tolerance. Mussels did not burrow beneath the top stratum of sediment (0 - 2.5 cm); thus we

were unable to fully elucidate the vertical thermal gradient effect. Our findings suggest that rising stream temperature and altered flow may directly impact freshwater mussel diversity by causing mortality, and may have indirect impacts via sublethal effects. Reduced burrowing capacity may hamper ability to escape predation or unfavorably high or low flows, and decreased byssus production may negatively affect attachment and dispersal capabilities in juveniles.

Keywords: burrowing, byssus, climate change, freshwater mussels, LT50

Introduction

Laboratory research offers many advantages and yields findings that may not be attainable by research conducted afield, such as strict application of treatment factors, replication, and quality control. Such laboratory experiments, however, often lack the ecological complexity and realism of field research, and their applicability may be diminished or lost when multiple field conditions must be considered (Odum, 1984). Mesocosm experiments are a useful tool for increasing environmental complexity while maintaining experimental control in many fields of study (Odum, 1984). While mesocosm experiments have become increasingly popular in terrestrial and aquatic research, we are aware of relatively few such experiments incorporating freshwater mussels (e.g., Downing, Van Leeuwen & Di Paolo, 2000; Spooner & Vaughn, 2006, Allen & Vaughn, 2009), and only one that examined temperature effects on mussels (Spooner & Vaughn, 2012).

Freshwater mussels are a critically imperiled taxon; an assessment of North American mussel fauna, where approximately half of the worldwide unionid diversity exists, concluded that 71.7% of species were endangered, threatened, or of special concern (Williams et al., 1993). Freshwater mussels are integral to stream ecology and surface water quality (Vaughn & Hakencamp, 2001; Howard & Cuffey, 2006; Vaughn, Nichols & Spooner, 2008; Spooner & Vaughn, 2012; Haag, 2012). Because they perform many ecological services and often comprise a substantial proportion of benthic biomass (Vaughn & Hakencamp, 2001; Vaughn, Gido & Spooner, 2004; Vaughn et al., 2008; Haag, 2012), identifying and quantifying threats to freshwater mussels is important for conserving biodiversity and ecological integrity of aquatic systems, two environmental benchmarks that typify management goals (Downing, Van Meter & Woolnough, 2010; Kwak & Freeman, 2010). Two such threats are elevated stream temperatures and altered hydrologic flows due to climate change and other anthropogenic activities.

The global climate is warming at a much faster pace than at any other time in recent geologic history (IPCC, 2007). The National Oceanic and Atmospheric Administration (NOAA) reported that July 2012 ranked as the warmest month ever recorded in the history of record keeping in the United States (US), and 2001 – 2011 rank among the 13 warmest years ever recorded globally (NOAA, 2012a, 2012b). These climate statistics and many other climate records broken elsewhere in recent years exemplify climate trends of warming and extreme weather that are impacting terrestrial and aquatic habitats alike. Concurrent changes in land use may also have deleterious consequences to aquatic habitats by contributing to

additional heated point- and non-point-source effluents (Hester and Doyle, 2011), thus exacerbating thermal stress to freshwater mussels and other aquatic organisms.

Thermal inputs to aquatic systems are common, and despite extensive thermal research associated with fisheries and fish populations, research on thermal stressors to freshwater mussels has gained momentum only in the past decade. Few investigators have evaluated the effects of thermal stress on native freshwater mussels, and to date, quantitative information on lethal temperatures is limited to about 15 species (Dimock & Wright, 1993; Pandolfo et al., 2010; Archambault, 2012a). Most of these studies were restricted to the water-only standard method for toxicity testing (ASTM, 2006a), and thus did not incorporate any environmental components that may affect mussel thermal sensitivity in situ. In a companion study to this one, we developed and applied a new method for conducting thermal tests in sediment with juvenile freshwater mussels (Archambault, 2012a). Because we found that acute (96-h) median lethal temperatures (LT50) were similar among experiments conducted in water-only and in sediment, we suggested that more complex factors may be influential in determining thermal sensitivity in streams, and ultimately in mitigating mortality during periods of excessive heat.

Few studies have focused on potential sublethal effects of thermal stress in freshwater mussels [e.g., Pandolfo, Cope & Arellano, 2009 (heart rate); Galbraith, Blakeslee & Lellis, 2012 (shell gaping)]. Burrowing behavior is central to the ecology of these endobenthic organisms, but little is known about the effects of environmental stressors on burrowing (e.g., Nichols & Wilcox, 1997). Waller, Gutreuter, and Rach (1999) considered the effects of common stream temperatures on righting and burrowing behaviors, but studies quantifying

the effects of extreme temperatures on burrowing behavior are rare (e.g., Bartsch et al., 2000), and to our knowledge, no one has done so with juvenile mussels.

Research on stressors to byssus production has concentrated primarily on efforts to control the nonnative zebra mussel (*Dreissena polymorpha*) (Clark & McMahon, 1996; Cope, Bartsch & Marking, 1997). Although some investigators have attempted to elucidate the significance of byssus in native freshwater bivalves, few have explored potential stressors to byssus production. Clark and McMahon (1996) found that invasive zebra mussels produced more byssus at higher temperatures, with the highest rate at 30°C, which is interestingly, near their upper lethal limit.

We postulated that thermal gradients typically found in stream substrates may influence lethal and sublethal thermal sensitivity endpoints in freshwater mussels, and may be especially important in mitigating thermal stress in juveniles. Building on our work that established a reliable and repeatable method for conducting thermal toxicity tests with freshwater mussels in sediment (Archambault 2012a), here we incorporate a vertical thermal gradient as an additional ecological factor. In this research, we investigate the effects of thermal stress, thermal refuge, proxy flow, and acclimation on survival, burrowing behavior, and byssus production in juvenile native unionids by incorporating multiple environmental variables toward enhancing ecological relevance in controlled laboratory experiments.

Methods

In previous research, we developed a standardized method for conducting thermal exposures to freshwater mussels in sediment, with two acclimation temperatures (22 and

27°C), five temperature treatments per acclimation group, and two experimental water treatments (low water and dewatered) that served as surrogates for different flow regimes (moderate and severe drought conditions; Archambault, 2012a). In this study, we expanded on our earlier design to include a vertical temperature gradient in the sediment and pore water. A standard protocol for conducting toxicity tests with freshwater mussels in sediment does not currently exist, but exposures in sediment were conducted following the same standards (ASTM, 2006a) as for water-only exposures, to the extent practical, to ensure data quality and comparability.

Experimental design

We employed a dual-chamber static-renewal design for thermal exposure of juvenile mussels in sediment. To enable direct comparison with results from the companion study (Archambault, 2012a), we followed a similar design; however, we used an improved inner chamber in this study and slightly increased the volume of sediment to achieve a sufficient depth in the top layer of the vertical component. A 12.7-cm long by 4.4-cm diameter cylindrical irrigation filter (Peaceful Valley Farm Supply, Inc., Grass Valley, California, USA) composed of 100-µm nylon mesh and plastic frame comprised the inner chamber. Two surrogates for flow regime (hereafter called flow regime) were simulated by controlling the amount of reconstituted hard water (ASTM, 2006b) added to the test chambers. A low water treatment, intended to simulate low-flow stream conditions (e.g., a reach with a patchy distribution of water or slack-water), included 350 mL of overlying water. A dewatered treatment served to simulate extensive drought conditions and included just enough water to

wet the sediment, and a maximum of 50 mL overlying water to mitigate evaporative loss during the experiments.

We developed and constructed a novel apparatus to achieve a vertical sediment temperature gradient. Three replicate chambers for each of the two flow regime treatments were sealed into holes cut in the bottom of a 42.5-L Lifoam® insulated cooler for each of six temperatures, consisting of the acclimation temperature and five corresponding experimental temperatures (Figure 1). The coolers were floated in temperature-controlled artificial streams (Model LS-700, Frigid Units, Inc., Toledo, Ohio, USA) set to the test acclimation temperature (22 or 27°C), thus the cooler containing the acclimation temperature treatment served as a control within the artificial streams. A submersible thermostatically-controlled heater (IHC Model HTS1 or JH500, Aquatic Ecosystems, Inc., Apopka, Florida, USA), a water pump (Maxi-Jet® Pro, Aquatic Ecosystems, Inc.), and approximately 4-L of deionized water were placed in each cooler; the water bath circulated around the outsides of the beakers, and the contents of the beakers (i.e., mussels, sediment, and water) were isolated from direct contact with the water bath. The top 2.5 cm of sediment and overlying water in each chamber were exposed to the treatment temperatures inside the coolers. The middle stratum of sediment (2.5 to 6 cm) in each chamber was encased by the foam bottom of the coolers and served as a thermal gradient zone. The bottom stratum of sediment (6 to 8 cm) of each chamber protruded from the bottom of the cooler and was exposed to the acclimation temperature of the artificial stream (Figure 2). Beakers were covered with watch-glasses, and coolers were loosely covered with plastic wrap and a foam lid to reduce evaporation and heat exchange with ambient laboratory air temperatures, thus minimizing thermal variation in

the experiment. Large holes were cut in the foam lids to maintain a natural, ambient light:dark cycle (14:10 h) throughout the experiment. Three replicates in each of two flow regime treatments were also held in a light- and temperature-controlled environmental chamber (Precision Model 818, Thermo Fisher Scientific, Marietta, Ohio, USA) at 20°C as an experiment-wide control.

Sediment.— Commercially available, contaminant-free filter sand (Southern Products and Silica Co., Inc. Hoffman, North Carolina, USA) served as the substrate for the experiments. This silica sand is widely used in applications, such as drinking water filtration, meets or exceeds the current American Water Works Association Standard for Filter Material (Southern Products, 2011), and was suited for use in this application (i.e., the materials did not introduce any confounding influences of organic matter, parasites, pathogens, or chemical toxicants). Before use, the sand was dry sieved to achieve a uniform size range of 500 – 850 µm and was heated to 200°C in a drying oven to ensure the lack of organisms and low starting moisture content.

Test Organisms

We tested two species of mussels in the Lampsilini tribe of the Unionidae family, *Lampsilis abrupta* (Say) and *Lampsilis radiata* (Gmelin). All juveniles were propagated via host-fish infection in facilities at Missouri State University (Springfield, Missouri) or the Harrison Lake National Fish Hatchery (Charles City, Virginia), using standard propagation and culture methods (Barnhart, 2006).

Test species were chosen based on native range, conservation status, and availability. *Lampsilis abrupta* is federally-listed as endangered (US Fish and Wildlife Service, 1985) and

occurs in the Interior Basin of the central US. *Lampsilis radiata* has a native range primarily in the Atlantic Slope Basin, and occurs in some northern watersheds of the Interior Basin. It is considered a globally secure species and has wide distribution, but is classified as imperiled in several states and is listed as threatened in North Carolina (NatureServe, 2012; NC Wildlife Resources Commission, 2011).

Test Conditions

We conducted acute (96 h) thermal exposures in the low water and dewatered sediment treatments that consisted of seven temperature treatments as follows: a control held at 20°C (ASTM, 2006a), an acclimation temperature (22 or 27°C), and five experimental temperatures, four of which were similar between the two acclimation groups (Figure 1). Test temperatures in the 22°C acclimation exposures ranged from 27 to 37°C, and test temperatures in the 27°C acclimation exposures ranged from 31 to 39°C (Figure 1). Similar temperature treatments between the two acclimation regimes facilitated the identification and analysis of any acclimation-related effect.

Mussels used in the thermal tests ranged in age from 12 to 17 months. Average shell lengths were 7.58 mm (± 1.63 mm, SD) for *L. abrupta* and 4.78 mm (± 0.72 mm) for *L. radiata*. Mussels within a species for a given test type differed in age by two weeks at most. Juvenile mussels were acclimated to the test acclimation temperature by adjusting their shipping temperature upon arrival by 2.5°C/d, with a minimum 24-h acclimation period once the target temperature was attained (ASTM 2006a, Pandolfo et al. 2010). Shipping temperatures averaged 19.3°C (± 3.5°C, SD) from June through August 2012. Experiments were nonaerated static-renewal tests with reconstituted hard water renewed (90% volume) at

48 h (ASTM 2006a, 2006b). Seven mussels were in each of three replicates per treatment and 10 mussels per replicate in controls.

Burrowing data were recorded upon completion of 96-h thermal exposures in all tests. The number of mussels visible on the sediment surface in each chamber was recorded. Mussels were considered not burrowed if they were lying flat or relatively flat on the sediment surface and no attempt at burrowing was apparent. Mussels were considered burrowed if they were visibly upright and in position for siphoning at the sediment-water interface, as indicated by the observation of mantle tissue or the anterior edge of the shell or were not visible beneath the sediment-water interface. Burrowing depth of mussels was further assessed by inspecting the top (0 – 2.5 cm), middle (2.5 – 6 cm), and bottom (6 – 8 cm) strata of sediment and recording the number of mussels present in each layer. The sediment within the mussel enclosure (inner chamber) was extruded into the 3 depth strata and searched for the presence of juvenile mussels with a magnifying lamp. The presence of byssal threads on juvenile mussels in each chamber at the end of tests was assessed visually using a magnifying lamp and was recorded using a dichotomous dependent variable index, with 1 representing "byssus detected" and 0 representing "byssus not detected".

Quality assurance and control were ensured by conducting all tests according to the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM, 2006a), as modified for sediment testing. Thermometers used for daily temperature monitoring in the control incubator were certified for accuracy by the National Institute of Standards and Technology (NIST). Daily temperatures of circulating water in the coolers and artificial streams were monitored with partial-immersion thermometers (Fisherbrand®

Red-Spirit®, Fisher Scientific, Pittsburgh, Pennsylvania, USA) that met NIST tolerances for accuracy. Target test temperatures in the water baths and artificial streams were ± 1°C (*n* = 466) for 90.3% of trials and ± 2°C for 97.0% of trials. Sediment temperatures within treatment beakers were monitored with iButton® iBCod (Model 22L) submersible temperature data loggers and OneWireViewer software (version 0.3.15.50; Alpha Mach, Inc., Mont St-Hilaire, Quebec, Canada), with one logger placed in each of the three depth strata in one low water and one dewatered replicate per temperature treatment. The temperature loggers were placed at the surface in the top stratum, at approximately 4 cm (half of the sediment depth) in the middle stratum, and at bottom of the beaker (8 cm depth) in the bottom stratum. Substrate temperature in the 20°C controls was monitored only in the top and bottom strata. Mean water quality conditions among all tests were 102.5 mg $CaCO_3$/L alkalinity, 137.5 mg $CaCO_3$/L hardness, 464.5 µS/cm conductivity, 8.30 pH, and 7.27 mg/L dissolved oxygen (*n* = 4 for alkalinity and hardness, *n* = 32 for all other variables).

Statistical Analysis

The lethal effects of temperature treatments on mussels were analyzed using survival data to calculate LT50s and LT05s with the Trimmed Spearman-Karber method (Comprehensive Environmental Toxicity Information Software (CETIS)™, v1.8.0.12, Tidepool Scientific, LLC, McKinleyville, California, USA). The LT50 was defined as the temperature that caused mortality in 50% of the individuals in the exposed sample, and the LT05 caused mortality in 5% of the sample. LT50s and their 95% confidence intervals (CI) were compared between acclimation temperatures, test types (low water vs. dewatered), and species, to detect significant differences when 95% CIs did not overlap.

The effects of temperature, flow regime, thermal refuge, and acclimation treatment on burrowing and byssus production were analyzed with logistic regression (SAS PROC LOGISTIC; SAS version 9.2; SAS Institute, Inc., Cary, North Carolina, USA). The most parsimonious models explaining burrowing and byssus production for each species with the fewest parameters were selected from all possible models using Akaike's Information Criterion adjusted for low sample sizes (AIC_C; Burnham & Anderson, 2002). An additional analysis of the effect of age on byssus production was performed for *Lampsilis abrupta* by including a numeric term for age in the logistic regression model and comparing data from this study and a companion study (Archambault 2012b). Because of the nature of the byssus data (i.e., one datum per replicate), analysis of interactive effects was not possible, and only main effects on byssus production were interpreted.

Results

We found that elevated temperatures had a negative effect on sublethal measures of thermal stress in both *L. abrupta* and *L. radiata* (Tables 1 and 2; Figure 3). Increasing temperatures significantly reduced burrowing (Table 1) and byssus production (Table 2) in both species, and effects of flow treatment were apparent in *L. radiata*. Acute median lethal temperatures (96-h LT50s) averaged 32.8°C, and acute LT05s averaged 26.8°C (Tables 3 and 4). In eight experiments conducted with *L. abrupta* and *L. radiata*, including a low water and dewatered test in each of the two acclimation groups for each species, we achieved 100% (n = 597) recovery of juvenile mussels from the sediment chambers among all sediment tests. Temperature monitoring in the sediment columns revealed good maintenance of consistent

temperatures throughout the vertical column in control (20°C) and acclimation (22 and 27°C) treatments with an average differential of 0.3°C, and establishment of a vertical thermal gradient in all other temperature treatments during each experiment (Table 5, Figure 4). All mussels were recovered from the top stratum (0 – 2.5 cm) of sediment upon test termination, and most were burrowed in an upright position for siphoning; no mussels were found in the middle and bottom sediment strata.

Survival

Acute (96 h) LT50s ranged from 29.9 to 35.6°C, with a grand mean of 32.8°C, and low water and dewatered treatment means of 31.5 and 35.2°C, respectively (Table 3). No significant effects of acclimation temperature or flow regime treatment were detected in *L. abrupta*, and no significant effect of acclimation temperature was detected for *L. radiata*. In the 22°C acclimation temperature tests, *L. radiata* was more thermally sensitive in the low water treatment, compared to the dewatered treatment. No significant differences were detected between species for a given acclimation and flow regime treatment combination. LT50s could not be determined in two cases due to lack of mortality (Table 3).

LT05s ranged from 18.7 to 32.5°C, with a low water treatment mean of 26.2°C, a dewatered treatment mean of 27.5°C, and a grand mean of 26.8°C (Table 4). LT05s could not be determined from survival data in one case due lack of mortality, and 95% CIs could not be estimated in three cases. No significant differences were detected among tests that could be compared within or between species for a given acclimation temperature and flow regime (Table 4).

Burrowing behavior

The most parsimonious logistic regression model explaining burrowing behavior in *L. abrupta* included treatment temperature and acclimation temperature (Table 1). Treatment temperature significantly affected the burrowing behavior of *L. abrupta* ($p < 0.0001$) (Figure 3). For a given acclimation, every degree rise in temperature decreased the odds of burrowing by a factor of 0.917, or approximately 8%. Acclimation temperature was included in the most parsimonious model explaining burrowing behavior in *L. abrupta*, but was not significant ($p = 0.1278$), and a model that contained only temperature had an equivalent AIC_C.

The most parsimonious logistic regression model explaining burrowing behavior in *L. radiata* included main effects from all three experimental factors and two interactions (Table 1; Figure 3). The flow regime treatment interacted with both acclimation temperature ($p = 0.0194$) and treatment temperature (0.0719), but the latter interaction was only nearly significant. Despite the interactive effects of the treatments, increasing temperatures always reduced burrowing, regardless of the flow regime and acclimation treatment combination [i.e., when values for acclimation temperature (22 or 27°C) and flow regime (0 or 1) were included in the logistic model, the slope of the temperature coefficient (β_1) remained negative, regardless of the flow/acclimation treatment combination].

Siphoning.— Of the mussels that were burrowed at the end of the 96-h acute exposures, we observed a substantial proportion in position for siphoning surface water. In *L. abrupta*, 87.2% of burrowed mussels were observed in an upright siphoning position, and in *L. radiata*, 81.6% of those burrowed were in siphoning position. The grand mean

percentage of burrowed mussels that were siphoning in juveniles of both species was 84.4%. In both species, more than half of the chambers had 100% of the burrowed mussels siphoning. Because siphoning was not a pre-determined sublethal endpoint, the specific effects of acclimation, treatment temperature, or flow regime to siphoning were not analyzed.

Byssus

The most parsimonious logistic regression model explaining byssus production in *L. abrupta* included treatment temperature and flow regime (Table 2). After controlling for the flow treatment, each degree increase in temperature reduced the odds of byssus production ($p = 0.0080$) by a factor of 0.826, or approximately 17%. While flow regime was included in the model as a negative effect, it did not significantly affect byssus production ($p = 0.1305$). In a second analysis that included data from our companion study (Archambault 2012b) and age as a factor, age explained a significant amount of the variation in byssus production ($p < 0.0001$), causing a reduction by a factor of 0.776, or approximately 22%, per month (Table 6). In addition to showing an effect of age, the most parsimonious model that used the two-study combined dataset also yielded significant negative effects of flow regime ($p < 0.0001$) and acclimation ($p = 0.0376$) on byssus production, in addition to temperature ($p < 0.0001$) (Table 6), similar to the incubator-only byssus model for *L. abrupta* in previous research (Archambault 2012b).

The most parsimonious logistic regression model explaining byssus production in *L. radiata* included treatment temperature and acclimation temperature; however, like flow regime in *L. abrupta*, the effect acclimation temperature on byssus production was not significant ($p = 0.1313$) (Table 2). Treatment temperature significantly reduced byssus

production in *L. radiata* ($p < 0.0001$). Every unit increase in temperature reduced the odds of byssus production by a factor of 0.803, or approximately 20%.

Discussion

We successfully established an ecologically realistic vertical thermal gradient within the sediment column during our laboratory sediment tests with juvenile mussels. Temperatures in the middle stratum of sediment within the five main treatment temperatures for each acclimation group averaged 1.8°C cooler than temperatures in the top stratum (Table 5, Figure 4). Within the control and acclimation treatments, the temperature differential averaged only 0.3°C. In a study conducted in streams of the Tar River Basin within the Piedmont region of North Carolina, temperatures 5 cm beneath the sediment-water interface averaged 1.9°C cooler than temperatures in the water column 10 cm above the sediment-water interface from July to October 2011 (T. Pandolfo, unpubl. data), similar to the conditions we tested in the laboratory.

Though juvenile mussels regularly burrow deeper than 5 cm, and even as deep as 20 cm (Schwalb & Pusch, 2007), the mussels in our study never descended deeper than the top stratum of sediment (2.5-cm). Schwalb & Pusch (2007) found that mussel surface densities of three unionid species were positively correlated with temperature and day length within the ranges studied (13.5 – 26.2°C, and 12.0 – 16.8 h day light, respectively) and negatively correlated with flow discharge, with discharge as the dominant factor, explaining 53% of burrowing behavior. Negishi et al. (2011) found juvenile (< 20 mm) *Pronodularia japanensis* (Lea), a freshwater unionid, near the surface (0 – 3 cm) in spring and summer,

and that 70% of all mussels studied – both juveniles and adults – descended to greater depths in winter (3 – 8 cm). Amyot & Downing (1997) also observed seasonal vertical movements, finding that *Elliptio complanata* (Lightfoot) had a peak surface abundance in July that was closely correlated with water temperature. Our experiments were conducted from June through August, so the lack of deeper burrowing in our observations may have been caused by environmental cues, such as summer day length, water treatment temperature, or a lack of flow. A recent study during a 15-week drought in Alabama, USA, found that when mussels in a river responded to stranding away from water by burrowing, they burrowed to only 3 – 4 cm depth (Gough, Gascho Landis & Stoeckel, 2012). If freshwater mussels, including juveniles, are more likely to remain near the surface in warmer summer months because of environmental cues, as evidenced in previous field research (Amyot & Downing, 1997; Schwalb & Pusch, 2007; Negishi et al., 2011), they may have limited drive or ability to escape excessively low flows, high water temperatures, or drying streams when such conditions are most likely to occur. The drive to escape unfavorable environmental conditions may be diminished by possible entrainment of their molecular clock by these environmental cues into a seasonal shallow burrowing pattern, and may explain why mussels in our study did not seek out more favorable conditions in the cooler refugia of the middle (2.5 – 6 cm) and bottom (6 – 8 cm) strata of sediment.

Another factor that may have precluded deeper burrowing by juvenile mussels in our study is the ratio of substrate size to body size. The mean body size of all mussels in our experiments (5.90 mm ± 1.80 mm) compared to the substrate particle size (0.500 – 0.850 mm) yields a body:particle size ratio range of 11.8 (smaller particles) to 6.9 (larger particles).

The large particles relative to body size and lack of fine particles may have been difficult for small mussels to negotiate to any appreciable depth. At least one study suggests that vertical movements in unionids may not be affected by particle size (Troia & Ford, 2010), but the finding is from one species, and related research has not been conducted with juveniles.

Survival

Evidence is mounting that freshwater mussels have a predictable and relatively narrow range of thermal tolerances, regardless of exposure conditions. Among the few published studies on acute lethal temperatures for early life stages of freshwater mussels, Pandolfo et al. (2010) reported 35.8°C (32.5 – 38.8°C) as the mean and range of 96-h LT50s for juveniles, and Dimock and Wright (1993) reported LT50s for juvenile *Utterbackia imbecillis* (Say) and *Pyganodon cataracta* (Say) as 31.5 and 33.0°C, respectively. In a companion study (Archambault, 2012a), we reported a mean juvenile LT50 of 35.6°C that ranged 33.3 – 37.2°C in acute exposures of five species, both in water-only and in tests with sediment and no vertical thermal gradient. The mean (32.8°C) and range (29.9 – 35.6°C) of LT50s for all experiments presented here, perhaps due the lack of burrowing to the available cooler depth strata, are similar to those studies, further substantiating the narrow range of upper lethal thermal limits and suggesting that many freshwater mussel species may respond similarly.

Lampsilis radiata was more thermally sensitive in the low water treatment, compared to dewatered, at the 22°C acclimation; the reaction was possibly similar in the 27°C acclimation, but a comparison was not possible because the LT50 could not be determined in the dewatered treatment due to lack of partial mortality responses. This finding is

counterintuitive based on our previous research in thermal tolerance experiments in incubator settings, where we found either no difference in survival among the flow regime treatments, or greater thermal sensitivity of species in the dewatered treatments (Archambault, 2012a). Moreover, field studies of mussel responses to severe drought conditions support the inference that extreme drought conditions are clearly more detrimental to survival and mussel community composition than moderate drought conditions (Haag & Warren, 2008; Galbraith, Spooner & Vaughn, 2010; Gough et al., 2012). The apparent greater tolerance in the dewatered experimental treatments may have been related to micro-scale thermal environments within test chambers, which may be further explored in future studies.

Effects of acclimation temperature on survival for a given species and flow regime treatment were not detected in our study. Acclimation effects on survival were also largely absent in our companion study (Archambault, 2012a) and in research by Pandolfo et al. (2010). These findings together further support the hypothesis that acclimation may be unimportant in acute lethal tests when acclimation temperatures are relatively high and proximate in range (22 and 27°C), despite our use of more conservative protocols for test organism acclimation than those recommended by the ASTM (2006a). However, recent thermal research with adult mussels, not following the ASTM (2006a) mussel early life stage acute guidelines, detected differences in temperature sensitivity between divergent cool and warm (15°C and 25°C) acclimation temperatures (Galbraith et al., 2012) when mussels were fed and held for 7 d prior to testing. Related future research with lethal temperatures and juvenile freshwater mussels may benefit from a longer acclimation period to determine any pattern of effect, but it may be impractical to hold early life stages of mussels for longer

periods while following the ASTM (2006a) guidelines for acute tests (i.e., not feeding test organisms). Such research should consider ecologically relevant acclimation temperatures (i.e., an average spring stream temperature for a given species and region would be most applicable to climate change research).

Burrowing

Increasing temperature significantly reduced burrowing in *L. abrupta* and *L. radiata*. In *L. radiata*, the effect was exacerbated in the dewatered treatment, compared to low water. These findings are consistent with results of burrowing analyses for the five species evaluated in the incubator-based temperature exposures of our companion study (Archambault, 2012b), and support previous research by Bartsch et al. (2000), who found that adult unionids took longer to upright in sediment and had lower survival after emersion for up to 60 minutes in high (45°C) air temperatures.

This is our second series of temperature studies in which we regularly observed juvenile mussels siphoning at the sediment-water interface. A majority of *L. abrupta* and *L. radiata* were observed siphoning (87.2 and 81.6% of those burrowed, respectively), and both species exhibited a maximum of 100% siphoning for a given treatment. In our companion study, we observed a grand mean of 53% of burrowed juveniles siphoning among all experiments, and means for individual species ranging from 32.2 – 68.4% (Archambault 2012b). Findings of several previous studies suggest that juvenile unionids remain burrowed in the sediment for the first two to four years of life, garnering their nutrition primarily from sediment pore water by employing a pedal feeding strategy (Yeager, Cherry & Neves, 1994; Balfour & Smock 1995; Strayer et al. 2004; Schwalb & Pusch 2007). Our results suggest

that juvenile freshwater mussels may regularly siphon at the sediment-water interface, and that future research and applications on diet, contaminant exposure, or other parameters with juvenile mussels should perhaps not rule out surface water as a potential source.

Byssus

Like burrowing, byssal thread production was negatively affected by elevated temperatures. Increasing temperatures reduced byssus production by 17 – 20% per degree Celsius. This finding supports those of our companion study, which evaluated the byssus production of three species, including *L. abrupta*, under similar conditions, and found a reduction of byssus by 18 – 35% per degree Celsius (Archambault, 2012b). Native freshwater mussels apparently use byssus chiefly for attachment to the substrate and for drift, typically as juveniles (Bradley, 2011). Diminished byssus production due to elevated stream temperatures may reduce the ability of young mussels to disperse, or conversely, to retain position within a stream bed. An environmental condition common to southeastern streams during summer is the combination of very low flows due to seasonal drought, followed by flashy stream conditions caused by strong thunderstorms (NOAA 2012a); our findings suggest that this combination may reduce byssus production, thereby inhibiting attachment, and then sweep juveniles downstream, resulting in mortality or dispersal to potentially unsuitable habitats.

Byssus production in both *L. abrupta* and *L. radiata* was not significantly affected by flow regime or acclimation temperature in this study. In our companion study of thermal experiments conducted in uniform temperature incubators, we observed that flow regime not only affected byssus production, but was the factor with the most drastic negative effect,

reducing byssus in the dewatered treatments by 93 – 99%, compared to low water, in the three species evaluated (Archambault, 2012b). While byssal thread production in unionids is poorly understood, scientists postulate that freshwater mussels form byssal threads via a series of chemical reactions, including oxidation reactions requiring water, similar to their marine bivalve counterparts (Waite, 1983; Cope et al., 1997). Therefore, despite a finding of no statistical effect of proxy flow in this study, we maintain that severe drought should be considered as a threat to byssus production and subsequent ability for juveniles to disperse or properly attach to substrates.

In a review of freshwater mussel byssus literature, Haag (2012) reported the byssus production in most species generally senesces by one or two years of age. We evaluated the byssus production behavior of *L. abrupta* in two studies where test organisms were from the same cohort and separated in age, affording the opportunity to directly compare behavior between very young (5 months old) and older (17 months old) juveniles. Our analysis of the apparent effect of age on byssus production explained a significant amount of the variation in byssus production ($p < 0.0001$). This finding supports the currently accepted concept that small, very young juveniles are the most prolific producers of byssus in most species and that production tapers with age and growth (Bradley, 2011; Haag, 2012). Moreover, the models substantiate the negative impacts of high temperatures and low flows to byssus production in native freshwater bivalves, and therefore, impacts to their attachment and drift capabilities.

Conclusions

We expanded upon our recently developed method of conducting thermal toxicity tests with freshwater mussels in sediment (Archambault, 2012a), and successfully incorporated a realistic vertical thermal gradient, a benthic environmental component found in nature. We observed a burrowing pattern in juveniles of remaining near the sediment surface, consistent with previous research suggesting that mussels remain near the sediment-water interface during warmer months (Amyot & Downing, 1997; Negishi et al., 2011; Gough et al., 2012). We report acute median lethal temperatures similar to those reported in recent research (Dimock & Wright, 1993; Pandolfo et al., 2010; Archambault 2012a, 2012b), and found that elevated temperatures were detrimental to burrowing behavior and byssus production in both species tested, and that flow may be important to these activities in some species, consistent with our previous research (Archambault, 2012b). Finally, the effect of age on byssus production showed that increasing age had a negative correlation with byssus production, consistent with recent findings by Bradley (2011).

Because no mussels burrowed beneath the top stratum of sediment, we were unable to assess whether the cooler sediment refugium strata may mitigate mortality or sublethal effects of thermal stress. Future studies may consider conducting thermal tests during early spring to elucidate effects of phenology on burrowing in juveniles, and using sediments collected from known mussel beds to test any effect of particle size on burrowing of very small juveniles.

Several recent studies, including our work here, have highlighted the sensitivity of freshwater mussels to excessive temperatures and drought in both lethal and sublethal

contexts (Dimock & Wright, 1993; Haag & Warren 2008; Pandolfo et al., 2009; Pandolfo et al., 2010; Archambault, 2012a, 2012b; Galbraith et al., 2012; Gough et al., 2012). The impetus for most of these studies is the status of the current climate trends of warming and increased stochasticity of precipitation patterns. The consequences of global climate change to aquatic ecosystems will likely result from altered intensity, variability, and distribution of precipitation, and more frequent flooding and droughts (Bates, 2008). Further, stream temperature in urbanized areas is greatly affected by reduction of riparian vegetation, modified flow regimes, and alteration of stream geomorphology (LeBlanc, Brown & FitzGibbon, 1997). While we suggest ways to refine future research approaches on these topics, it is perhaps more important for this knowledge to be promptly applied by resource managers in an effort to stem the ongoing decline of freshwater mussel abundance and diversity. Given the body of findings on freshwater mussel thermal sensitivity coupled with the knowledge of their importance to aquatic ecosystem functioning and a global climate forecast of increasing heat and more frequent and severe droughts in some regions, proactive management is warranted for avoiding additional losses of ecological integrity. Establishing clear fundamental objectives (e.g., protecting imperiled species or diverse mussel assemblages) and means objectives (e.g., limit thermal inputs to surface waters) for realizing the fundamental goals will enable resource managers to more clearly identify management actions (e.g., restore/maintain/protect riparian habitats; limit impervious area; initiate/maintain minimum flow standards) that are likely to conserve not only freshwater mussel diversity, but also ecological integrity of aquatic systems (Kwak & Freeman, 2010).

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Table 1. Burrowing behavior of *Lampsilis abrupta* and *L. radiata* tested in vertical-gradient thermal exposure experiments, as explained by the most parsimonious logistic regression models, selected using Akaike's Information Criterion, corrected for small samples (AIC_C). Let t = treatment temperature, f = flow regime, and a = acclimation temperature. Then let $x_1 = t$; $x_2 = f$; $x_3 = a$; $x_4 = t * f$; $x_5 = t * a$; $x_6 = f * a$; and $x_7 = t * f * a$. Coefficients for proxy flow regime are for the dewatered treatment with respect to low water (reference level), and all models = logit (burrowed/exposed), or the log odds of burrowing.

		***p*-values for partial slopes**						
Species	**Model**	x_1 **(temp)**	x_2 **(flow)**	x_3 **(acc)**	x_4 **(temp* flow)**	x_5 **(temp* acc)**	x_6 **(flow* acc)**	x_7 **(temp* flow* acc)**
Lampsilis abrupta	$y = 1.5615 – 0.0870x_1 + 0.0527x_3$	<0.0001		0.1278				
Lampsilis radiata	$y = 2.8958 – 0.1272x_1 – 5.6491x_2 + 0.0487x_3 + 0.0539x_4 + 0.1728x_6$	<0.0001	0.0019	0.3481	0.0719		0.0194	

Table 2. Byssus production behavior of *Lampsilis abrupta* and *L. radiata* in vertical gradient thermal exposures, as explained by the most parsimonious logistic regression models, selected using Akaike's Information Criterion, corrected for small samples (AIC_C). Let x_1 = treatment temperature, x_2 = flow regime, and x_3 = acclimation temperature. Coefficients for flow regime are for dewatered with respect to low water (reference level); all models = logit (byssus), or the log odds of byssus occurring; and odds ratios are point estimates, with 95% confidence intervals in parentheses.

		***p*-values for partial slopes**			**Odds Ratio Estimates**		
Species	**Model**	x_1 **(temp)**	x_2 **(flow)**	x_3 **(acc)**	x_1 **(temp)**	x_2 **(flow)**	x_3 **(acc)**
Lampsilis abrupta	$y = 3.5481 - 0.1908x_1 - 1.3608x_2$	0.0080	0.1305		0.826 (0.718 – 0.951)	0.256 (0.044 – 1.496)	
Lampsilis radiata	$y = 3.3183 - 0.2194x_1 + 0.1620x_3$	<0.0001		0.1313	0.803 (0.719 – 0.896)		1.176 (0.953 – 1.451)

Table 3. Median lethal temperatures (LT50) causing 50% mortality (with 95% confidence intervals) in juvenile mussels at 22°C and 27°C acclimation temperatures in low water and dewatered sediment exposures (96 h). LT50 values with the same letter for a given species are not significantly different. LT50 values between species for a given acclimation temperature and proxy flow regime treatment did not differ in any case. ND = value could not be determined.

	22°C Acclimation LT50		27°C Acclimation LT50	
Species	Low Water	Dewatered	Low Water	Dewatered
Lampsilis abrupta	31.9 A (30.7 – 33.3)	ND	33.3 AB (32.1 – 34.7)	35.6 B (34.7 – 36.5)
Lampsilis radiata	29.9 A (29.1 – 30.7)	34.8 B (34.1 – 35.6)	31.0 A (29.9 – 32.2)	ND

Table 4. Protection-level lethal temperatures (LT05) causing 5% mortality (with 95% confidence intervals) in juvenile mussels at 22°C and 27°C acclimation temperatures in low water and dewatered sediment exposures (96 h). LT05 values with the same letter for a given species are not significantly different. LT05 values between species for a given acclimation temperature and proxy flow regime treatment did not differ in any case that could be compared. ND = value could not be determined.

	22°C Acclimation LT05		27°C Acclimation LT05	
Species	Low Water	Dewatered	Low Water	Dewatered
Lampsilis abrupta	25.1 (ND)	18.7 (ND)	27.0 A (17.3 – 29.8)	31.3 A (29.1 – 32.6)
Lampsilis radiata	27.1 A (22.2 – 28.9)	ND	25.6 A (14.3 – 28.7)	32.5 (ND)

Table 5. Mean sediment temperatures (°C) with standard deviation in parentheses recorded every 30 minutes during 96-h experiments demonstrating establishment of a vertical temperature in each of the five treatment temperatures per acclimation group in the 22°C and 27°C acclimation tests. No vertical temperature gradient was intended in either the control (20°C) or acclimation (22 and 27°C) control treatments in each experiment.

		22°C Acclimation Test Treatment Temperatures (°C)													
		20		**22**		**27**		**31**		**33**		**35**		**37**	
	Sediment Strata	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**
L. abrupta	Top	20.6 (0.5)	20.9 (0.5)	23.0 (0.2)	23.3 (0.4)	26.3 (0.8)	26.8 (0.9)	27.8 (1.2)	27.0 (0.9)	31.8 (0.3)	28.6 (0.1)	32.6 (0.3)	31.0 (0.4)	35.7 (0.4)	32.3 (0.3)
	Middle	--	--	22.9 (0.2)	22.7 (0.2)	25.6 (0.6)	26.3 (0.8)	26.1 (0.8)	25.5 (0.7)	30.3 (0.4)	26.7 (0.1)	29.0 (0.4)	28.5 (0.4)	34.4 (0.5)	29.1 (0.2)
	Bottom	20.5 (0.5)	21.2 (0.4)	22.1 (0.3)	22.6 (0.3)	23.6 (0.3)	23.8 (0.4)	22.6 (0.3)	22.6 (0.2)	25.8 (0.4)	23.1 (0.2)	23.8 (0.3)	23.9 (0.3)	26.2 (0.4)	23.6 (0.2)
L. radiata	Top	20.6 (0.5)	20.5 (0.5)	23.1 (0.2)	23.2 (0.1)	26.9 (0.4)	27.1 (0.3)	31.0 (0.4)	30.0 (0.4)	31.8 (0.7)	30.9 (0.5)	34.0 (0.3)	33.5 (0.3)	35.5 (0.4)	35.4 (0.5)
	Middle	--	--	23.1 (0.1)	22.9 (0.3)	25.6 (0.3)	26.6 (0.4)	29.8 (0.3)	28.4 (0.3)	29.3 (0.5)	28.7 (0.3)	31.8 (0.3)	30.8 (0.3)	32.5 (0.5)	32.8 (0.5)
	Bottom	20.5 (0.5)	20.9 (0.5)	22.0 (0.3)	22.5 (0.3)	23.1 (0.2)	23.1 (0.1)	24.2 (0.1)	23.6 (0.1)	24.9 (0.3)	23.3 (0.2)	24.9 (0.4)	24.9 (0.9)	26.2 (0.6)	25.2 (0.4)

Table 5 Continued

		27°C Acclimation Test Treatment Temperatures (°C)													
		20		**27**		**31**		**33**		**35**		**37**		**39**	
	Sediment Strata	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**	**LW**	**DW**
L. abrupta	Top	20.9 (0.4)	20.1 (0.6)	26.4 (0.4)	26.9 (0.3)	30.0 (0.4)	30.0 (0.3)	31.9 (0.9)	31.5 (0.9)	34.7 (1.3)	34.6 (1.4)	36.9 (0.7)	35.8 (0.7)	37.8 (0.3)	38.1 (0.4)
	Middle	--	--	27.1 (0.4)	26.9 (0.3)	29.0 (0.3)	29.4 (0.3)	31.1 (0.8)	30.2 (0.5)	32.9 (0.9)	33.0 (0.9)	35.7 (0.6)	33.0 (0.4)	35.1 (0.3)	36.9 (0.4)
	Bottom	20.8 (0.4)	20.7 (0.6)	27.4 (0.2)	27.2 (0.2)	27.7 (0.2)	27.9 (0.3)	28.2 (0.2)	28.0 (0.3)	29.6 (0.3)	29.7 (0.3)	30.2 (0.3)	29.6 (0.3)	29.8 (0.2)	30.9 (0.4)
L. radiata	Top	20.8 (0.5)	20.4 (0.4)	27.5 (0.5)	27.8 (0.6)	31.4 (0.4)	31.1 (0.4)	31.5 (0.3)	31.6 (0.4)	33.7 (0.6)	34.3 (0.6)	36.0 (0.5)	35.0 (0.5)	37.2 (0.4)	36.1 (0.8)
	Middle	--	--	28.0 (0.4)	27.7 (0.5)	30.5 (0.3)	31.0 (0.4)	29.5 (0.2)	30.5 (0.4)	32.2 (0.5)	33.4 (0.6)	33.1 (0.5)	32.8 (0.8)	35.0 (0.5)	32.9 (1.2)
	Bottom	20.6 (0.6)	21.1 (0.4)	27.7 (0.2)	27.5 (0.2)	27.7 (0.5)	27.8 (0.5)	27.6 (0.5)	27.7 (0.5)	28.0 (0.2)	29.0 (0.3)	28.3 (0.6)	28.1 (0.6)	28.5 (0.2)	28.8 (0.4)

Table 6. Byssus production behavior of *Lampsilis abrupta* in 2011 (age 5 months) and 2012 (age 17 months) thermal exposures, as explained by the most parsimonious logistic regression models, selected using Akaike's Information Criterion, corrected for small samples (AIC_C). A highly significant effect of age on byssus production was detected. Let x_1 = treatment temperature, x_2 = flow regime, x_3 = acclimation temperature, and x_4 = age. Coefficients for flow regime are for dewatered with respect to low water (reference level); all models = logit (byssus), or the log odds of byssus occurring; and odds ratios are point estimates, with 95% confidence intervals in parentheses.

	***p*-values for partial slopes**				**Odds Ratio Estimates**			
Model	x_1 **(temp)**	x_2 **(flow)**	x_3 **(acc)**	x_4 **(age)**	x_1 **(temp)**	x_2 **(flow)**	x_3 **(acc)**	x_4 **(age)**
$y = 12.9959 - 0.1894x_1 - 2.2558x_2 - 0.1995x_3 - 0.2660x_4$	<0.0001	<0.0001	0.0376	<0.0001	0.827 (0.760-0.900)	0.105 (0.037-0.297)	0.819 (0.679-0.989)	0.766 (0.695-0.845)

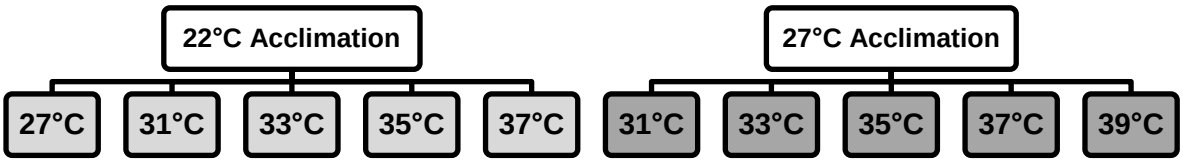


Figure 1. Schematic diagram of experimental design showing acclimation temperatures (22 and 27°C) and experimental temperature treatments for all juvenile and adult mussel exposures. All experiments employed a control temperature of 20°C.

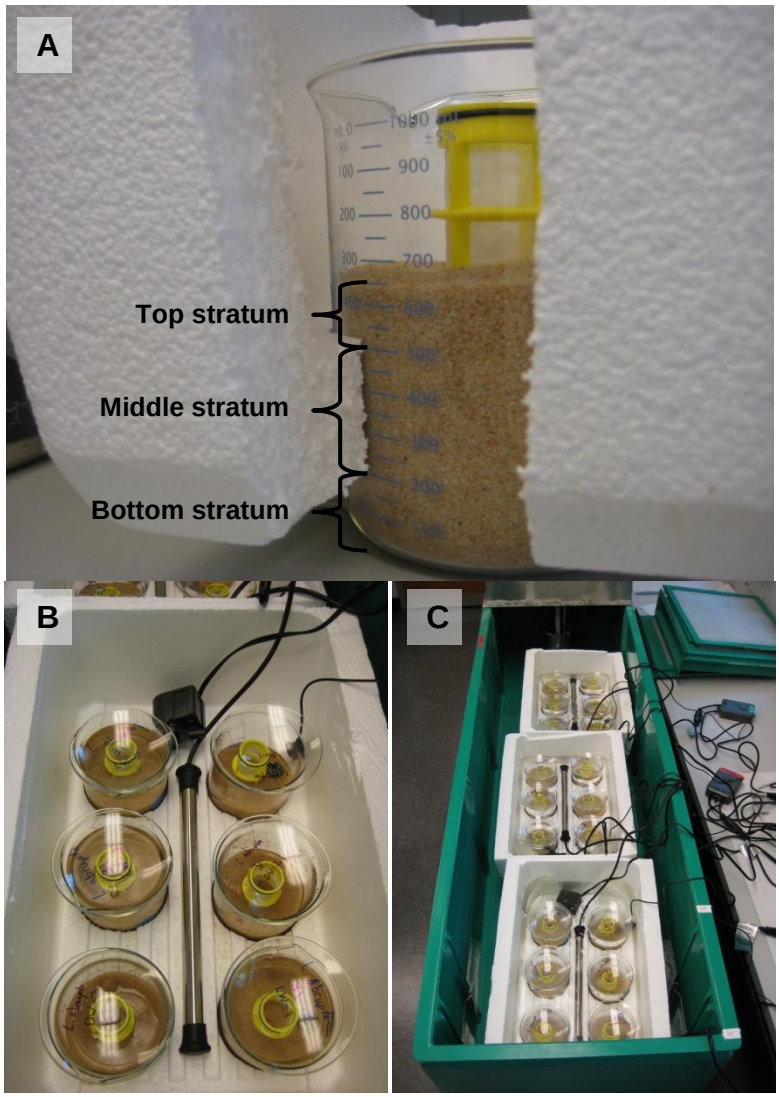


Figure 2. To create a vertical sediment temperature gradient, experimental chambers were sealed into coolers with the bottom protruding under the cooler and top held inside the cooler (A – cut-away side view of chamber and sediment strata). Experimental temperatures were employed inside the coolers using submersible thermostatically-controlled heaters (B) while coolers were floated in artificial streams (C), exposing the bottom sediment stratum to the experimental acclimation temperature.

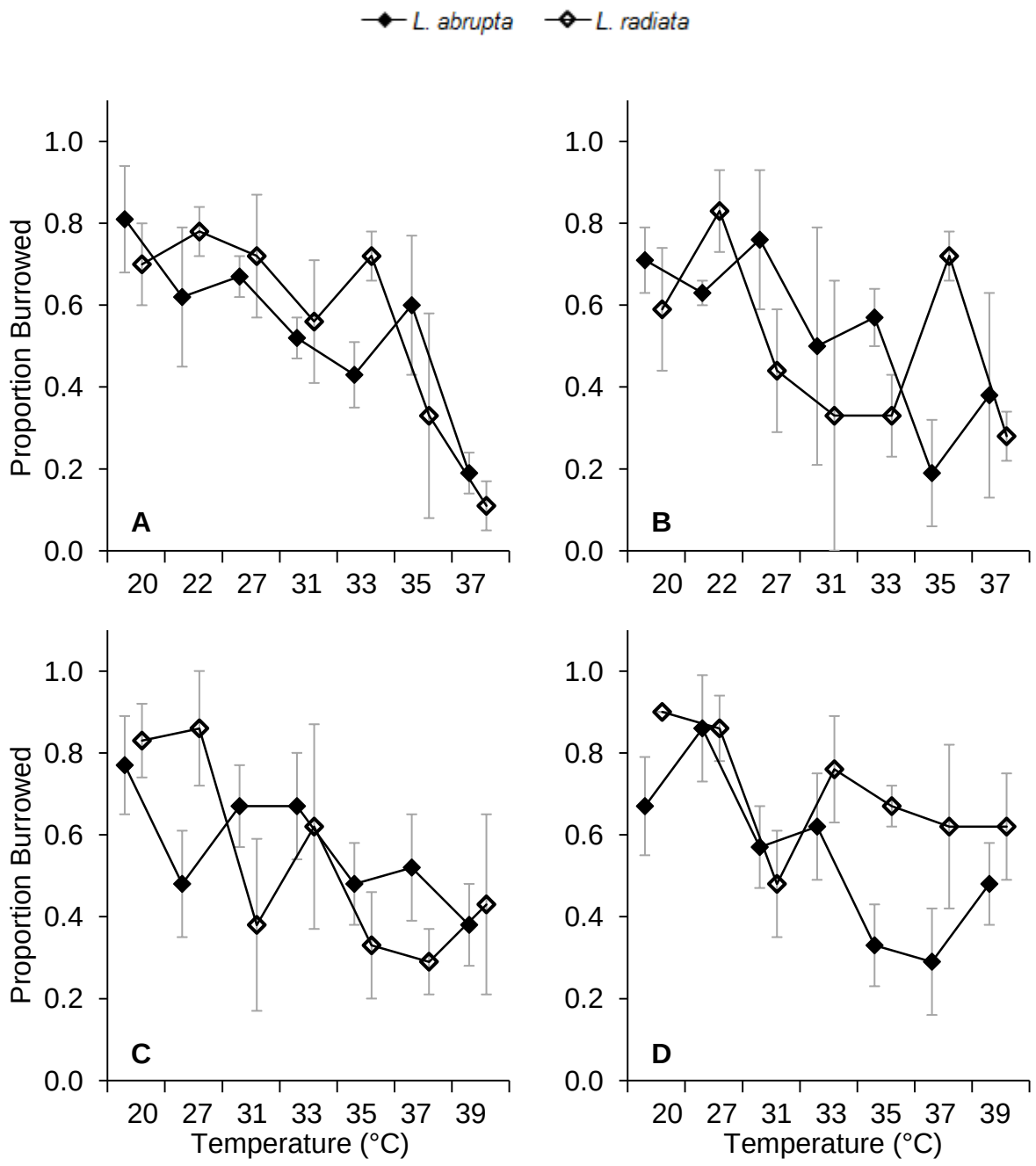
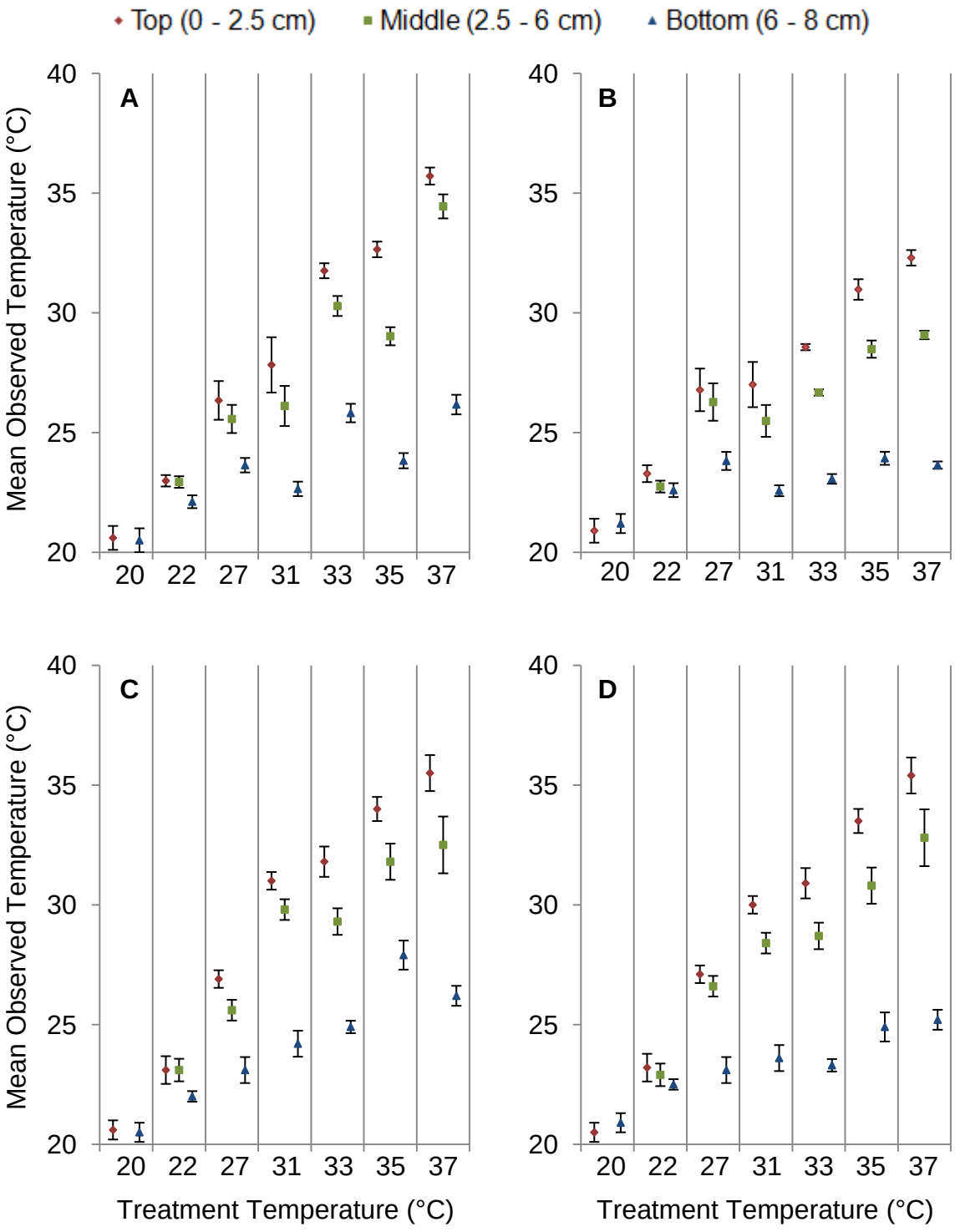
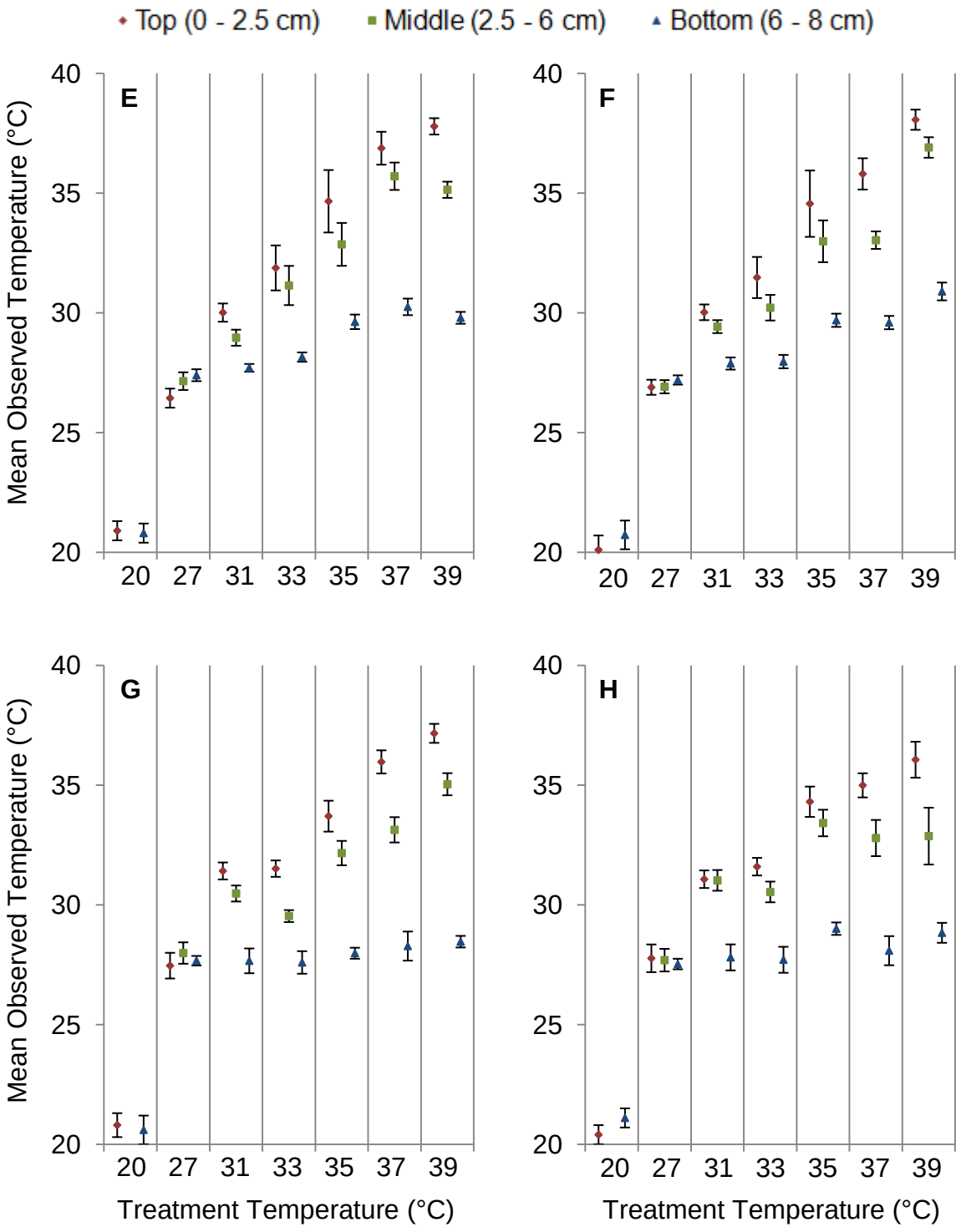


Figure 3. The mean (± SE) proportion of mussels that were burrowed at the end of the acute (96 h) exposures in the (A) 22°C acclimation, low water; (B) 22°C acclimation, dewatered; (C) 27°C acclimation, low water; and (D) 27°C acclimation, dewatered experiments.

Figure 4. The mean observed sediment temperatures (± SE) in the top, middle, and bottom sediment strata for each of seven treatment temperatures in the 22°C acclimation (A, *Lampsilis abrupta*, low water treatment; B, *L. abrupta*, dewatered treatment; C, *Lampsilis radiata*, low water treatment; D, *L. radiata*, dewatered treatment) and 27°C acclimation (E, *L. abrupta*, low water treatment; F, *L. abrupta*, dewatered treatment; G, *L. radiata*, low water treatment; H, *L. radiata*, dewatered treatment) vertical thermal gradient experiments.


Top (0 - 2.5 cm)
Middle (2.5 - 6 cm)
Bottom (6 - 8 cm)
A
B
C
D
Mean Observed Temperature (°C)
Treatment Temperature (°C)
40
35
30
25
20
20
22
27
31
33
35
37


Top (0 - 2.5 cm)
Middle (2.5 - 6 cm)
Bottom (6 - 8 cm)
E
F
G
H
Mean Observed Temperature (°C)
Treatment Temperature (°C)
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Appendices

Appendix A. Candidate models and selection criteria for analyses of burrowing behavior data of freshwater mussels in experiments discussed in Chapter 2. Legend: temp = temperature; flow = surrogate flow regime (low water, dewatered); acc = acclimation temperature. All models = logit (burrowed/exposed), or the log odds of burrowing.

Table 1. Candidate models and selection criteria for *Amblema plicata* (*n* = 42). Acclimation temperature was excluded because *A. plicata* were only tested at the 27°C acclimation.

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp	186.683	2	186.991	0.000	0.4836
temp + flow	186.747	3	187.379	0.388	0.3984
temp + flow + temp*flow	188.729	4	189.810	2.819	0.1181

Table 2. Candidate models and selection criteria for *Lampsilis abrupta* (*n* = 83).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + flow + acc + temp*acc	382.099	5	382.878	0.000	0.5720
temp + flow + acc + temp*acc + acc*flow	383.432	6	384.537	1.659	0.2496
temp + flow + acc + temp*acc + acc*flow + temp*flow	385.382	7	386.875	3.997	0.0775
temp + flow	387.365	3	387.669	4.791	0.0521
temp + flow + acc	388.671	4	389.184	6.306	0.0244
temp + flow + acc + temp*acc + acc*flow + temp*flow + acc*temp*flow	387.262	8	389.208	6.330	0.0242
temp	402.451	2	402.601	19.723	0.0000

Table 3. Candidate models and selection criteria for *Lampsilis cariosa* (*n* = 84).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + acc + temp*acc + flow + temp*flow	277.120	6	278.211	0.000	0.5269
temp + acc + temp*acc + flow + temp*flow + acc*flow	278.151	7	279.625	1.414	0.2599
temp + acc + temp*acc + flow + temp*flow + acc*flow + acc*temp*flow	278.100	8	280.020	1.809	0.2132
temp + acc + temp*acc + flow	299.438	5	300.207	21.996	0.0000
temp + acc + temp*acc	307.987	4	308.493	30.282	0.0000
temp + acc	313.378	3	313.678	35.467	0.0000
temp	352.218	2	352.366	74.155	0.0000

Table 4. Candidate models and selection criteria for *Lampsilis fasciola* (*n* = 112).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + flow + temp*flow	287.374	4	287.748	0.000	0.4138
temp + flow + temp*flow + acc	288.048	5	288.614	0.866	0.2683
temp + flow + temp*flow + acc + acc*flow	289.016	6	289.816	2.068	0.1471
temp + flow + temp*flow + acc + acc*flow + acc*temp +acc*temp*flow	289.722	8	291.120	3.372	0.0766
temp + flow + temp*flow + acc + acc*flow + acc*temp	290.163	7	291.240	3.492	0.0722
temp + flow	293.404	3	293.626	5.878	0.0219
temp	310.753	2	310.863	23.115	0.0000

Table 5. Candidate models and selection criteria for *Lampsilis siliquoidea* (*n* = 84).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + acc + flow + temp*flow + temp*acc + acc*flow + acc*temp*flow	602.135	8	604.055	0.000	0.8163
temp + acc + flow + temp*flow	607.433	5	608.202	4.147	0.1026
temp + acc + flow + temp*flow + temp*acc	608.119	6	609.210	5.155	0.0620
temp + acc + flow + temp*flow + temp*acc + acc*flow	610.092	7	611.566	7.511	0.0191
temp	635.898	2	636.046	31.991	0.0000
temp + acc	635.747	3	636.047	31.992	0.0000
temp + acc + flow	635.700	4	636.206	32.151	0.0000

Appendix B. Candidate models and selection criteria for analyses of freshwater mussel byssus production data in experiments discussed in Chapter 2. Legend: temp = temperature; flow = surrogate flow regime (low water, dewatered); acc = acclimation temperature. All models = logit (byssus), or the log odds of byssus occurring.

Table 1. Candidate models and selection criteria for *Lampsilis abrupta* (*n* = 84).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
flow + temp + acc	82.528	4	83.034	0.000	0.8560
flow + temp	86.305	3	86.605	3.571	0.1436
flow	104.276	2	104.424	21.390	0.0000

Table 2. Candidate models and selection criteria for *Lampsilis cariosa* (*n* = 84).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + flow	53.224	3	53.524	0.000	0.6748
temp + flow + acc	54.477	4	54.983	1.459	0.3253
temp	83.533	2	83.681	30.157	0.0000

Table 3. Candidate models and selection criteria for *Lampsilis siliquoidea* (*n* = 42).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + flow	35.934	3	36.566	0.000	0.9962
temp	47.317	2	47.625	11.059	0.0040

Appendix C. Candidate models and selection criteria for analyses of burrowing behavior data of freshwater mussels in experiments discussed in Chapter 3. Legend: temp = temperature; flow = surrogate flow regime (low water, dewatered); acc = acclimation temperature. All models = logit (burrowed/exposed), or the log odds of burrowing.

Table 1. Candidate models and selection criteria for *Lampsilis abrupta* in the vertical temperature gradient experiments (n = 84).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + acc	786.650	3	786.950	0.000	0.3663
temp	786.981	2	787.129	0.179	0.3349
temp + acc + temp*acc	787.711	4	788.217	1.267	0.1944
temp + acc + temp*acc + flow	789.440	5	790.209	3.259	0.0718
temp + acc + temp*acc + flow + temp*flow	791.371	6	792.462	5.512	0.0233
temp + acc + temp*acc + flow + temp*flow + acc*flow	793.363	7	794.837	7.887	0.0071
temp + acc + temp*acc + flow + temp*flow + acc*flow + acc*temp*flow	795.178	8	797.098	10.148	0.0023

Table 2. Candidate models and selection criteria for *Lampsilis abrupta* using data from experiments conducted in incubators and in vertical temperature gradient studies (n = 167).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + flow + temp*flow	1359.568	4	1359.815	0.000	0.3764
temp + flow	1359.944	3	1360.091	0.276	0.3278
temp +flow + temp*flow + acc	1361.474	5	1361.847	2.032	0.1363
temp + flow + temp*flow + acc + temp*acc	1362.636	6	1363.161	3.346	0.0706
temp	1364.235	2	1364.308	4.493	0.0398
temp + flow + temp*flow + acc + temp*acc + acc*flow	1363.866	7	1364.570	4.755	0.0349
temp + flow + temp*flow + acc + temp*acc + acc*flow + acc*temp*flow	1365.468	8	1366.379	6.564	0.0141

Table 3. Candidate models and selection criteria for *Lampsilis abrupta* using data from experiments conducted in incubators and in vertical temperature gradient studies, with an indicator variable for experiment type ("exp") (n = 167).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
exp + temp + flow + temp*flow	1224.389	5	1224.762	0.000	0.3913
exp + temp + flow	1224.942	4	1225.189	0.427	0.3160
exp + temp +acc + temp*flow + acc	1226.272	6	1226.797	2.035	0.1414
exp + temp + flow + temp*flow + acc + acc*flow	1227.296	7	1228.000	3.238	0.0775
exp + temp + flow + temp*flow + acc + acc*flow + temp*acc	1228.875	8	1229.786	5.024	0.0317
exp + temp	1229.947	3	1230.094	5.332	0.0272
exp + temp + flow + temp*flow + acc + acc*flow + temp*acc + acc*temp*flow	1230.184	9	1231.330	6.568	0.0147
exp	1351.684	2	1351.757	126.995	0.0000

Table 4. Candidate models and selection criteria for *Lampsilis radiata* (*n* = 84).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + acc + flow + acc*flow + temp*flow	739.922	6	741.013	0.000	0.4554
temp + acc + flow + acc*flow	741.177	5	741.946	0.933	0.2856
temp + acc + flow + acc*flow + temp*flow + temp*acc	741.385	7	742.859	1.846	0.1810
temp + acc + flow + acc*flow + temp*flow + temp*acc + acc*temp*flow	743.367	8	745.287	4.274	0.0537
temp + acc	747.848	3	748.148	7.135	0.0129
temp + acc + flow	747.873	4	748.379	7.366	0.0114
temp	759.794	2	759.942	18.929	0.0000

Appendix D. Candidate models and selection criteria for analyses of freshwater mussel byssus production data in experiments discussed in Chapter 3. Legend: temp = temperature; flow = surrogate flow regime (low water, dewatered); acc = acclimation temperature. All models = logit (byssus), or the log odds of byssus occurring.

Table 1. Candidate models and selection criteria for *Lampsilis abrupta* (*n* = 84).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + flow	47.677	3	47.977	0.000	0.4666
temp	48.283	2	48.431	0.454	0.3718
temp + flow + acc	49.595	4	50.101	2.124	0.1613

Table 2. Candidate models and selection criteria for *Lampsilis abrupta* using data from experiments conducted in incubators and in vertical temperature gradient studies (*n* = 167).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + flow + acc	170.210	4	170.455	0.000	0.6413
temp + flow	171.474	3	171.620	1.165	0.3582
temp	186.070	2	186.143	15.688	0.0003

Table 3. Candidate models and selection criteria for *Lampsilis abrupta* using data from experiments conducted in incubators and in vertical temperature gradient studies, including a variable for age ("age") (n = 167).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
age + temp + flow + acc	128.424	5	128.794	0.000	0.7685
age + temp + flow	130.948	4	131.193	2.399	0.2316
age + temp	151.015	3	151.161	22.367	0.0000
age	173.093	2	173.166	44.372	0.0000

Table 4. Candidate models and selection criteria for *Lampsilis radiata* (n = 84).

Candidate models	**AIC**	**K**	**AIC_C**	**Δ_i**	**w_i**
temp + acc	96.573	3	96.873	0.000	0.4401
temp	96.949	2	97.097	0.224	0.3935
temp + acc + flow	98.312	4	98.818	1.945	0.1664