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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 temperature 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 (22 and 27 °C) and two experimental water levels (watered and dewatered) as proxies for flow regime. Increasing temperature significantly reduced burrowing in all five 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 watered treatment (a proxy for low flow conditions): increasing temperature alone reduced byssus by 18-35%. Alanine aminotransferase and aspartate aminotransferase were significantly affected by treatment temperature in the 27 °C acclimation, watered 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.

**Keywords:** climate change; benthic fauna; stream flow; mussels; Unionidae; LT50; endangered species

#### Introduction

Aquatic fauna and flora are potentially affected by a myriad of stressors. Freshwater mussels (Unionida) are especially vulnerable to disturbance because they are incapable of escaping detrimental habitat 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;

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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.

Climate change will affect freshwater ecosystems through increased water temperature and altered intensity, variability, and distribution of precipitation, including more frequent flooding and droughts (Bates et al. 2008). Hydrology in the USA during 2011 was characterized by the NOAA as a year of extremes - areas that were extremely wet combined with other extremely dry areas accounted for a record high combination of 58% of the US land area affected (NOAA 2012a); global hydrology followed a similar trend in 2012, with some regions experiencing record drought while others were wetter than average (NOAA 2012b). The agency also reported that all 12 years in the twenty-first century (2001–2012) ranked among the 14 warmest in the history of the instrumental record of global surface temperature tracked since 1850, and 2012 was ranked as the warmest year ever recorded in US history and the tenth warmest year, globally (NOAA 2012a, 2012b). These climate statistics and other climate records in recent years exemplify climate trends of warming and extreme weather. Concurrent changes in land use, such as land clearing and urbanization, may also have deleterious consequences to freshwater habitats by contributing to additional heated point- and non-point-source effluents (Hester & Doyle 2011), thus exacerbating thermal stress to aquatic organisms. Stream temperature in urbanized areas is greatly affected by reduction of riparian vegetation, prevalence of impervious surfaces, modified flow regimes, and alteration of stream geomorphology (LeBlanc et al. 1997).

Though thermal inputs into freshwater systems are common, and despite extensive thermal research associated with fisheries and fish populations, research on thermal stress in 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 & Wright 1993; Pandolfo et al. 2010; Archambault 2012), but few studies have focused on sublethal effects of thermal stress (e.g., Spooner & Vaughn 2008 (physiological rates and condition); Pandolfo et al. 2009 (heart rate); Galbraith et al. 2012 (gaping)).

## Burrowing

Research on freshwater mussel burrowing has examined horizontal and vertical movements (Yeager et al. 1994; Schwalb & Pusch 2007; Allen & Vaughn 2009; Negishi et al. 2011), effects of sediment particle size on burrowing (Lewis & Riebel 1984; Troia & Ford 2010), and ecosystem services provided by bioturbation (Vaughn & Hakencamp 2001). Few studies have considered burrowing in juveniles (e.g., Yeager et al. 1994; Rogers 1999; Schwalb & Pusch 2007; Negishi et al. 2011), and research on the effects of stressors on burrowing is largely lacking (but see Nichols & Wilcox 1997; adults and zebra mussel (Dreissena polymorpha) infestation). 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. Because mussels are infaunal organisms, and juveniles may spend their first two to four years of life fully burrowed in the sediment (Yeager et al. 1994; Balfour & Smock 1995; Strayer et al. 2004; Schwalb & Pusch 2007), understanding the effects of thermal stress and other stressors on burrowing behavior is vital to mussel ecology and conservation.

## **Byssus production**

Research on stressors to byssus production has concentrated primarily on efforts to control the nonnative zebra mussel (D. polymorpha) (Clarke & McMahon 1996; Cope et al. 1997). Byssus is a thread-like material secreted from a series of glands in the foot of bivalve mollusks, allowing attachment to substrates and other surfaces (Cope et al. 1997). While byssal thread production in unionids is poorly understood, scientists postulate that freshwater mussels form byssal threads via a series of chemical reactions, similar to their marine bivalve counterparts (Cope et al. 1997). Native freshwater mussels apparently use byssus chiefly for attachment to the substrate and for drift, typically as juveniles, but also in some adults (Bradley 2011). Although some investigators have attempted to elucidate the role of byssus in unionid ecology, few have explored potential stressors to byssus production. Clarke 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. They concluded that thermal stress did not inhibit the physiological processes governing byssus production in zebra mussels. We investigated the effects of thermal stress on byssus production in juvenile native unionid mussels.

## **Biomarkers**

There are extensive examples of non-lethal techniques for assessing stress in marine and freshwater bivalves, but the vast majority of studies have focused on stress due to chemical contaminants (Gagne & Blaise 2003; Boutet et al. 2005) or events such as hypoxia (Lee et al. 2008), starvation (Patterson et al. 1999), and relocation (Naimo & Monroe 1999). While there have been advances in using biomarkers as a non-lethal means of evaluating stress in marine mollusks (Corporeau & Auffret 2003; Liu et al. 2004; Chen et al. 2007; An & Choi 2010), few studies have examined this question with freshwater bivalves (e.g., Greseth et al. 2003). 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 and low flow) in freshwater bivalves (2012 email from A. Fritts to JMA, unreferenced, see 'Acknowledgments'). 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.

## Materials and 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 (watered 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 guidelines (ASTM 2006a) as for water-only exposures to the extent practical, to ensure data quality and comparability.

# Test organisms

We tested five species of mussels representing two tribes in the Unionidae family, including *Amblema plicata* (Say) (tribe Amblemini); and *Lampsilis abrupta* (Say),

Lampsilis cariosa (Say), Lampsilis fasciola (Rafinesque), and Lampsilis siliquoidea (Barnes) (all Lampsilini). All juveniles and adults 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 USA that represented distribution in the Atlantic Slope and Interior Basins and that spanned a range of conservation statuses, from secure to federally endangered. *A. plicata* and *L. siliquoidea* are both widely-distributed species that are ubiquitous to the Interior Basin. *L. 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 USA (NatureServe 2011, North Carolina Wildlife Resources Commission 2011). *L. cariosa* is vulnerable or imperiled across much of its range and is further listed under some state wildlife Resources Commission 2011). *L. carolina* Wildlife Resources Commission 2011). *L. carolina* Wildlife Resources Commission 2011). *L. shupta* is federally-listed as endangered (US Fish and Wildlife Service 1985).

### Test chambers

We employed a novel nested-chamber static-renewal design for thermal exposure of juvenile mussels in sediment (Figure 1). The nested-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, which allowed for efficient recovery of juvenile mussels. The outer chamber was a 1-L glass beaker



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

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 maximum depth of 2.5 cm. Eighteen evenly-spaced 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 watered treatment, intended to simulate stagnant/low-flow stream conditions had 400 mL (5-cm depth) of overlying water. The total water volume added to the watered treatments averaged 508 ( $\pm$ 38, SD) mL. A dewatered treatment served to simulate drought conditions and included 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 ( $\pm$ 20) mL.

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, and meets or exceeds the current American Water Works Association Standard for Filter Material (Southern Products 2011). Before use, the sand was dry sieved to a more uniform size range of  $500-850 \,\mu\text{m}$  and heated to  $200 \,^{\circ}\text{C}$  in a drying oven to ensure the lack of organisms and low starting moisture content. Using this substrate may have practical limitations (e.g. it is unknown whether the lack of organics or varied particle sizes may affect burrowing), but it also does not introduce any confounding influences (e.g. parasites, pathogens, or chemical toxicants), making it well-suited for this application. In at least one unionid burrowing study, investigators noted that the natural sediment in the study area "consist[ed] almost exclusively of sand (mean particle size [D50]=0.42  $\pm 0.12 \,\text{mm}$ )" (Schwalb & Pusch 2007), indicating that the substrate we used sufficiently represents at least some natural sediments.

#### **Test conditions**

We conducted acute 96-h thermal exposures in the watered 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 2). Optimal acclimation and test temperatures were informed by results of Pandolfo et al. (2010), encompassed a range of probable summer stream temperatures, and ensured sufficient mortality in the highest treatments for calculating median lethal temperatures. Test temperatures in the 22 °C acclimation exposures ranged from 27 to 37 °C, and test



Figure 2. 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.

temperatures in the 27 °C acclimation exposures ranged from 31 to 39 °C (Figure 2). 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. Mussels used in the juvenile tests ranged in age from three to five months to ensure that they were large enough to recover from the sediment (>3 mm). 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 arrival temperature by 2.5 °C/d, with a standard minimum 24-h acclimation period once the target temperature was attained (ASTM 2006a; Pandolfo et al. 2010). The acclimation procedure used here is considerably more conservative than the recommended rate of 3 °C/h (ASTM 2006a). Shipping temperatures averaged 23 °C ( $\pm$ 1 °C, SD) from May through August, and 18 °C ( $\pm$ 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 placed in each of three replicates per treatment, with 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 ( $\pm$ 2 °C). Acute (96 h) experiments 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=1206) for 97.1% of trials and  $\pm 2$  °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  $\leq 1.1$  °C in watered exposures (n = 494) and by  $\leq 0.9$  ° C in dewatered exposures (n = 500). Mean water quality conditions among all juvenile tests were 108.2 mg CaCO<sub>3</sub>/L alkalinity, 143.3 mg CaCO<sub>3</sub>/L hardness, 534.9  $\mu$ S/ 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  $105.2 \text{ mg CaCO}_3/\text{L}$  alkalinity (n = 2),  $149.0 \text{ mg CaCO}_3/\text{L}$  hardness (n=2), 568.3 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 if they were not visible beneath the sediment-water interface. Those that were burrowed in the siphoning position as described were recorded as burrowed and siphoning. The presence of byssal threads on juvenile mussels in each chamber was assessed visually at the end of the tests using a magnifying lamp and was recorded using a 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 most parsimonious 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<sub>C</sub>; 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 sinus cavity of the anterior adductor muscle of each adult L. fasciola surviving the 96-h thermal exposures. To collect hemolymph, the mussel was gently pried open with a thin-blade knife just far enough to insert a 5 mm wide flat-end forceps to keep the shell open and expose the anterior adductor muscle, and a small sterile, 25-gauge needle on a 1.0 mL syringe (PrecisionGlide<sup>™</sup>, Becton Dickinson and Company, Franklin Lakes, New Jersey) was inserted into the anterior adductor muscle sinus. Up to 250 µL of hemolymph was extracted per mussel and expelled from the syringe (with the needle removed to prevent any potential physical damage to hemocytes) into a 2 mL cryogenic vial (Wheaton Science Products, Millville, New Jersey). Hemolymph taken from mussels in each replicate was combined 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, calcium, and bicarbonate in each hemolymph sample were analyzed by standard methods at 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)<sup>TM</sup> (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. watered 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 in juveniles, and that the dewatered flow regime simulation treatment also depressed these behaviors, compared to the watered 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 3). 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 & 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 watered and dewatered flow regime treatments in the 27 °C acclimation test, but were statistically significant only in the watered test; biomarkers were apparently unaffected in the 22 °C acclimation tests, even in the elevated treatment temperatures (Figure 4).

### **Burrowing behavior**

Treatment temperatures affected burrowing behavior in all five species observed (Figure 3). 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% CI, 0.645–0.807), or approximately 28%. Flow regime treatment did not significantly affect the burrowing behavior of *A. plicata* (Table 1 and Figure 3).

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 watered and dewatered flow regime treatments (Table 1 and Figure 3). The negative effect of temperature on burrowing was somewhat

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Species	Model	$x_1$ (temp)	$x_2$ (flow)	$x_3$ (acc)	$x_4$ (temp × flow)	$x_5$ (temp × acc)	$x_6$ (flow $ imes$ 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 siliauoidea	$y = 73.9041 - 2.1036x_1 - 83.6619x_2 - 2.3302x_3 + 2.4625x_4 + 0.0659x_5 + 2.9265x_6 - 0.0862x_7 + 2.0262x_7 + 2.026x_7 + 2.026$	0.0055	0.0020	0.0187	0.0019	0.0222	0.0048	0.0046
and the second second								
Notes: Let $t=t_1$	reatment temperature, $f$ =flow regime, and $a$ = acclimation temperature. The	an let $x_1 =$	$t; x_2 = f;$	$x_3 = a; x$	$_{4}=t\times f;\ x_{5}=t$	$\times a; x_6 = f \times a$	; and $x_7 = t \times x_7$	$f \times a$ . Models are

listed in order of increasing model complexity, coefficients for flow regime are for dewatered with respect to watered (reference level), and in all models,  $y = \log i t$  (burrowed/ exposed), or the log odds of burrowing.

		n-d	alues for partial slo	sade		Odds ratio estimates	
Species	Model	$x_1$ (temp)	$x_2$ (flow)	x <sub>3</sub> (acc)	$x_1$ (temp)	$x_2$ (flow)	x3 (acc)
Lampsilis siliquoidea Lampsilis cariosa	$y = 10.9634 - 0.3241x_1 - 3.66801x_2$ $y = 13.6241 - 0.4285x_1 - 5.1814x_2$	0.0032 0.0002	0.0071 0.0005	Not tested	$\begin{array}{c} 0.723 & (0.583 - 0.897) \\ 0.651 & (0.520 - 0.817) \end{array}$	$\begin{array}{c} 0.025  (0.002 - 0.367) \\ 0.006  (<\!\! 0.001 - 0.102) \end{array}$	
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)

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 watered (reference level); in all models, y = logit (byssus), or the log odds of byssus occurring; and odds ratios are point estimates, with 95% CI in parentheses.



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

Table 3.	Median	lethal	temperatures	(LT50)	causing	50%	mortality	and	protec	ction-le	vel	lethal
temperatu	ires (LTO	5) caus	sing 5% mor	tality (w	ith 95%	CI) i	in adult L.	fase	ciola 1	nussels	at	22 °C
and 27 °C	c acclima	tion ter	nperatures in	watered	and dew	vatered	d sediment	exp	osures	(96 h).		

	LT	50	LT05			
Lampsilis fasciola	Watered	Dewatered	Watered	Dewatered		
22 °C Acclimation 27 °C Acclimation	34.4 (33.5 – 35.4) 34.7 (33.9 – 35.7)	33.7 (32.1 – 35.4) 33.7 (32.5 – 34.9)	ND ND	26.6 (20.6 – 29.0) 28.4 (19.0 – 31.0)		

Notes: 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.



Figure 4. The mean concentrations of (ALT; light grey bars) and (AST; dark grey bars) ( $\pm$ SE) in composite samples of hemolymph extracted from surviving adult *L. fasciola* at the end of acute (96 h) exposures in the (A) 22 °C acclimation, watered; (B) 22 °C acclimation, dewatered; (C) 27 °C acclimation, watered; and (D) 27 °C acclimation, dewatered experiments. The vertical dashed line indicates the acute (96-h) median lethal temperature (LT50).

mitigated in the dewatered treatment compared to watered. 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 were reduced in the dewatered flow regime (p < 0.0001), compared to watered, 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 ( $\beta_1$ ) remained negative) (Figure 3). 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,  $\beta_1$  remained negative, regardless of the flow/acclimation treatment combination) (Table 1 and Figure 3). The interactive effects of temperature and flow regime were mitigative, as was shown in *L. fasciola*; however, as 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 3). 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 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 the 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 on 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. 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 watered treatment. Increasing temperature alone reduced byssus production in all species analyzed by 18–35%. Acclimation temperature reduced by succimation 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 watered treatment (Table 2).

Byssus production in *L. siliquoidea* was 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 watered (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 watered (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 watered 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 watered 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 4). ALT (p = 0.04) and AST (p = 0.02) concentrations were significantly increased by treatment temperature in the 27 °C acclimation, watered test. 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 4(C)).

Concentrations of ALT and AST showed a qualitatively similar response to temperature in the dewatered treatment of the 27 °C acclimation test (Figure 4(D)); 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 4(A) and (B)). Mean ALT concentrations in the watered and dewatered treatments ranged 7.5-11.2 and 3.8-7.0 IU/L, respectively, and mean AST concentrations ranged 12.6-17.0 and 6.0-13.4 IU/L, respectively.

## Discussion

Overall, we found that increasing temperature 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 watered 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  $\geq 93\%$  for all three 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 min in high (45 °C) air temperatures. The mitigative effects of some of the treatment interactions complicate the 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 the main effects. Mussels may burrow to escape high-velocity currents (e.g. flash floods) (Schwalb & Pusch 2007), to remove zebra mussel infestations (Nichols & Wilcox 1997), or to feed (Rogers 1999), and while adult mussels have evolved impressive adaptations for surviving emersion during lowflow events, survival time depends on temperature, humidity, and duration of the event (Byrne & 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, thinshelled 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 & Pusch 2007), and reproductive timing (Amyot & Downing 1997; Negishi et al. 2011), stream temperature regimes altered by climate change, point source, and nonpoint 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 life, garnering nutrients primarily from sediment pore

water by employing a pedal feeding strategy (Yeager et al. 1994; Balfour & Smock 1995; Strayer et al. 2004; Schwalb & 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. It is plausible that the lack of organic material and microbes in the filter sand used here may have affected this behavior, but food was also unavailable in the overlying water. 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 (Cope et al. 2008).

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 temperature, but flow regime had a greater effect than temperature. The dewatered flow regime reduced byssus by 93–99% among species, compared to watered – an intuitive finding, because water is used in the production of byssus (Waite 1983; Cope et al. 1997). Increasing temperature alone 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 USA 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 watered 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 4(C)), may be biologically meaningful. The LT50 for the 27 °C acclimation watered experiment was 34.7 °C (33.9-35.7 °C, 95% 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 4(D)) 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 (e.g. altered resource assimilation/excretion rates, Spooner & Vaughn 2008), 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 watered treatment). A recent severe drought in 2000 caused mussel density to decline as much as 83% in some southeastern USA streams. All species suffered losses, resulting in a reduction in species richness in these systems, primarily through the loss of rare species at rates similar to common species (Haag & 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 holding tanks weeks before the 27 °C acclimation and testing period were approximately 19 °C. The mussels were then ramped to the test acclimation temperature at a much more conservative rate than recommended by the ASTM (2006a) guideline. As no guidance for acclimation of adults currently exists, the protocols for juveniles (ASTM 2006a) were referenced 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. (2008) 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 & 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 and metabolomics (Corporeau & 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 on 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 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 (Galbraith et al. 2012), even when a short-duration acclimation period like that recommended by the ASTM (2006a) is applied. 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 et al. 2008), and because surface water temperatures are regularly impacted by anthropogenic activities (LeBlanc et al. 1997; Hester & 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 & 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 USA, where dams are plentiful and global mussel biodiversity is greatest (Bogan 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., 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 & 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 & 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 dewatering 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 USA 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 watered experiment, and showed a qualitatively similar response in the 27 °C acclimation dewatered experiment. The results of those 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 occurs in streams in the eastern USA, 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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