Health assessment of freshwater mussels using metabolomics

DISSERTATION

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By

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Abstract

Effective conservation of freshwater mussels (Mollusca: Bivalvia: Unionidae), one of the most endangered groups of animals in North America, is compromised by limited knowledge of their health. We address this gap in knowledge by characterizing the metabolic profile of *Amblema plicata* in the wild and in response to relocation, captivity, and food limitation.

Eight mussels brought into captivity from the wild were isolated for 18 days without a food source. Hemolymph samples were taken prior to, and 9 and 18 days after the start of the experiment; these samples were analyzed by gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. A total of 71 biochemicals were detected and identified in the hemolymph of freshwater mussels; of these, 49 showed significant changes during captivity and food limitation. Fasting resulted in severe metabolite depletion. Captive (but fed) mussels experienced changes similar to (albeit less severe than) fasting mussels, suggesting that mussels may experience nutritional deficiency under common captive conditions. *Amblema plicata* responded to food limitation stress by preferentially using energy reserves for maintenance rather than growth. Carbohydrate and energy metabolism exhibited down-regulation in captive, food-limited, and wild mussels. Lipid metabolism was up-regulated in captive/food-limited mussels and unchanged in wild mussels. Amino acid metabolism was up-regulated in wild mussels and down-regulated in captive/food-limited mussels.

Nucleotide metabolism was up-regulated in the wild mussels, down-regulated in food-limited mussels, and unchanged in captive mussels. The different responses between treatment groups suggest potential for nucleotide metabolism as a biomarker of health status for freshwater mussels.

Metabolomics techniques were also used to assess the physiological state of freshwater mussels relocated form the Muskingum River in Ohio to a conservation facility and to another stream in Ohio. Hemolymph samples were taken from mussels in all groups in September 2012, November 2012, May 2013, and August 2013. A total of 95 biochemicals were identified during the 2012 sampling period and 104 biochemicals during the 2013 sampling period. Glucose and lipid metabolism remained similar between all groups and sampling times. Differences between wild and relocated mussels were observed in altered amino acid, polyamine, methionine, and nucleotide metabolism. Decreased levels of these metabolites are likely responsible for the decreased growth rates and higher mortality observed in relocated mussels. The results are interpreted as indicative of a general stress response.

Climate change, pollution and other anthropogenic activities increase temperatures, decrease levels of dissolved oxygen, and acidify freshwater ecosystems. These changes have negative impacts on freshwater mussels. Effects on freshwater mussels include changes in behavior, respiration, filtration and excretion, heart rate, enzyme activity, biochemical composition, reproduction, and heavy metal uptake. This review focuses on the effects of temperature, dissolved oxygen, and pH stress on the

behavior and physiology of freshwater mussels in the families Unionidae and Margaritiferidae.

Dedication

For my husband, Brandon Sinn.

Thank you for all the love and support you have given me.

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I would like to thank Dr. Marymegan Daly for the friendly mentorship and thought-provoking instruction. Her help and dedication to my work is deeply appreciated. I would like to express my gratitude to Dr. G. Thomas Watters, who influenced my decision to come to The Ohio State University and who continually impresses me with his vast knowledge of mollusks. I would also like to thank Dr. Barbara A. Wolfe for her patient guidance and for inspiring me to pursue the field of metabolomics.

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Publications

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Fields of Study

Major Field: Evolution, Ecology and Organizmal Biology

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Chapter 1: Nontargeted metabolomics reveals biochemical pathways altered in response to captivity and food limitation in the freshwater mussel *Amblema plicata*

Introduction

Freshwater mussels (Mollusca: Bivalvia: Unionidae) are one of the most endangered faunal groups in North America (Lydeard et al., 2004). Of the 281 species native to North America, 232 are endangered, threatened, or of special concern (see the *IUCN Red List*: iucnredlist.org). These aquatic animals are exposed to a variety of threats, including the construction of impoundments, invasive species, agricultural runoff, and other nonpoint-source pollution (Strayer, 1999; Watters, 2000). The decline of freshwater mussels has spurred great interest in conserving, propagating, and restoring mussel populations to pre-anthropogenically altered levels (Bishop et al., 2007).

Conservation success depends critically on a detailed understanding of the physiology and biology of freshwater mussels. However, visual inspection of individuals is often insufficient to determine physiological condition. Hemolymph, the internal fluid that comprises the bulk constituent of mollusc circulatory fluid, can provide information relevant to health assessment. Non-invasive collection of hemolymph from the adductor muscle sinus (Gustafson et al., 2005a) has been used to study the chemistry and cellular components of freshwater mussel hemolymph to provide standardized approaches to the interpretation of health (Gustafson et al., 2005b; Burkhard et al., 2009). Knowledge of mussel hemolymph composition and its relationship to health, however, remains limited.

In addition to their status as an imperiled group, freshwater mussels are an excellent model for studying how invertebrates respond to environmental stress because, as relatively immobile organisms, they must rely primarily on physiological adaptations to survive in their environment. The few behavioral modifications available to mussels, such as closing the shell or burrowing deeper in sediment, may affect physiology through a reduction in feeding rates or a reliance upon less energy-deriving anaerobic respiration. A solid understanding of their physiology is crucial because they are often used as indicator organisms of aquatic ecosystem health (Williams et al., 1993).

Within the past decade, nearly a dozen mussel conservation facilities have become operational in the US. Some facilities have access to natural water sources (stream-side flow-through) and the mussels in these are assumed to feed on natural food sources. In the wild, the diet of freshwater mussels includes bacteria, algae, zooplankton, and detritus, although the exact proportions and between-species differences are still debated (Nichols and Garling, 2000; Gatenby et al., 2003). Compared to wild mussels, captive mussels often exhibit higher mortality (Cope and Waller, 1995) and stunted growth rates (Watters, unpubl.). Nutrition is one explanation: food may be diminished or even destroyed by the pumps, plumbing, and other equipment necessary to maintain flow. Some facilities attempt to grow their own food sources through extensive algal cultures, but these necessarily limit the available, and perhaps necessary, range of food available. In either case, artificially maintained systems also may not supply the food quality or quantity or water chemistry required by mussels to survive and reproduce. Further confounding the problem is the limited knowledge of freshwater mussel dietary

requirements (Nichols and Garling, 2000; Newton et al., 2013). At present, lack of food or otherwise inadequate nutrition is implicated as the primary reason of sub-optimal health in cultivated freshwater mussels. Effects of food limitation have been studied on other mollusks such as gastropods (Rosenblum et al., 2005; Moschovaki-Filippidou et al., 2013) and marine mussels (Tuffnail et al., 2009), but less research has focused on freshwater mussels (Vaughn et al., 2008). Increased knowledge about the effects of food limitation on freshwater mussel metabolism would improve current feeding and health assessment practices in captivity.

The objective of this study was to investigate differences in metabolism between wild, captive, and food-limited freshwater mussels using metabolomics. Our nontargeted metabolomics approach provides information on changes in small biochemicals, which are intermediates and products of various metabolic processes and are often first to respond to any environmental stimulus (Peñuelas and Sardans, 2009). Metabolomics provides a direct link between metabolic measurements and the organism's physiological status at a known time point (Viant, 2007). Identification and quantification of these metabolites through chromatography and spectrophotometry have been used to study the effects of toxicant exposure (Hines et al., 2010; Booth et al., 2011; Leonard et al., 2014), pathogen exposure (Lin et al., 2006), and responses to environmental stressors (Hines et al., 2007; Schock et al., 2010). Our study provides information on how the metabolism of the freshwater mussel *Amblema plicata* is altered in response to captivity and food limitation.

Methods

Experimental design

A total of eight freshwater mussels of the species *A. plicata* (Threeridge mussel) were collected in the Muskingum River in Washington Co., OH below Devola Lock & Dam #2 (39.468703 N, -81.489303 W) on June 28, 2012. The mussels ranged in length from 91.5 – 131.9 mm. Sex of mussels was not identified. Mussels were transported live in coolers filled with water from the Muskingum River to the Freshwater Mussel Conservation and Research Center (FMCRC) near Shawnee Hills, Delaware Co., OH. At the FMCRC, mussels were housed in tanks supplied with stream-side flow-through water from the Scioto River. After a seven week acclimation period, mussels were moved to a 76-L aquarium to eliminate incoming food sources. The aquarium was filled with the same water and equipped with an air pump and pebbles for substrate. There was no filtration system used so mussels could feed and deplete food at a natural rate.

Hemolymph samples from each mussel were taken in the wild immediately upon collection (T_0), after 7 weeks in captivity (T_1), and 9 days (T_2) and 18 days (T_3) after the start of food limitation. Mussels were sampled at T_0 to provide a measure of baseline hemolymph parameters to which all subsequent sample periods were compared. A seven week acclimation period was used in order to clearly differentiate between stress of captivity and of food limitation. As part of a separate project, hemolymph of a different group of *A. plicata* in the Muskingum River was sampled on June 28, 2012 and on September 12. Although the samples are taken from different individuals, the data are mentioned to provide information on how the metabolic profile of wild mussels changes during the study period. Samples were obtained from the anterior adductor muscle by

gently prying open the shell and penetrating the muscle with a 25 G hypodermic needle (Gustafson et al., 2005a). Approximately 200 µL of hemolymph was drawn from each individual. The number of mussels and volume of hemolymph sampled was determined in consultation with Metabolon, Inc. to provide statistically significant results via mass spectrometry analysis. The samples were transferred to 2-mL screw-cap cryotubes, snap-frozen in liquid nitrogen, and stored at -80^{0}C (Dunn and Ellis, 2005). The animals were returned to the Muskingum River after completion of the study. The Institutional Animal Care and Use Committee does not regulate use of freshwater mussels.

Sample analysis

Samples were shipped on dry ice to Metabolon, Inc. (Durham, NC) to be analyzed by gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS and LC/MS/MS). Further sample preparation and analyses were performed at Metabolon, Inc.; see Lawton et al. (2008) and Evans et al. (2009) for a complete description of their proprietary methods. Protein extraction was carried out using the MicroLab STAR system (Hamilton Company, Reno, NV). The samples were split into two aliquots, one for analysis on each platform. Organic solvents used in the extractions were removed by placing samples on a TurboVap LV Evaporator (Zymark Corp., Hopkinton, MA).

The LC/MS platform used an ACQUITY UPLC (Waters Corp., Milford, MA) and Finnigan LTQ mass spectrometer (Thermo Electron Corp., Waltham, MA). Samples analyzed on this platform were further split into two aliquots and reconstituted in either acidic or basic solvents containing at least 11 injection standards for quality control. For

the positive ion mode, extracts were gradient eluted with water and methanol each containing 0.1% formic acid. For the negative ion mode, extracts were gradient eluted with water and methanol each containing 6.5 mM ammonium bicarbonate.

Samples analyzed on the GC/MS platform were derivatized under dried nitrogen using N,O-Bis(trimethylsilyl)trifluoroacetamide and loaded on a 5% phenyl column with a temperature ramp of 16.25^0C/minute up to 300^0C. Samples were analyzed on a Finnigan Trace DSQ single-quadrupole gas chromatograph/mass spectrometer (Thermo Electron Corp., Waltham, MA) using electron impact ionization.

Statistical analysis

Data extraction, analysis, and visualization were completed using the Metabolon Laboratory Information Management System. Compound identification was obtained by comparison to the more than 1000 commercially available standards. Data were normalized to correct day-to-day instrument variation; the median of each compound was assigned a value of one. Two-way analysis of variance (ANOVA) was used to test for significant differences among treatments ($\alpha = 0.05$). As part of a separate project, pairwise comparisons between mussels sampled in the wild in September and those sampled in June were performed using Welch's t-tests ($\alpha = 0.05$).

Results

A total of 71 biochemicals of known identity were detected in the hemolymph of freshwater mussels. Of these, 49 showed significant changes during captivity and/or food limitation ($p < 0.05$). Changes were observed in biochemicals involved in energy, amino acid, carbohydrate, fatty acid, and nucleotide metabolism (Table 1). Most of these

changes were observed in captivity both prior to and during food limitation, with food limitation often eliciting a greater relative change.

Carbohydrate/ energy metabolism

Glucose, lactate, and succinate, which are indicators of energy production status, decreased when mussels were brought into captivity (Fig. 1) and changed by a factor of 0.32 ($p = 0.07$), 0.43 ($p < 0.001$), and 0.08 ($p < 0.001$), respectively (Table 1). During the food limitation experiment, lactate and succinate levels remained low while glucose levels increased slightly (Fig. 1). Wild mussels sampled in September exhibited significantly decreased levels of all these metabolites, including glucose, compared to June (T_0) (Table 1).

Lipid metabolism

Levels of the medium-chain saturated fatty acid heptanoate and the long-chain polyunsaturated fatty acids eicosapentanoate, and arachidonate increased in captivity and during food limitation (Fig. 2). Food limitation also caused an increase in levels of the long-chain polyunsaturated fatty acid eicosenaote (Fig. 2). The ketone body 3-hydroxybutyrate decreased significantly during captivity by a factor of 0.52 (T_1) and remained low during food limitation (T_2 and T_3). Levels of acetylcarnitine, an important indicator of energy reserves, exhibited a significant decrease in response to food limitation (T_2 and T_3).

	T_1	T_2	T_3	Wild in Sep.
Amino acid				
Serine	1.03	0.39**	0.28***	11.69***
Betaine	0.44*	0.67	0.32***	
Alanine	0.57*	0.55**	0.59*	0.78
N-6-trimethyllysine	1.48*	1.54*	1.56*	
N-6-acetyllysine	0.55*	0.46**	0.55*	1.03
Phenylalanine	0.38**	0.42**	0.51**	1.62*
Tyrosine	0.44*	0.50	0.46*	2.77*
Kynurenine	0.84	0.33***	0.38***	3.11***
Tryptophan	0.64*	0.51**	0.61*	1.87*
C-glycosyltryptophan	1.63***	0.93	0.79*	1.33**
Isoleucine	0.71	0.60*	0.56**	3.02**
Leucine	0.66	0.60*	0.55**	2.82**
Valine	0.86	0.55**	0.54**	2.92**
S-adenosylhomocysteine	0.73*	1.02	1.11	0.56*
Arginine	1.07	0.78	0.79	2.66***
Ornithine	0.55*	0.47	0.53	2.99*
Putrescine	0.41**	0.45*	0.47*	1.05
Agmatine	0.18*	0.55	0.50	1.13
4-guanidinobutanoate	1.01	0.27***	0.32***	1.56**
Carbohydrate/ energy				
Glucose	0.32	0.80	0.54	0.12**
Lactate	0.43***	0.42***	0.43***	0.29***
Succinate	0.08***	0.09***	0.26*	0.03***
Lipid				
Eicosapentaenoate	1.90**	1.45*	1.42	1.38
Heptanoate	1.48*	1.93***	1.91***	0.57***
Eicosenoate	1.92	1.19	2.30*	1.29
Arachidonate	2.03*	1.99*	2.11**	
Acetylcarnitine	0.80*	0.39***	0.32***	2.09**
3-hydroxybutyrate	0.52*	0.50*	0.53*	
Nucleotide				
Inosine	0.58**	0.13***	0.11***	2.31**
2'-deoxyadenosine	0.67	0.34*	0.42	0.60
Adenosine 5'-monophosphate	0.44	0.61	0.29**	1.17
Guanosine	1.78	0.46**	0.48**	2.74***
Allantoin	1.32	0.20***	0.26***	2.45**
Cytidine	0.67	0.28***	0.20***	1.30
Thymidine	0.79*	0.62**	0.62**	
Uridine	0.40**	0.19***	0.20***	1.12
Pseudouridine	0.85	0.26***	0.32***	2.05**

Table 1. Relative change in concentration of selected metabolites between wild mussels sampled in June compared to captive (T_1), one week food-limited (T_2), two week food-limited (T_3) mussels, and wild mussels sampled in September. Two-way analysis of variance (ANOVA) was used to test for significant differences among treatments (α =

0.05); as part of a separate project, pairwise comparisons between mussels sampled in the wild in September and those sampled in June were performed using Welch's t-tests (α = 0.05): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

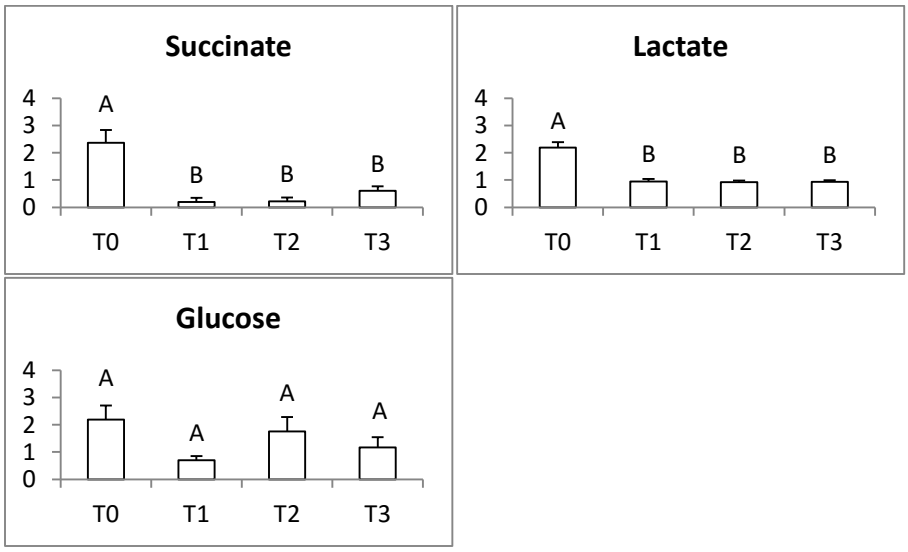

Figure 1. Metabolites associated with energy and carbohydrate metabolism in wild (T_0), captive (T_1), one week food-limited (T_2), and two week food-limited (T_3) mussels. The y-axis indicates the normalized relative level (+1 SE). Values with the same upper case letter are not significantly different ($p < 0.05$).

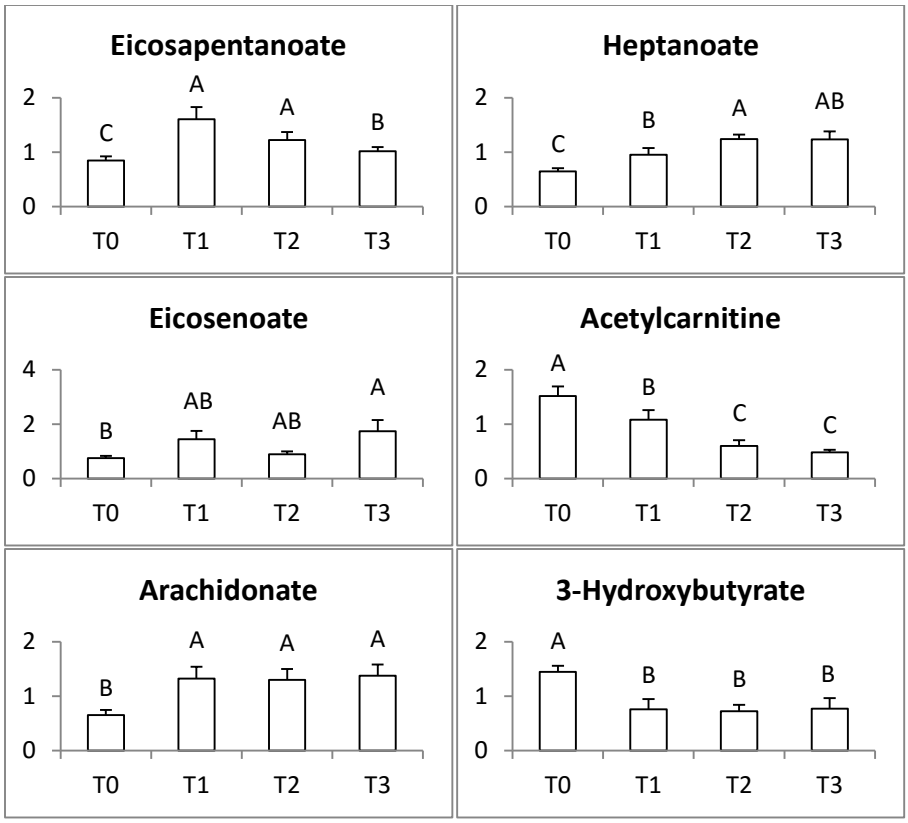

Figure 2. Metabolites associated with lipid metabolism in wild (T_0), captive (T_1), one week food-limited (T_2), and two week food-limited (T_3) mussels. The y-axis indicates the normalized relative level (+1 SE). Values with the same upper case letter are not significantly different ($p < 0.05$).

Amino acid metabolism

A conspicuous decrease was observed in the amino acids alanine, phenylalanine, tyrosine, and tryptophan (relative change: 0.57, 0.38, 0.44, and 0.64, respectively) in captive mussels (Fig. 3). These amino acids remained low during food limitation (T_2 and T_3) and were accompanied by significant decreases in levels of isoleucine and leucine (Fig. 3). Significant changes between sampling periods ($p < 0.05$) were observed in

metabolites associated with polyamine synthesis. Ornithine, putrescine and agmatine decreased when mussels were brought into captivity (T_1). Putrescine levels remained low during the food limitation experiment and were accompanied by decreased levels of 4-guanidinobutanoate (T_2 and T_3). In contrast, in wild mussels, amino acids and their products showed significantly elevated levels in September when compared to June (Table 1).

Nucleotide metabolism

Nucleotide metabolism exhibited significant shifts ($p < 0.05$) between sampling periods. Specifically, inosine, thymidine, and uridine levels decreased during captivity (T_1), changing by a factor of 0.58, 0.79, and 0.40, respectively (Fig. 4). These metabolites remained low during food limitation (T_2 and T_3). At T_2, decreases were observed in inosine, 2'-deoxyadenosine, guanosine, allantoin, cytidine, and pseudouridine, accompanied by a decrease in adenosine 5'-monophosphate (AMP) at T_3. Levels of inosine, guanosine, allantoin, and pseudouridine increased in wild mussels between June and September (Table 1).

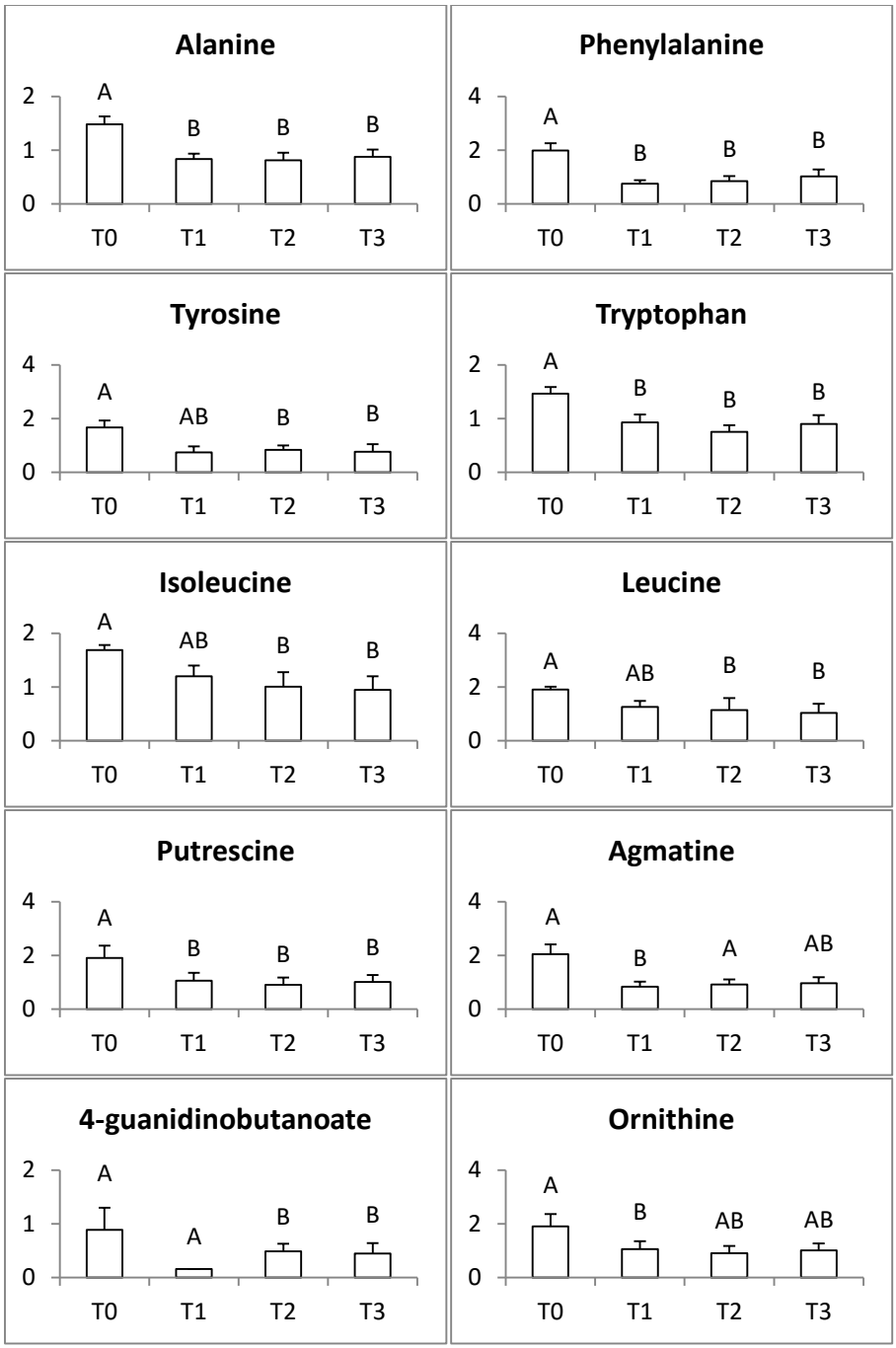

Figure 3. Metabolites associated with amino acid metabolism in wild (T_0), captive (T_1), one week food-limited (T_2), and two week food-limited (T_3) mussels. The y-axis indicates the normalized relative level (+1 SE). Values with the same upper case letter are not significantly different ($p < 0.05$).

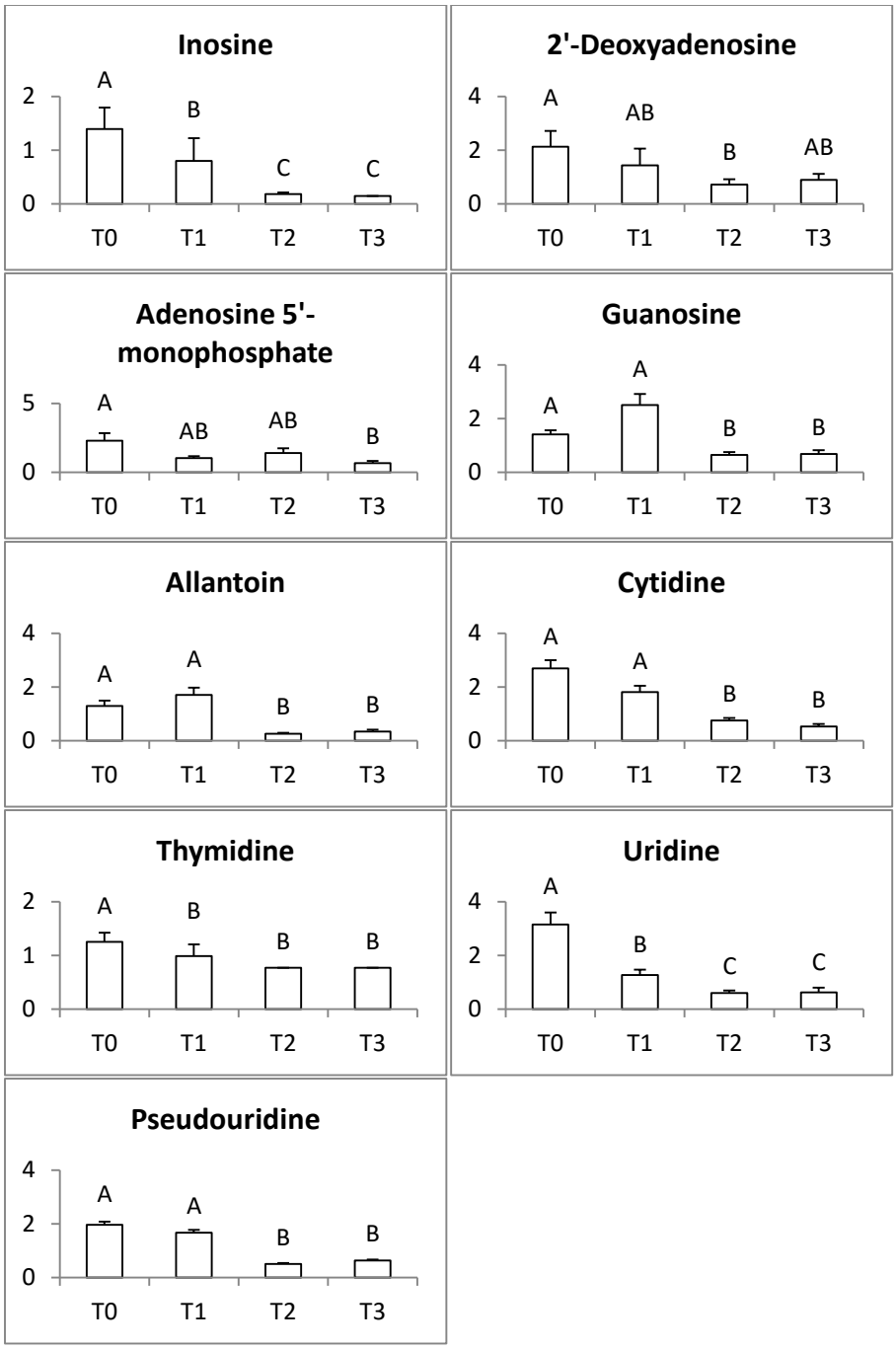

Figure 4. Metabolites associated with nucleotide metabolism in wild (T_0), captive (T_1), one week food-limited (T_2), and two week food-limited (T_3) mussels. The y-axis indicates the normalized relative level (+1 SE). Values with the same upper case letter are not significantly different ($p < 0.05$).

Discussion

Carbohydrate/ energy metabolism

The metabolites glucose, lactate, and succinate are involved in the Krebs cycle, which breaks down glucose for energy production. The observed increase in glucose during food limitation (Fig. 1) in the treatment group could be caused by the production of glucose through gluconeogenesis. During the initial stages of fasting, circulating glucose levels typically decrease, inducing animals to break down glycogen to produce glucose (McCue 2010). Patterson et al. (1999) show that mantle glycogen concentration in *A. plicata* is significantly lower in quarantined starved mussels than in fed mussels, suggesting that glycogen is mobilized during food limitation. Glycogen is a very limited resource, and resources are likely to be depleted if food limitation is prolonged. Rather than breaking down glycogen, animals can use gluconeogenesis to synthesize glucose from other sources, including lactate, pyruvate, and various amino acids. Our results suggest that *A. plicata* undergoes gluconeogenesis in response to food limitation. The initial drop in glucose in captivity is reversed over time, despite the absence of food. At the same time, and consistent with the expectations of gluconeogenesis, the levels of numerous amino acids decreased significantly in *A. plicata* (Table 1).

Restoration of glucose to pre-fasting levels has been observed in species such as Japanese quail *Coturnix japonica* (see Lamosova et al., 2004) and emperor penguins *Aptenodytes forsteri* (see Groscolas, 1986), a response referred to by McCue (2010) as "overcompensation". The spike in glucose is usually temporary and followed by a gradual decrease over the fasting period. We infer that *A. plicata* exhibits a similar

response under fasting. Under sustained fasting, levels will become dangerously low as the amino acid constituents are also depleted and the mussels may eventually break down proteins for energy. Mussels kept their shells gaped during the study and the low levels of succinate at T_1 and T_2 suggest that mussels were not undergoing anaerobic metabolism, although levels were higher at T_3 (Table 1).

Although measuring glucose levels is a common procedure for determining the health and energy status of many organisms, it is important to note that for *A. plicata*, the pattern is more complex, making glucose a poor marker for general health. Glucose levels may reflect seasonal changes (Roznere, unpubl. data), and do not respond linearly to food shortage, making it difficult to infer condition from glucose level. Furthermore, as highlighted above, the gluconeogenesis response masks nutritional status. Consequently, although energy and carbohydrate metabolites may seem like an appropriate choice for use as biomarkers, they do not provide a reliable measure of food limitation or condition.

Lipid metabolism

As fasting continues, animals often start to use fats as an energy source (Reshef et al., 2003). The significant elevation in free fatty acids in the hemolymph of *A. plicata* during captivity (T_1) and food limitation (T_2 and T_3) indicates that the mussels were beginning to break down stored triglycerides (Fig. 2). The significant decrease in acetylcarnitine (Table 1) also suggests that mussels were using this energy reserve for production of acetyl CoA.

The oxidation of fatty acids produces acetyl CoA, which is directly connected to the Krebs cycle and can also form ketone bodies through ketogenesis. Ketone bodies are

another important source of energy (Moyes et al., 1990) and an increase in ketone bodies is typically an indication that fats are being used for energy in endothermic animals (McCue, 2010) such as rats (Bates et al., 1968) and humans (Robinson and Williamson, 1980). Our finding that one of these critical ketone bodes, 3-hydroxybutyrate, decreased significantly (Fig. 2), is consistent with results of other studies of ectothermic animals (McCue, 2010). Studies of the red sea bream *Chrysophrys major* (Woo and Murat, 1981) and the green lizard *Ameiva ameiva* (Pontes et al., 1988) found that circulating levels of ketone bodies were reduced during food limitation. Our results highlight this difference between ectotherms and endotherms with respect to lipid metabolism, a difference that may reflect differences in average cardiac and neural tissue mass (McCue, 2010).

Amino acid metabolism

An increase in concentrations of free amino acids has often been attributed to degradation of proteins for fuel (e.g., Gillis and Ballantyne, 1996); that we observe a decrease in amino acids in *A. plicata* suggests that *A. plicata* is not switching from a lipid- to protein-based catabolism after two weeks of fasting. Instead, the decrease in circulating amino acid levels may be caused by the utilization of free amino acids in the Krebs cycle for energy production without a corresponding replenishment by proteolysis. Levels of amino acids in tissues are reported to increase in freshwater mussels such as *A.plicata* and *Elliptio complanata* after exposure to contaminants (Gardner et al., 1981; Day et al., 1990) but the relationship between energy demand and mode of action of toxicants is not clear. The effect that food limitation has on amino acid dynamics can also

be unpredictable and species-specific (e.g., Cook et al., 1972; Maity et al., 2012) and would benefit from further research.

Changes in polyamine levels may be indicative of nutritional stress (Stuck et al., 1996). The polyamine putrescine and the metabolites ornithine, 4-guanidinobutanoate, and agmatine that are associated with putrescine synthesis (Fig. 5) decreased significantly during the captivity or food limitation period. Polyamines interact electrostatically with the negative phosphate ions of DNA to facilitate the compaction of DNA during cell division (Iacomino et al., 2012). Because polyamines play this crucial role in cell proliferation, the significant decrease of putrescine and other metabolites in this pathway could explain the previously noted reduced growth rates of captive freshwater mussels. Decreased cell proliferation could also result in a reduced capacity to replace aging and damaged cells, possibly shortening an organism's life span. Although polyamines can be synthesized endogenously in many organisms, a large portion is obtained from diet (Kalač and Krausová, 2005; Larqué et al., 2007). The importance of an exogenous source of polyamines in freshwater mussels is unknown, but the presence of these compounds in algae (Tarakhovskaya et al., 2007) and bacteria (Busse et al., 1996), and the observed decrease here of putrescine and agmatine in *A. plicata* subjected to food limitation indicate that dietary intake may be crucial for mussel health. Alternatively, mussels may be conserving energy by sacrificing production of ornithine, agmatine, and putrescine from arginine in favor of maintaining synthesis of phospho-L-arginine (Fig. 5), which functions as an energy storage compound and regulates intracellular energy transport (Takeuchi et al., 2004). Levels of arginine showed a 0.78 fold decrease during food

limitation but these changes were not significant (Table 1) and, although phospho-L-arginine was not detected in this study, it is the only phosphagen found in mollusks and may play a critical role in times of reduced food availability due to its regulating effects on glycogenolysis (Ellington, 2001).

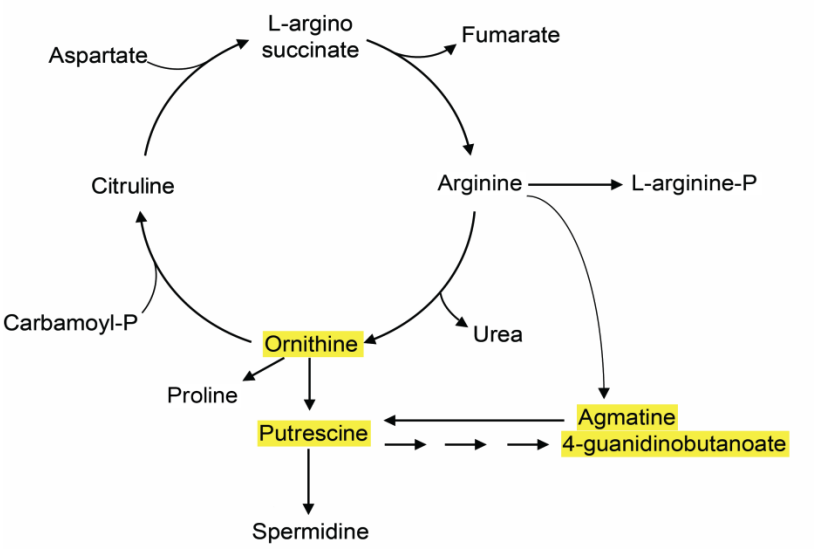

Figure 5. The arginine and proline metabolism pathway is involved in polyamine (putrescine and spermidine) synthesis. Metabolites that exhibited significant changes in response to captivity or food limitation are highlighted in yellow.

Nucleotide metabolism

Further evidence for reduced cell proliferation capacity was observed in changes in nucleotide metabolism, especially during food limitation. Decreased levels of numerous nucleosides (inosine, guanosine, cytidine, thymidine, and uridine) at T_2 (Fig. 4) indicated restricted synthesis of purine and pyrimidine bases. Adenosine 5'-

monophosphate (AMP) levels were significantly lower after two weeks of food limitation (T_3). Phosphoadenylate nucleotide values in the marine mussel *Mytilus galloprovincialis* correlate with seasonal changes in chlorophyll *a* concentrations (Blanco et al., 2006); chlorophyll *a* concentrations are expected to roughly reflect the density and composition of planktonic food sources. The effect of food limitation on amounts of RNA and DNA in tissues has been documented for the American oyster *Crassostrea virginica* (Wright and Hetzel, 1985) and the Japanese turban shell *Turbo cornutus* (Okumura et al., 2002). In both of these mollusks, a decrease in RNA: DNA ratios were good indicators of nutritional stress and reduced growth. In *A. plicata*, changes in levels of individual nucleotide bases show potential as biomarkers for nutritional status, as these tracked experimental condition and were generally significant between treatment groups (Table 1).

Conclusions

Metabolites associated with energy production were significantly reduced during captivity and food limitation. During food limitation, *A. plicata* appears to increasingly rely on gluconeogenesis and switch to fat (versus carbohydrate) as the energy source. Although captivity and food limitation elicited similar changes in metabolites, nucleotide metabolism was affected most strongly by food limitation. *Amblema plicata* responded to food limitation stress by preferentially using energy reserves for maintenance rather than growth. A decrease in metabolites participating in polyamine and nucleotide synthesis revealed lower cell proliferation capacity, which may account for reduced growth rates and earlier mortality observed in conservation facilities and relocation projects. Amino

acid and lipid metabolism differentiated the wild mussels from the captive and food-limited mussels; nucleotide metabolism exhibited a different response in all groups, showing potential as a biomarker of freshwater mussel health status. Metabolomics provides a means to study the effects of various diets that are provided for mussels in captivity and could be used to maximize growth and survival rates by adjusting environmental parameters.

Chapter 2: Health assessment of relocated freshwater mussels using metabolomics

Introduction

Freshwater unionid mussels (Mollusca: Bivalvia: Unionidae) are one of the most imperiled groups of animals in the world (Lydeard et al. 2004). Unionids, whose biomass can exceed that of all other benthic organisms in freshwater streams, play important roles in stream nutrient cycling through consumption, excretion, and biodeposition processes (Vaughn et al. 2004). However, their numbers are dramatically decreasing due to habitat alterations such as damming and dredging, point and non-point source pollution, and introduction of invasive species (Lydeard et al. 2004). Some of the more common conservation strategies include bringing mussels into captivity into research facilities or relocating them to other habitats (Kesler et al. 2007). Although critical to the success of this endeavor, knowledge of the impact of captivity and relocation on freshwater mussel health and appropriate health assessment techniques remains extremely limited.

It is difficult to assess the health of a mussel because the animal is enclosed between two shells and exhibits very few signs of stress or ill health that can be identified by non-invasive observation alone. Despite being potentially long-lived organisms with life expectancies exceeding 100 years, approximately half of the freshwater mussels that are relocated as part of conservation projects die, most within the first year (Cope and

Waller 1995). Reduced health, evidenced by low survival and low growth rates, is not uncommon in many captive facilities for mussels (Henley et al. 2001; Newton et al. 2001).

The health of a mollusk can be assessed through analysis of hemolymph, the bulk constituent of its circulatory fluid. Because it is non-lethal, collection of hemolymph from the adductor muscle sinus and heart ventricle (Gustafson et al. 2005a) offers an important advantage in health assessment studies with endangered or threatened species. This technique has been used to study the chemistry and cellular components of freshwater mussel hemolymph to provide standardized approaches to the interpretation of health (Gustafson et al. 2005b; Burkhard et al. 2009; Roznere et al. 2014).

The relatively slower growth and high mortality of captive mussels suggest that captivity is stressful for them. We aim to assess the physiological effects of relocation and of captivity on freshwater mussels and do this using metabolomics, the analysis of all the low molecular weight compounds present within a cell, tissue, or biofluid of an organism at a certain time period (Bundy et al. 2009). Because metabolites are interconverted by enzymes that are the products of gene expression, and the metabolites themselves regulate the biochemistry of cells, metabolomics provides a link between the genotype and phenotype. It provides information about the metabolic status of an organism and its phenotypical response to a particular environmental stimulus (Sardans et al. 2011). Metabolites dissolved in hemolymph are a direct link to physiological status at a known time point.

We evaluate metabolic profiles for wild, captive, and relocated (but not captive) populations of *Amblema plicata* (Threeridge) to determine whether captivity or the act of relocation can be localized as the source of decreased health for captive mussels. We use the metabolic profiles to understand the nature of the detrimental effect on their health. We see differences between the relocated and captive mussels, compared to wild populations, that are independent of seasonal fluctuations and highlight those aspects of physiology likely responsible for increased mortality and decreased growth of mussels moved or brought into captivity.

Methods

Experimental Design

We collected 66 freshwater mussels of *A. plicata* from the Muskingum River in Washington County, Ohio, USA, below Devola Lock and Dam #2 (39.468703 N, -81.489303 W) on June 28, 2012. We chose *A. plicata* because it is common, not listed by state or federal agencies, found in a wide variety of habitats, and is usually considered to be a tolerant species (Haag 2012). Mussels were transported live in coolers filled with water from the collection site to the Columbus Zoo and Aquarium Freshwater Mussel Conservation and Research Center (FMCRC) near Shawnee Hills, Delaware County, Ohio. At the FMCRC, mussels were housed in tanks supplied with stream-side flow-through water from the Scioto River. Half of the mussels were kept at the FMCRC for the duration of the experiment. The other half were transported to Big Darby Creek in Franklin County, Ohio (39.884722 N, -83.218256 W) after 15 days at FMCRC. To facilitate relocation during subsequent sampling dates, mussels released into Big Darby

Creek were tagged with Passive Integrated Transponder tags with underwater repair epoxy. A unique identifying number also was etched onto the outer shell. Hemolymph samples from each mussel were taken in September 2012 (T_1) and November 2012 (T_2). As part of a separate study, samples were also taken in May 2013 (T_3), and August 2013 (T_4). During sampling, mussels were collected haphazadly from the same location in the Muskingum River. Among the mussels brought into captivity, hemolymph samples were collected from the same eight mussels during each sampling period except at T_4, when mortality necessitated the use of three different mussels (originally collected from the Muskingum River at the same time). Among the mussels relocated to Big Darby Creek, the same eight mussels were sampled at T_1 and T_2 but mortality necessitated the use of six different mussels at T_3 and two different mussels at T_4 with a sample size of seven at T_4. It has been shown that repeated hemolymph sampling does not have a negative effect on freshwater mussels (Gustafson et al. 2005a), so we do not think mortality was due to sampling. Samples were obtained from the anterior adductor muscle by gently prying open the shell and penetrating the muscle with a 25 G hypodermic needle (Gustafson et al. 2005a). Approximately 200 µL of hemolymph were drawn from each individual. The number of mussels and volume of hemolymph sampled was determined in consultation with Metabolon, Inc. to provide statistically significant results via mass spectrometry analysis. The samples were transferred to 2-mL screw-cap cryotubes, snap-frozen in liquid nitrogen, and stored at -80^{0}C (Dunn and Ellis 2005). The animals were returned to the Muskingum River after

completion of the study. The Institutional Animal Care and Use Committee of the Ohio State University does not regulate use of freshwater mussels.

Metabolite analysis

Samples were shipped on dry ice to Metabolon, Inc. (Durham, NC) to be analyzed by gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS and LC/MS/MS). Further sample preparation and analyses were performed at Metabolon, Inc.; see Lawton et al. (2008) and Evans et al. (2009) for a complete description of their proprietary methods. Samples from 2012 and 2013 were analyzed separately. Protein extraction was carried out using the MicroLab STAR system (Hamilton Company, Reno, NV). The samples were split into two aliquots, one for analysis on each platform. Organic solvents used in the extractions were removed by placing samples on a TurboVap LV Evaporator (Zymark Corp., Hopkinton, MA).

The LC/MS platform used an ACQUITY UPLC (Waters Corp., Milford, MA) and Finnigan LTQ mass spectrometer (Thermo Electron Corp., Waltham, MA). Samples analyzed on this platform were further split into two aliquots and reconstituted in either acidic or basic solvents containing at least 11 injection standards for quality control. For the positive ion mode, extracts were gradient eluted with water and methanol each containing 0.1% formic acid. For the negative ion mode, extracts were gradient eluted with water and methanol each containing 6.5 mM ammonium bicarbonate.

Samples analyzed on the GC/MS platform were derivatized under dried nitrogen using N,O-Bis(trimethylsilyl)trifluoroacetamide and loaded on a 5% phenyl column with a temperature ramp of 16.25^0C/minute up to 300^0C. Samples were analyzed on a

Finnigan Trace DSQ single-quadrupole gas chromatograph/mass spectrometer (Thermo Electron Corp., Waltham, MA) using electron impact ionization.

Data extraction, analysis, and visualization were completed using the Metabolon Laboratory Information Management System. Compound identification was obtained by comparison to the more than 1000 commercially available standards. Data were normalized to correct day-to-day instrument variation; the median of each compound was assigned a value of one. Samples from groups T_1 and T_2 and groups T_3 and T_4 were analyzed separately using generalized linear models in SPSS.

Water chemistry and chlorophyll analysis

Water temperature, pH, dissolved oxygen, and conductivity were measured at each site at T_3 and T_4. Two liters of water were collected at each site at T_4 for Chlorophyll *a* (Chl *a*) analysis. The two liters from each site were split into four 500 ml aliquots and filtered through 47-mm Whatman GF/C glass microfiber filters. The filters were stored in photographic film containers at -20^0 C. Chlorophyll analysis was conducted using a modified EPA Method 446.0 with a correction for pheophytin (Arar 1997).

Results

A total of 95 biochemicals of known identity were detected in the hemolymph of freshwater mussels during the 2012 sampling period and a total of 104 biochemicals were detected during the 2013 sampling period. Detected biochemicals included compounds associated with carbohydrate/energy, amino acid, polyamine, methionine, and nucleotide metabolism (Table 2). Salinity, dissolved oxygen, temperature and pH at T3 and T4 in

the Muskingum River, FMCRC, and Big Darby Creek are shown in Table 3. Chl *a* averaged 23.0 (+ 1.6 SE) µg/L in the Muskingum River, 6.5 (+ 0.3 SE) µg/L in FMCS, and 1.0 (+ 0.1 SE) µg/L in Big Darby Creek.

Lipid and carbohydrate metabolism

At T_1, levels of fructose, galactose, glucose, lactate, arabinose, succinate, and malate were all similar between captive and Muskingum River (wild) mussels. Levels of fructose were significantly higher in Big Darby Creek mussels compared to wild mussels by a factor of 4.30 ($p < 0.01$) (Table 2) but all other metabolites were similar. By T_2, all metabolites associated with carbohydrate and energy metabolism in Big Darby Creek mussels were not significantly different from those of the wild mussels and remained so at T_3 and T_4. Most lipid metabolites were also similar across the groups. One exception was the decreased levels of acetylcarnitine in Big Darby Creek mussels at T_1 and T_2 by a factor of 0.24 ($p < 0.01$) and 0.18 ($p < 0.001$), respectively (Table 2).

Amino acid metabolism

At T_1 for captive mussels and T_1 and T_2 for Big Darby Creek mussels, levels of multiple amino acids were significantly lower compared to wild mussels (Table 2). Amino acid levels remained stable in the wild mussels between T_1 and T_2 and between T_3 and T_4. For example, levels of leucine were lower in the captive mussels at T_1 and in Big Darby Creek mussels at T_1 and T_2 than in wild mussels and remained constant in wild mussels over time (Fig. 6). By T_4, levels of most amino acids were similar to those found in the wild (Table 2).

	FMCRC				Big Darby Creek			
	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4
Amino acid								
Serine	0.11	0.74	0.52	2.19	0.04*	0.09	0.25*	1.15
Threonine	0.35	1.55	0.52	1.78	0.30**	0.46*	0.34***	1.41
Asparagine	0.15*	0.91	1.21	1.98	0.05*	0.11*	0.42	1.33
Alanine	0.62	1.90	0.69**	1.00	0.96	2.50	0.69*	0.86
Glutamate	0.58	0.75	0.39	0.44	0.71	0.37**	0.38	0.55
Glutamine	0.89	0.85	1.08	0.88	0.53	0.44	0.64	0.98
Histidine	0.22	3.14	1.12	2.95	0.08	0.08	0.32**	1.81
Formiminoglutamate			0.99	1.26			1.14	16.34
Lysine	0.22*	0.66	0.65	1.00	0.22**	0.19	0.43*	2.57
Phenylalanine	0.30**	0.57	0.52	0.69	0.20***	0.44	0.38*	1.26
Tyrosine	0.22	0.82	0.46	0.78	0.18	0.39	0.39	2.52
Tryptophan	0.41**	0.90	0.65	1.41	0.18***	0.51*	0.42**	1.19
Isoleucine	0.28**	1.08	0.58	1.34	0.17**	0.34	0.39*	1.03
Leucine	0.19**	1.18	0.40	1.37	0.13***	0.34*	0.27	1.19
Cysteine	0.39	0.75	0.53	0.76	0.39*	0.49	0.15*	0.75
S-methylcysteine	0.33**	0.33***	0.27**	0.67	0.34***	0.28***	0.20***	0.72
S-adenosylhomocysteine	0.58	0.71	0.49	0.44*	1.09	0.56	0.57	1.24
Methionine	0.08**	0.78	0.24*	0.88	0.10**	0.16*	0.17*	0.91
Arginine	0.38***	0.78	0.80	1.09	0.34***	0.45**	0.61**	0.92
Ornithine	0.24**	0.51	0.40*	0.36	0.27*	0.28	0.40*	0.76
Proline	0.64	0.68	1.02	1.02	0.35***	0.45*	0.61	1.00
2-aminobutyrate	0.44**	0.72	0.64	0.71	0.61	0.45*	0.45	0.66
5-methylthioadenosine	0.55*	0.82	1.09	1.04	0.73	0.58	0.61	0.55
Putrescine	0.41***	0.46*	0.24*	0.54	0.49**	0.39*	0.26*	0.50
Agmatine	0.90	0.28	0.36	0.29	1.11	0.47	0.17	1.19
4-guanidinobutanoate	0.65	0.29	0.98	1.57	0.17**	0.21**	0.53	0.40**
Carbohydrate/ energy								
Fructose	1.45	0.41	0.74	1.20	4.30**	0.60	1.38	0.69
Galactose	1.41	0.34	1.30	0.69	1.77	0.63	1.93	0.88
Glucose	1.90	0.47	1.37	1.47	5.84	0.48	3.44	0.91
Lactate	0.74	0.72	0.72	0.40	0.77	1.03	1.06	0.47
Arabinose	1.13	2.93	1.76	2.21	0.91	1.00	1.35	0.28
Succinate	1.21	0.28	5.33	0.41	2.24	5.48	0.74	0.23
Malate	0.43	0.86	4.10	0.31	0.74	1.44	3.40	0.31
Lipid								
Caproate (6:0)	1.19	1.07	1.28	0.97	1.15	1.26	1.29	0.84
Heptanoate (7:0)	1.17	0.87	1.15	1.43	1.18	1.31*	1.04	1.38
Caprylate (8:0)	0.99	1.01	0.80	0.88	0.93	0.98	0.82	0.99
Acetylcarnitine	0.45	0.45	0.89	1.01	0.24**	0.18***	0.54	0.36

Table 2. Relative difference in concentration between medians of selected metabolites of captive and relocated mussels compared to wild mussels at specified time points. Data were analyzed using generalized linear models: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

continued

Table 2 continued

Choline	0.54**	0.93	0.39	1.07	0.57**	0.96	0.58	0.90
Stigmasterol	1.05	0.98	0.84	0.66	1.15	0.95	0.71	0.94
Campesterol	0.83	0.92	0.70	0.87	1.10	0.84	0.96	1.26
Ergosterol	0.93	0.93	1.04	0.91	0.71	0.51*	0.67	0.74
Nucleotide								
Xanthine	0.40	2.66	1.02	1.16	0.17	0.41	0.66	0.37
Inosine	0.38	1.03	0.25	0.74	0.27*	0.29*	0.42**	0.44**
Adenosine	0.64	0.22	0.41	0.24	0.75	0.15	0.76	0.58
2'-deoxyadenosine	1.43	0.44	0.64	0.20	1.72	0.37	1.32	0.92
Guanine	0.36	1.17	0.56	1.38	0.32	0.36	0.40	0.36
Guanosine	0.37	0.70	0.29	1.26	0.22**	0.18**	0.51	0.51
2'-deoxyguanosine	0.39	0.58	0.59	0.86	0.68	0.23*	1.51	1.05
Allantoin	0.64	1.65	0.95	0.99	0.17*	0.26*	0.44	0.34**
Cytidine	0.60	0.87	0.76	0.92	0.40	0.20	0.45*	0.38
3-aminoisobutyrate	0.19**	1.49	0.44	1.11	0.36	0.67	0.23	0.96
Pseudouridine	0.46**	1.15	1.25	2.13	0.20***	0.29***	0.45**	0.81

	Muskingum River		FMCRC		Big Darby Creek	
	T3	T4	T3	T4	T3	T4
Salinity (µS)	520	454	650	370	730	596
Dissolved O_2 (%)	108.2	109.1	89.3	90.2	112.5	103.4
Temperature (°C)	17.9	24.3	17.9	24.3	18.0	22.5
pH	8.34	8.39	7.90	8.03	8.25	8.17

Table 3. Salinity, dissolved oxygen, temperature, and pH at T_3 and T_4 in the Muskingum River, FMCRC, and Big Darby Creek.

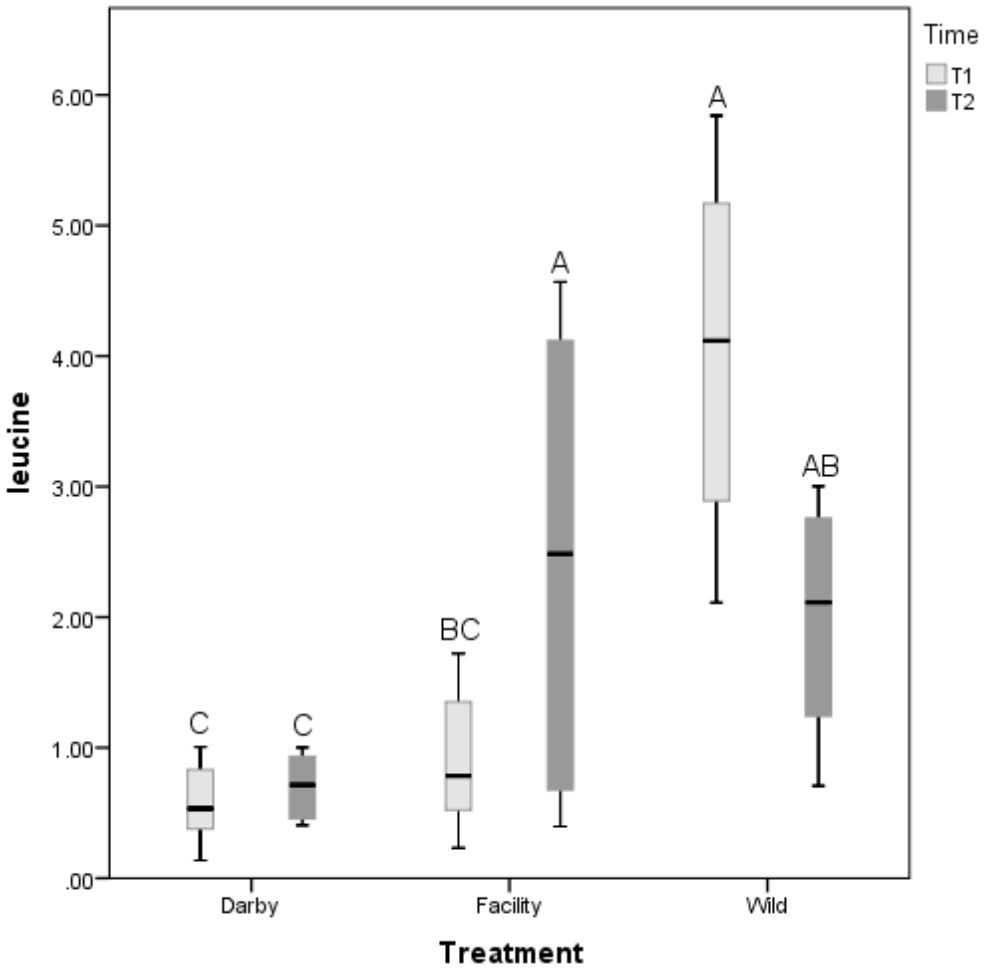

Figure 6. Levels of leucine in 2012. Data were analyzed using generalized linear models. The y-axis indicates the normalized relative level. Values with the same upper case letter are not significantly different ($p < 0.05$).

Polyamine metabolism

At T_1, captive mussels had significantly lower levels of the metabolites methionine, arginine, ornithine, and putrescine than wild mussels, and Big Darby Creek mussels had significantly lower levels of methionine, arginine, proline, and putrescine (Table 2). By T2 in captive mussels and T4 in relocated mussels, most of these metabolites were similar to those in the wild mussels. An exception was putrescine, which remained lower in the captive and Big Darby Creek mussels than in the wild mussels until T_4. Levels of metabolites associated with polyamine metabolism did not change between T_1 and T_2 or between T_3 and T_4 in wild mussels.

Methionine metabolism

Many of the metabolites involved in methionine metabolism were significantly lower in captive and Big Darby Creek mussels than wild mussels between T_1 and T_3 (Table 2). Cysteine levels in Big Darby Creek mussels were significantly lower than in wild mussels at T_1 and T_3. Levels of S-methylcysteine were significantly lower at T_1, T_2, and T_3 for captive and Big Darby Creek mussels. Levels of S-adenosylhomocysteine (SAH) were the same as those in the wild for all groups except for captive mussels at T_4. Levels of 2-aminobutyrate were significantly lower in captive mussels at T_1. Methionine levels were significantly lower for both relocated groups at T_1, for Big Darby Creek mussels at T_2, and for all relocated groups at T_3. By T_4, levels of all of these metabolites, except for SAH, were similar to those found in the wild mussels. Levels of metabolites associated with methionine metabolism did not change between T_1 and T_2 or between T_3 and T_4 in wild mussels. Formiminoglutamate was only detected in mussels sampled at T_3

and T_4 and levels in Big Darby Creek at T_4 were higher than in the Muskingum River mussels by a factor of 16.34 (Table 2).

Nucleotide metabolism

Levels of nucleotides and their degradation products, such as xanthine, guanosine, allantoin, cytidine, and pseudouridine were significantly lower in Big Darby Creek mussels than wild mussels (Table 2). Captive mussels exhibited decreased levels of 3-aminoisobutyrate and pseudouridine at T_1.

Discussion

Lipid and carbohydrate metabolism

The biochemicals fructose, galactose, glucose, lactate, arabinose, succinate, and malate, are involved in the Krebs cycle and are therefore good indicators of energy metabolism. The lack of significant differences between captive, Big Darby Creek, and wild mussels indicates that neither carbohydrate nor energy metabolism is affected by relocation. If mussels were relocated to environments with drastically different temperatures, these metabolites may have exhibited changes that are atypical of the pattern that we observed. There is evidence of decreased filtration rates (Vanderploeg et al. 1995; Loayza-Muro and Elias-Letts 2007) and decreased respiration rates of many freshwater mussels, including *A. plicata* (Huebner 1981; Baker and Hornbach 2001) at lower temperatures. Enzymatic activities of pyruvate kinase, citrate synthase, NADH dehydrogenase, cytochrome c reductase, and cytochrome c oxidase, all involved in either glycolytic metabolism or mitochondrial aerobic capacity, decrease in freshwater mussels exposed to colder temperatures (Doucet-Beaupré et al. 2010) and is probably the cause of

seasonally reduced growth rates in the wild (Versteegh et al. 2010). However, water temperature between the Muskingum River, Big Darby Creek, and FMCRC did not differ by more than 1.0^0C in May 2013 and 1.8^0C in August 2013 and levels of metabolites associated with carbohydrate and energy metabolism remained stable and similar in all groups.

Amino acid metabolism

Initial levels of amino acids tended to be lower in the captive and relocated mussels than the wild mussels. Seasonal changes in levels of free amino acids has been reported for other aquatic organisms, such as the freshwater amphipod *Gammarus pseudolimnaeus* (Graney and Giesy 1986) and the marine bivalves *Crassostrea virginica* (Feng et al. 1970), *Macoma balthica*, and *Mytilus* spp. (Sokolowski et al. 2003; Kube et al. 2007). The authors of these studies suggest that variation in free amino acid pools is related to environmental and physiological factors, including salinity, diet, reproductive cycle, and anaerobiosis. *Amblema plicata* develop glochidia from June – August (Ortmann 1919; Baker 1928; Heath et al. 1998), and the sharp increase in levels of amino acids was recorded in September, when spawning has presumably been completed. However, spawning in *M. balthica* resulted in a decrease in glycine concentrations (Sokolowski et al. 2003) and it is unknown whether marine and freshwater bivalves share the same patterns of free amino acid fluctuation.

Chl *a* concentrations were lower in the FMCRC and Big Darby Creek than in the Muskingum River, and a previous study of *A. plicata* revealed that food limitation causes a decrease in levels of hemolymph free amino acids (Roznere et al. 2014). However, food

limitation was also interpreted to cause an increase in hemolymph free fatty acids, a response not observed in this study (Table 2). Because we do not see any of the changes expected of food-limited mussels, it is unlikely that the low levels of amino acids in captive and relocated *A. plicata* were due to lack of food. Freshwater bivalves use free amino acids for osmoregulation, especially alanine and glutamate, and an increase in hemolymph amino acid content has been studied in *Ligumia subrostrata* in response to dehydration (Hanson and Dietz 1976). Dissolved oxygen levels were high in all study locations and, therefore, unlikely to have caused any significant changes in amino acids. Although salinity showed slight differences among locations, there was no consistent trend between salinity and an increase/decrease in levels of amino acids. The differences in amino acid levels between wild mussels and those in FMCRS and Big Darby Creek are likely a stress response due to relocation.

Polyamine metabolism

Levels of metabolites involved in polyamine synthesis also tended to be lower in captive and relocated mussels than wild mussels. Protein turnover is often depressed in response to stress (Hand and Hardewig 1996) and the increase in amino acids in the hemolymph may be caused by decreased rates of protein synthesis. Arginine metabolism produces amino acids such as proline and polyamines such as putrescine. Polyamines are essential for condensing new DNA with histone proteins prior to cell division (Iacomino et al. 2012). The significant decrease in the polyamine putrescine and metabolites connected to putrescine synthesis (arginine, ornithine, and agmatine) suggests decreased cell growth and proliferation, which in the long-term may impair tissue maintenance and

cause decreased growth rates. Seasonal variation in energy allocation for maintenance, reproduction, and growth has been observed in the freshwater mussel *Anodonta piscinalis* (Jokela 1996). Jokela and Mutikainen (1995) showed that, when subjected to the stress of relocation and decreased resources, *A. piscinalis* limits energy allocation for growth in favor of reproduction and maintains allocation of energy for maintenance. If *A. plicata* has a similar response, it is possible that relocation has negatively impacted growth but not necessarily reproductive capacity.

Methionine metabolism

The amino acid methionine is converted to S-adenosylmethionine (SAM), which serves as a methyl-donor and is converted to SAH (Fig. 7). Methionine is therefore an important source of methyl groups for transmethylation reactions (Finkelstein 1990). One pathway to methionine regeneration involves the methylation of homocysteine by methionine synthase using 5-methyltetrahydroflorate as a methyl donor (Fig. 7). Formiminoglutamate, a histidine breakdown intermediate, is a biomarker for nitrous oxide exposure (Deacon et al. 1983) and nitrous oxide is an inhibitor of methionine synthase (Koblin et al. 1981). Gut denitrification and nitrification during shell-coating biofilm production are two reactions that produce large amounts of nitrous oxide (Svenningsen et al. 2012) and are driven by environmental exposure to ammonium, nitrite, and nitrate (Heisterkamp et al. 2013). It is possible that the increased levels of formiminoglutamate in mussels at Big Darby Creek at T_4 are due to exposure to nitrogen fertilizer residues, which may also be the cause of the significant mortality of mussels at this time and location.

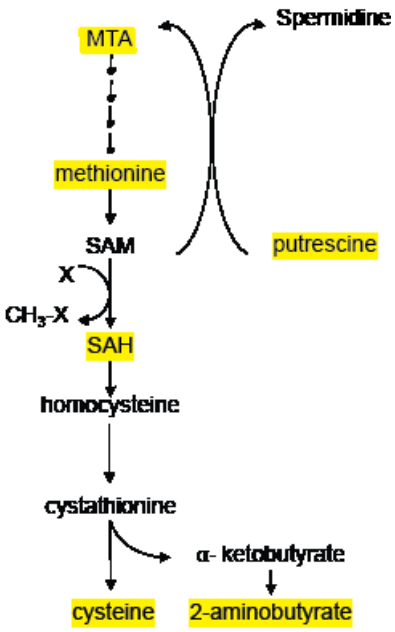

Figure 7. Methionine metabolism pathway. Metabolites that exhibited significant changes in response to relocation are highlighted in yellow.

Nucleotide metabolism

The altered profile of nucleotide metabolism for captive and relocated mussels could indicate stress-induced nucleotide degradation. DNA damage has been reported in *M. edulis* in response to heavy metals (Emmanouil et al. 2007), in the freshwater mussels *Elliptio complanata* and *U. tumidus* in response to municipal effluent (Gagné et al. 2011) and plant polyphenols (Labieniec and Gabryelak 2006), respectively.

Conclusions

This study assessed the impact of relocating mussels to a conservation facility or to another location to understand how general stress affects mussel physiology. When

studying a general stress response, it is necessary to look at the combined effect of all stressors over time and between locations, rather than the effect of any individual factor. The treatment groups varied in terms of metabolites associated with amino acid, polyamine, methionine, and nucleotide metabolism, which is indicative of reduced capacity for growth and cell maintenance, while levels of carbohydrate and lipid metabolites remained the same between groups. The observed responses are inconsistent with experimentally determined responses to food limitation but are consistent with other reports of general physiological stress. Because the change seen in relocated mussels can only be explained by relocation, we conclude that relocation is stressful to mussels. The similarity in response between captive and Big Darby Creek mussels suggests that a general stress response that is paramount in the health of captive mussels. This stress response was still evident a year after relocation. Furthermore, because *A. plicata* is considered a tolerant species, these effects may be even more significant in more sensitive species.

Chapter 3: The effects of water temperature, oxygen level, and pH on the physiology and behavior of freshwater mussels: a review

Introduction

Freshwater mussels are important components of aquatic ecosystems. They are active filter-feeders that play crucial roles in connecting the nutrient cycles between water and sediment (Strayer, 2014). Their numbers, however, have been declining in North America and worldwide (Williams et al. 1993). The main causes of this decline include the construction of dams in the 1900s, point and non-point source pollution, poor land use management practices, and introduction of zebra mussels (Lydeard et al., 2004). Many environmental variables that impact their health (body condition), such as temperature, oxygen levels, and pH are directly and indirectly affected by human activities. Thus, it is important to know the effects of specific environmental variables on freshwater bivalve physiology and behavior.

Freshwater mussels are especially vulnerable to environmental perturbations due to their sessile life cycle: adults spend most of their lives almost completely buried in the benthos of rivers and lakes. As benthic, filter-feeding animals, they are continuously exposed to contaminants found in freshwater ecosystems (Naimo, 1995). Futhermore, the high degree of specificity required to complete their complex life cycle makes them further vulnerable changes in environmental conditions at multiple life cycle stages. The

larvae of freshwater mussels (glochidia) are unable to transform into juvenile mussels if they are unable to attach to an appropriate fish host, and almost all glochidia are obligate parasites on fish (Watters et al., 2009).

Spatial and temporal variation in temperature may be beneficial to mussel communities by increasing the number of thermal niches (Spooner and Vaughn, 2009). However, many mussels are already living near their thermal limits (Spooner and Vaughn, 2008) and increases in temperature due to climate change and anthropogenic activities such as thermal effluent discharge from power plants and land clearing may negatively affect freshwater mussel communities and, subsequently, ecosystem function. The effects of climate change on mussel biology are especially hard to predict, and may result in loss of certain less tolerant species and species distribution shifts.

The global mean surface temperature in 2014 was reported to be 0.27 – 0.29˚C above the 1981 – 2010 average (Blunden and Arndt, 2014) and is projected to continue increasing at unprecedented rates. Freshwater mussels may be exposed to low levels of dissolved oxygen due to habitat location (e.g. pools versus riffles), temperature fluctuations, or eutrophication. Juvenile freshwater mussels are especially vulnerable since they spend most of their early lives buried in sediments and concentrations of interstitial dissolved oxygen (DO) in streams may be as low as 1 mg/L (Sparks and Strayer, 1998).

The quality of aquatic habitats can be altered via atmospheric deposition of acidic compounds and municipal wastewater effluent, which decrease the pH of the water. Lowered pH may also result from yearly snowmelt and episodic rain events. Although

the shells of freshwater mussels exhibit daily and annual growth rates that co-vary with long-term trends in temperature, this correlation disappears in mussels from polluted rivers with anthropogenically lowered pH levels (Dunca et al., 2005), suggesting that pH exerts a great influence on the physiology of freshwater mussels.

This literature review synthesizes the effects of temperature, oxygenations, and pH on freshwater mussel behavior and physiology. Alteration of these variables is caused by myriad anthropogenic factors such as climate change as well as more localized, regional disturbances such as nonpoint-source pollution. This review emphasizes the effects of individual variables on freshwater mussels, but we recognize that some are difficult to decouple (e.g., DO and temperature). However, in order to better understand these synergistic effects, we find it useful to discuss the effects of each of these environmental variables independently. Lastly, contaminants such as heavy metals and pesticides greatly affect freshwater mussels; however, excellent reviews of their effects preexist in the literature, and we do not redouble the efforts of these here (see Havlik and Marking, 1987; Naimo, 1995; Keller et al., 2007).

Effects of thermal stress

Several species of freshwater mussels have been found to fall into two groups based on temperature tolerance: those that are thermally tolerant and exhibit greater metabolic anabolism than catabolism at high temperatures (e.g. *Amblema plicata*, *Megalonaias nervosa*) and those that are thermally sensitive and exhibit greater catabolism than anabolism at high temperatures (e.g., *Lampsilis cardium, Quadrula*

pustulosa; Spooner and Vaughn, 2008); these results suggest that strong, species-specific responses to temperature exist. Galbraith et al. (2010) noted that during a period of drought in the Kiamichi River in the southern United States, the freshwater mussel community structure shifted to thermally tolerant species as the mortality rate of thermally sensitive species rose, suggesting that thermal tolerance of individuals can indeed translate to effects realized on the level of community structure. Thermal tolerance is often assessed using the critical thermal maximum (CTM), which describes the point at which an organism loses its ability to maintain homeostasis. CTM varies not only between species but is also dependent on acclimation temperature and level of aeration (Galbraith et al., 2012). Most studies have looked at temperature tolerance in juvenile mussels in the form of LT50s. The 96-hour LT50 is approximately 31.5˚C for juvenile *Utterbackia imbecillis* and 33˚C for *Pyganodon cataracta* (Dimock and Wright, 1993). Similarly, Pandolfo et al. (2010) found that LT50s of juveniles for several species range from 32.5˚C to 38.8˚C and that the range for glochidia can be much broader: between 21.4˚C and 42.6˚C. Conversely, Archambault et al. (2013b) found that LT50s of glochidia are, in general, similar to those of juveniles. Temperature tolerance appears to be species specific and may depend on the life cycle stage.

Respiration, as measured by O_2 consumption rates, tends to be highest in summer, lowest in winter, and is positively correlated with temperature (Baker and Hornbach, 2001; Lurman et al., 2014). For example, when *Parreysia corrugata* is subjected to increasing temperatures from 16˚C to 34˚C, oxygen consumption increases by 70% (Lomte and Nagabhushanam, 1971). The log of O_2 uptake by *Pyganodon grandis* and

Lampsilis radiata increases linearly up to 24˚C, indicating that the speed of physiological processes increases with increasing temperature (Huebner, 1982). However, the smaller increase in O_2 uptake between 24˚C and 31˚C suggests that these higher temperatures are physiologically stressful.

One way to measure responses to thermal stress is by using the temperature coefficient (Q_{10}) which is the factor by which a physiological process changes with each 10˚C increase in temperature. For most physiological processes in marine mussels, Q_{10} ranges from one to three (Zippay and Helmuth, 2012). Both Huebner (1982) and Myers-Kinzie (1998) found that the Q_{10} of *L. radiata* is about 3.4 when water temperature is between 15˚C and 25˚C. This Q_{10} value is relatively high, indicating that the metabolism of freshwater mussels is highly sensitive to temperature change. The increase in O_2 uptake is likely in response to an increased need for ATP at higher temperatures (Ganser et al., 2015). However, DO concentrations in water decrease with increasing temperature; this creates a critical limit past which mussels are not capable of maintaining homeostasis. Although one may think that this critical limit may change according to the temperature the organism has been used to, there is no evidence of such temperature acclimation in freshwater mussels (i.e., the rate of respiration change in response to increasing temperature is the same in the winter and summer; Huebner, 1982). Lack of acclimation may be an energy-saving mechanism during periods of seasonally low food supply, when the cost of increased metabolic activity would outweigh the benefits of nutrient acquisition. More work needs to be done to determine whether differences in Q_{10}

values and seasonal acclimation exist in other species to reach a more resolute conclusion on the effects of temperature on mussel respiration physiology.

Similar to O_2 consumption, filtration rates of *L. radiata luteola* roughly parallel the trend in seasonal temperature, increasing in summer and decreasing in winter (Vanderploeg et al., 1994). Loayza-Muro and Elias-Letts (2007) found that filtration in the tropical *Anodontites trapesialis* is highest at 20˚C and significantly reduced at 5˚C and 10˚C, accompanied by valve closure and mucus formation. As can be expected from increased filtration and, therefore, increased nutrient acquisition, growth is strongly correlated with differences in temperature (Hanson et al., 1988). Mussels also have higher ammonia and phosphorus excretion rates with increasing temperature (Spooner and Vaughn, 2012). Similarly, Christian et al. (2008) found that biodeposition of carbon, nitrogen, and phosphorus from *Actinonaias ligamentina* and *Ptychobranchus occidentalis* is higher in summer than in spring or fall. This can have cascading effects on the aquatic ecosystem by changing the water column nitrogen to phosphorus ratio (Vaughn et al., 2008). This ratio may further be altered if changes in temperature due to global warming cause shifts in species assemblages, since different species have different nutrient excretion rates (Vaughn et al., 2007). Thus, climate change induced shifts in freshwater mussel community structure can have significant impacts on nutrient cycling.

Studies on the effects of thermal stress on heart rate in freshwater mussels have produced conflicting results. In general, there is large variation in heart rate among species, ranging from 38 bpm for juveniles of *Ligumia recta* to 65 bpm for juveniles of *Potamilus alatus* when acclimated to 20˚C (Pandolfo et al., 2009). Most species tend to

increase their heart rate with increasing water temperature. For example, Dietz and Tomkins (1980) found that *Ligumia subrostrata* shows an exponential increase in heart rate with increasing temperature and a plateau at 31˚C. Sommerville (1975) described a linear relationship between temperature and heart rate in the range of 3˚C to 28.5˚C for *Anodonta cygnea*. Motley (1934) studied 45 species and also concluded that the correlation between temperature and heart rate is linear. Heart rate in juvenile *Villosa delumbis* and *L. recta* increases even up to 36˚C (Pandolfo et al., 2009). However, increasing temperature from 20˚C to 35˚C has no effect on the heart rate of juvenile *L. r. luteola* and causes a decrease in the heart rate of *Lampsilis abrupta* (Ganser et al., 2013). Acclimation temperature also has variable effects depending on the species. Acclimation temperature did not affect heart rate in *Pyganodon grandis*, whereas *U. imbecillis* acclimated at 15˚C had a lower heart rate at 30˚C than when acclimated at 25˚C (Polhill and Dimock, 1996). The use of heart rate as in indicator of thermal stress is advisable for only some freshwater mussel species.

Activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicators of amino acid metabolism, may increase in response to stress and indicate tissue damage (Fritts et al., 2015a). While levels of these enzymes increase with increasing temperature for *Elliptio crassidens*, *Villosa vibex,* and *Villosa lienosa* (Fritts et al., 2015a; Fritts et al., 2015b), AST shows no significant change across temperatures for *P. grandis* and *Pyganodon fragilis* (Doucet-Beaupré et al., 2010). However, *P. grandis* and *P. fragilis* were only tested up to 25˚C whereas *E. crassidens* and *V. vibex* were subjected to temperatures up to 35˚C. Other enzymes that increase with increasing

temperature in *P. grandis* and *P. fragilis* include citrate synthase (indicator of mitochondrial aerobic metabolism), pyruvate kinase (indicator of glycolytic metabolism), and NADP-dependent isocitrate dehydrogenase (indicator of redox balance) (Doucet-Beaupré et al., 2010). The activity of catalase, the enzyme that catalyzes decomposition of hydrogen peroxide to water and oxygen, is highest at about 10˚C in *Elliptio complanata* (Hobden, 1970). Contrastingly, Vidal et al. (2002) found no effect of temperature on catalase activity in *Corbicula fluminea*. Acetylcholinesterase exhibits a U-shaped response to temperature, decreasing between approximately 3˚C and 17˚C and increasing between 17˚C and 27˚C (Robillard et al., 2003). Lipid peroxidation is also affected by temperature, increasing at temperatures up to 25˚C and 30˚C (Falfushynska et al., 2014). Missing are studies that look at hemocyte count, phagocytosis, and lysozyme activity, all of which are involved in the immune system in bivalves and have been shown to change in response to temperature in marine mussels (Matozzo and Marin, 2011).

Seasonal variation has been observed in the biochemical composition of freshwater mussels (Baker and Hornbach, 2001). As temperature increases in the summer, so does the level of calcium in hemolymph of *Anodonta anatina* (Pekkarinen, 1997) and of *E. crassidens* and *V. vibex* (Fritts et al., 2015a), possibly due to the mobilization of calcium necessary for shell growth. Archambault et al. (2013a) did not find any significant correlation between temperature and calcium levels in glochidia, juveniles, or adults. Total lipid composition in gills also varies seasonally, tending to be highest in fall and lowest in winter, although Hagar and Dietz (1986) attribute the variation to the reproductive cycle, where an increase in total lipids would occur right

before spawning. Acclimatization to higher temperatures, although not prevalent with other physiological parameters, does increase levels of free amino acids and chloride in mussels (Rao and Ramachandra, 1961).

Heat shock protein (HSP) expression represents a promising avenue for future research on thermal stress effects on freshwater mussels (Wang et al., 2012). These molecular chaperones ensure the correct folding of new proteins and assist in the refolding of stress-induced misfolded proteins (Gupta et al., 2010). The marine mussel *Mytilus californianus*, for example, has higher levels of Hsp70 when found in non-shaded areas compared to those living in shaded areas. The study of heat shock proteins in freshwater mussels could provide a useful set of biomarkers for various environmental stressors in addition to temperature, such as hypoxia and high or low pH levels.

Temperature affects freshwater mussel behavior in addition to physiology. The rate and frequency of reestablishment after dislodgement increases with increasing temperature (Waller et al., 1999). *Anadonta anatina* burrows faster in the summer than in the winter (Lurman et al., 2014) and Block et al. (2013) found that while *P. alatus* fails to burrow 66.6% of the time at 10˚C, the failure rate at 30˚C is only 23.5%. Contrastingly, two studies have reported that burrowing is reduced at higher temperatures (Archambault et al., 2013a; Archambault et al., 2014). The surfacing of mussel populations during the spawning season is also thought to be caused by temperature cues. Watters et al. (2001) found that, of the eight species studied, all surface in the spring when water temperature increases and that some exhibit a bimodal pattern by reburying during elevated temperatures and resurfacing again when water temperature decreases. Vertical

movement in the benthos by *E. complanata* is also strongly correlated with water temperature; lateral movement in this species appears to be driven by cues based on day length rather than temperature (Amyot and Downing, 1997).

Although many streams have experienced a rise in average temperature due to anthropogenic effects due to industrial discharge and/or global climate change, some mussel species now reside in environments that are cooler than those in which they evolved. Namely, the benthos of pools immediately upstream of dams can experience artificially reduced temperatures due simply to depth. Because timing of reproduction is largely regulated by water temperature for several mussel species (Galbraith and Vaughn, 2009), one may expect impoundments to disturb the natural reproductive cycle. For example, *Megalonaias nervosa* living in impoundments in the Cumberland River where temperatures rarely exceed 20˚C fail to reproduce, but develop mature glochidia once translocated to an embayment of Kentucky Lake, where temperatures are warmer (Heinricher and Layzer, 1999), suggesting that a certain threshold temperature must be reached in order for most mussels to reproduce. Watters and O'Dee (2000) suggest that freshwater mussels use one of two types of reproductive strategies: they are either winter releasers, which require temperatures to drop below a certain threshold for glochidial release, or summer releasers, which require the temperature to exceed a certain threshold. A change in temperature caused both by direct (e.g., construction of impoundments) and indirect (e.g., global climate change) anthropogenic effects can influence mussel reproduction and, therefore, the survival of a population.

Not only can ambient water temperature influence the ability of a mussel species to initiate reproduction, temperature can affect individual portions of that cycle, such as the survival of glochidia. Once expelled from the marsupium, survival of glochidia is much higher at 0 ˚C and 10˚C than at 25˚C (Zimmerman and Neves, 2002). In a study by Tedla and Fernando (1969), 50% of glochidia survive for 120 hours at 10˚C but only for 12 hours at 20˚C. Similarly, survival of *Margaritifera laevis* glochidia is significantly higher at 10˚C than at 15˚C or 20˚C (Akiyama and Iwakuma, 2007). Therefore, timing of glochidia release in winter or spring is likely due to the greater survival of glochidia at lower temperatures.

Timing of glochidial metamorphosis on fish hosts is also influenced by temperature. Once attached to an appropriate fish host, low temperatures may facilitate the successful transformation of glochidia by depressing the fish immune system (Roberts and Barnhart, 1999). Many freshwater mussel glochidia species have a $Q_{10} > 4$ for temperature effect on transformation period (Roberts and Barnhart, 1999). Such a high Q_{10} translates into slow development rates in cold temperatures, which may be beneficial during the less than ideal conditions of winter, and high development rates in warm temperatures, resulting in quick growth during spring. In general, growth of juveniles is positively correlated with temperature (Carey et al., 2013), and reaching a larger size faster could increase the probability of survival for juveniles, which are vulnerable to size-dependent predation. Metamorphosed juveniles of *Margaritifera margaritifera* require that water temperature be above 15˚C for at least 14 days to complete development and release from the fish host (excystment; Hruška, 1992); those

of *L. cardium* excyst from the fish when a temperature threshold between 10˚C and 15.5˚C is reached (Watters and O'Dee, 1999). *Quadrula fragosa* requires a thermal cue of 7˚C to 20˚C (Steingraeber et al., 2007), which is a broader, overlapping range compared to *L. cardium*. Thus, the temperature threshold for excystment appears to vary by species, and glochidia are able to complete metamorphosis at low temperatures when overwintering on fish hosts but require a threshold temperature as the main cue for excystment (Watters and O'Dee, 1999).

Effects of emersion and hypoxic stress

Even though the majority of freshwater mussel species evolved in highly oxygenated streams, in general, freshwater mussels exhibit great tolerance to hypoxia and emersion in air. Amazingly, *A. cygnea* can survive for at least five days in complete anoxia (Holwerda and Veenhof, 1984). Similarly, *Pleurobema sintoxia* has a 95% survival rate even after exposure to air for five days (Badman and Chin, 1973) and *L. subrostrata* continues to consume oxygen from water-saturated air even after 30 days (Dietz, 1974). Because mussels are relatively immobile, it is not surprising that they have adapted to tolerate such extreme environmental conditions, at least temporarily – an adaption presumable due to the ephemeral nature of their habitats due to naturally-occurring droughts.

The finding that *Villosa iris* and *V. constricta*, species which live in well-oxygenated riffle habitats, are less able to tolerate low DO levels than *E. complanata, Elliptio fisheriana,* and *Elliptio lanceolata*, species that live in lentic or lotic habitats that

experience lower summer dissolved oxygen levels, suggests that the ability to regulate oxygen is related to the degree of hypoxia experienced in the typical habitat of a species (Chen et al., 2001). *Anodonta cygnea*, a species often found in lakes, maintains constant O_2 consumption at levels of O_2 partial pressure as low as 1 kPa (Massabuau et al., 1991). Lomte and Nagabhushana (1971), however, found that the O_2 consumption of *P. corrugata,* a species often found in mud with little oxygen, is highly dependent on DO levels between 1.0-5.3 ml/L. An interesting avenue for further research would be investigating how O_2 consumption rates vary among evolutionary lineages and the natural habitats of their species.

Despite their evolved resilience to temporarily xeric conditions, mussel species still exhibit symptoms of hypoxic stress. Studies on the effects of hypoxic stress on heart rate are limited and contradictory. When subjected to 0% DO, adults of *P. cataracta* lower their heart rate by 55% while juveniles lower theirs by 70% (Polhill and Dimock, 1996), which suggests that adults tolerate anoxic conditions better than juveniles. Sommerville (1975), however, did not find any correlation between DO and heart rate in adult *A. cygnea*, suggesting that the response of heart rate to low dissolved oxygen may be species specific.

Hypoxic stress has a great influence on the extracellular pH of freshwater mussels. If the increase in ambient CO_2 is greater than the increase in bicarbonate level, acidosis would be expected. Otherwise, a greater increase in bicarbonate levels, which act as a buffer to counteract acidosis (Fritts et al., 2015b), would shift the equilibrium towards a more alkaline environment. The extracellular pH of *E. complanata* exhibits

increased alkalosis under moderate hypoxia and increased acidosis under severe hypoxia (Byrne et al., 1995). Silverman et al. (1983) also found that hemolymph pH drops significantly in *L. subrostrata* exposed to severe hypoxia. Accumulation of CO_2 may have a stronger effect on hemolymph pH than oxygen levels since the hemolymph pH of *A. grandis simpsoniana* only exhibits alkalosis under severe hypoxia (<0.5% O_2) but is much more responsive to CO_2 levels (Byrne and McMahon, 1990). *Margaritifera margaritifera* also exhibits mantle fluid alkalosis during emersion (Heming et al. 1988). This balance may be influenced by the degree of gaping a mussel species exhibits, since gaping would allow release of CO_2 and valve closure would result in CO_2 accumulation. It remains unclear as to whether low oxygen levels tend to induce alkalosis or acidosis and this may vary depending on the balance between increased CO_2 and bicarbonate levels.

Further effects of hypoxic stress on mussel physiology include changes in enzyme activity and biochemical composition. Catalase activity increases by 37% in hypoxic conditions (20% DO) compared to normoxic conditions (89% DO) in *C. fluminea* while glutathione S-transferase is not affected by oxygen levels (Vidal et al., 2002). In *A. cygnea,* the activities of glutathione S-transferase and catalase exhibit a U-shaped pattern with increases in DO (Robillard et al., 2003). Increased activity of glutathione S-transferase and catalase in hypoxic conditions probably aids in the detoxification processes in freshwater mussel tissues. During hypoxia, the degradation of glucose to phosphoenolpyruvate (PEP) and subsequently to lactate has long been considered as one of the main pathways of anaerobic metabolism. Freshwater mussels, in addition to

forming lactate as an anaerobic end product, also accumulate succinate (Dietz, 1974; Chen, 1998), which indicates that they are also converting PEP to succinate via oxaloacetate. Although there is some accumulation of lactate and alanine in mussels subjected to anaerobic conditions, the primary end products are succinate and the volatile fatty acids acetate and propionate (Gäde and Wilps, 1975). Mussels respond to hypoxic stress by altering enzyme activity and switching to anaerobic metabolism, both of which are adaptive mechanisms that allow the organism to maintain metabolic activity and convert toxic byproducts into less harmful substances.

Although freshwater mussels have limited mobility, and their behavior is not often studied, it is clear that at least some species exhibit changes in behavior in response to hypoxic stress. *Pleurobema sintoxia* subjected to oxygen levels below 0.4 mg/L at 20˚C show markedly different patterns of activity resulting in prolonged shell closure (Badman, 1974). During simulated predatory attack in hypoxia, the valves of *C. fluminea* remained closed on average 137 seconds shorter than those in conditions with high levels of DO (Saloom and Duncan, 2005). Juveniles of *E. complanata* that are exposed to low levels of DO exhibit other behavioral changes such as increased siphon extension and gaping and decreased burial (Sparks and Strayer, 1998). Adult *L. subrostrata* subjected to anoxia also exhibit gaping of the valves and foot extension, presumably as a means of increasing surface area for oxygen absorption (Dietz 1974). These hypoxia-induced behavioral changes may increase vulnerability to predation by exposing more soft tissue, and deserve further study.

There have been few studies that investigate the effects of hypoxic stress on reproductive capacity in freshwater mussels. Aldridge and McIvor (2003) found that a lack of oxygen induces premature release of under-developed glochidia in *U. pictorum* and *U. tumidus*. Premature release of glochidia may be induced due to physiological limitations imposed when glochidia are brooding in the demibranchs, which likely reduces the surface area of the gills therefore impeding oxygen absorption. For example, the oxygen consumption of *P. cataracta* females is lower than males during the brooding season (Tankersley and Dimock, 1993). Therefore, during the brooding season, females may be more sensitive to hypoxia and expel immature glochidia in an effort to maintain sufficient gaseous exchange.

Effects of pH

Metabolic acidosis in bivalves, caused by acid exposure, could be due to either anaerobic metabolism that accompanies valve closure or the direct influx of H^+ from the environment. In bivalves, there is a strong correlation between decreasing hemolymph pH and increasing hemolymph [Ca^+], which is most likely released from the $CaCO_3$ stores in shell and mantle to increase buffering capacity (Pynnönen, 1990a). In *A. anatina*, for example, calcium levels were unchanged down to a pH of 3.5 but increased 2-6 times at pH 2.3-3.0 (Mäkelä and Oikari, 1992). The high external H^+ may depolarize neurons and the increase in calcium ions may hyperpolarize neurons, thereby offsetting any damage to nerve function (Christoffersen, 1973). Specific responses may vary according to species, though, since Pynnönen (1991) found that exposure to pH 4-5

causes an increase in hemolymph [Ca^{2+}] after two weeks in *U. pictorum* and no further change during the third week, whereas *A. anatina* exhibits an increase in [Ca^{2+}] after only one week but a decrease to the same levels as in control mussels after three weeks. Other changes in hemolymph composition that accompany acid exposure include decreased levels of sodium and chloride and increased levels of potassium (Pynnönen, 1991; Mäkelä and Oikari, 1992), although a time period of several weeks may be required for such changes to occur (Pynnönen, 1990b).

Temporarily-low stream pH is often the result of periodic rainfall or snowmelt and is associated with a rapid increase in metal concentrations and speciation (transformation from organic to bioavailable inorganic forms). However, it appears that a decrease in pH may help to protect mussels from metal bioaccumulation. In a study of three Ontario streams with varying pH depression during snowmelt, *E. complanata* exposed to more acidic conditions had lower tissue concentrations of aluminum than those in less acidic conditions (Servos et al., 1987). Also, accumulation of cadmium by *U. pictorum* was lower at pH 4.0-5.0 than at pH 8.0-8.3 (Pynnönen, 1990b). There is the possibility that the lower temperatures associated with snowmelt decrease mussel metabolism and filtering rates, consequently reducing metal uptake. Decreased uptake of metals at low pH could be due to increased valve closure in acidic conditions or an altered mechanism of metal movement into and out of cells, especially across the gill epithelium.

Similar to the effects of temperature and hypoxia, effects of acid stress on mussels include changes in respiration, behavior, and reproduction. Oxygen consumption does not

vary much between pH 6.0 and 7.2 for *P. corrugata* but is reduced at lower and higher pH levels (Lomte and Nagabhushana, 1971). While *A. cygnea* open valves for feeding and respiration for an average of 3-5 hours at pH 8.0-8.3 to which they are acclimated, the time decreases to 0.5 hours when pH is lowered to 5.0 (Pynnönen and Huebner, 1995). The authors suggest that valve closure as a response to acid stress may be caused by irritation of adductor muscle by H^+ ions or the subsequent release of Ca^{2+} from the adductor muscle, which could alter contraction patterns. Low pH has a negative effect on the survival of *M. margaritifera* glochidia, such that none survive 24 hours at pH 4.5 and a lower effect on recently metamorphosed juveniles (Taskinen et al., 2011), indicating that the glochidial life stage may be more sensitive. Few studies have looked at the effects of acid stress on enzyme activity or biochemical composition. Vidal et al. (2002) found that catalase activity is 21% lower in *C. fluminea* held at pH 8-9 compared to pH 7. A decrease in catalase activity in unionids may indicate reduced capacity for protection from oxidative damage but to our knowledge, no studies to date have looked at this.

Conclusions

Based on our review of studies investigating the effects of temperature on freshwater mussels, we expect that increases in mean average temperature due to global warming will most likely negatively affect freshwater mussel physiology and behavior, especially those already identified as being thermally sensitive. Effects of increased temperature include changes in respiration, filtration, heartbeat, enzyme activity, biochemical composition, behavior, and reproduction. Although freshwater mussels

exhibit high tolerance to hypoxia and species found in less oxygenated waters may have developed certain adaptations that allow them to survive during low DO levels, decreases in oxygen are generally stressful to mussels. Hypoxia induces hemolymph acidosis or alkalosis, affects heart rate and behavior, and changes the activities of glutathione S-transferase and catalase. These physiological changes may well explain the increased mortality rates observed during drought at different locations throughout the United States (Golladay et al., 2004; Haag and Warren, 2008), since cessation of flow in rivers is often accompanied by decreased DO levels and increased temperatures. Furthermore, hypoxia may induce early release of immature glochidia, disrupting that natural reproductive cycle that could potentially result in losses of entire cohorts. In addition to temperature and hypoxia stress, some mussels are exposed to acid stress as well, but few studies have documented the effect of pH on mussel physiology and behavior. Effects of pH include changes in hemolymph pH, $[Ca^{2+}]$, heavy metal uptake, respiration, behavior, and reproduction.

Although we would be surprised to observe a change in one environmental variable in nature which does not have an accompanying and often compensatory influence on other such variables (i.e. increasing water temperature and decreasing DO levels), few studies have focused on the synergistic effects of temperature, DO levels, and/or pH. Future research on the individual and synergistic effects of various environmental variables should focus on the integration of a broad range of environmental variables and attempt to dissect their combined influence if we are to

predict the effects of human activities on freshwater mussel populations and species composition in our ever-changing world.

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