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Source: Freshwater Mollusk Biology and Conservation, 22(2) : 98-108

Published By: Freshwater Mollusk Conservation Society

URL: <https://doi.org/10.31931/fmbc.v22i2.2019.98-108>

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REGULAR ARTICLE

# EXPOSURE TO ELEVATED CONCENTRATIONS OF MAJOR IONS DECREASES CONDITION INDEX OF FRESHWATER MUSSELS: COMPARISON OF METRICS

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## ABSTRACT

Energy storage is critical for gametogenesis and successful spawning in bivalve mollusks. However, it often is overlooked as an endpoint in toxicological studies of freshwater mussels. Energy storage can be assessed through direct measurement of energy substrates or the use of the condition index (CI) as an indicator of overall nutritional status. Our study focused on the CI of adult *Lampsilis fasciola* exposed to treatment conditions designed to mimic the Powell River (Virginia, USA), which historically supported an exceptionally diverse freshwater mussel community. Coal mining operations have impacted the upper Powell River, and low-flow specific conductance frequently exceeds 900  $\mu\text{S}/\text{cm}$ . We used four treatments in a full-factorial design to evaluate mussel responses to diluted pond water (control), simulated Powell River water, control sediment, and Powell River sediment. We measured glycogen content of mantle tissue and CI and compared several CI metrics. Exposure to simulated Powell River water caused a significant decrease in several CI metrics compared to control water. There was no effect of sediment type, nor was there any effect of sex; both males and females lost body mass in simulated Powell River water. However, males had significantly lower glycogen content of mantle tissue, indicating females likely were using other sources of energy to compensate for salinity stress. Comparison of CI metrics demonstrated that dissection was necessary to discern the effect of major ions on energy storage and that the use of tissue weight (g)/shell cavity capacity (g) had lower variability than tissue weight (g)/shell cavity volume (mL). The observed decrease in CI of adult *L. fasciola* after exposure to elevated concentrations of major ions has implications for maintaining mussel populations in the Powell River and in other rivers with rapidly increasing salinity.

**KEY WORDS:** conductivity, mining, bivalve, Unionidae, condition, glycogen

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## INTRODUCTION

Central Appalachian streams and rivers that drain watersheds with intensive coal mining have undergone dramatic changes in water and sediment quality. Frequently, they have elevated concentrations of major ions ( $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{K}^+$ ) and trace elements, as well as substrate alterations, such as increased proportions of fine sediment and sand (Pond et al. 2008; Bernhardt et al. 2012; Griffith et al. 2012). The upper Powell River watershed (Virginia, USA) has experienced extensive disturbance from

coal mining. Surface mining has occurred in more than one-third of the watershed (Zipper et al. 2016), and deep mines are present under almost all of the surface-mined areas (DMME 2015). Since the 1960s, a steady increase in concentrations of major ions, measured as total dissolved solids (TDSs), has occurred in the Powell River (Zipper et al. 2016). As of the mid-2000s, measured specific conductance in the upper Powell River frequently exceeds 900  $\mu\text{S}/\text{cm}$  (VDEQ 2015). This value exceeds derived extirpation concentrations (95<sup>th</sup> centile) for the majority of mayfly genera in central Appalachian streams, and it is well above the proposed 300  $\mu\text{S}/\text{cm}$  benchmark for protection of aquatic life in this region (USEPA 2011). Sediment quality in the Powell River also has been affected by

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coal mining. The presence of excess fine sediment (measured as embeddedness) likely impairs benthic aquatic life in the upper Powell River (MapTech 2011). In addition, the sediment contains elevated concentrations of nickel and naphthalene (MapTech 2011), contaminants often associated with coal particles (Stout and Emsbo-Mattingly 2008; Van Aken et al. 2015), which are visible in the sediment and which make up 1–5% of it by weight (Wolcott 1990).

The Powell River is part of the Upper Tennessee River watershed, an area of exceptionally high freshwater mussel diversity. Long-term monitoring of the river has documented declining mussel species richness and densities, with the decline greatest in its upstream reaches (Wolcott and Neves 1994; Johnson et al. 2012; Ahlstedt et al. 2016). There is a spatial association between areas with the highest in-stream TDS concentrations and the greatest declines in freshwater mussel assemblages (Zipper et al. 2016). However, simulated Powell River water (944 mg/L TDS, 1190  $\mu\text{S}/\text{cm}$ ) did not cause a significant decrease in survival or growth of juvenile freshwater mussels compared to diluted pond water (Ciparis et al. 2015). These results suggest either that major ions are not the primary cause of the observed mussel declines in this river or that they cause physiological changes in mussels that were not captured by using only survival and growth in juvenile mussels as toxicological endpoints.

Energy storage is directly linked to gametogenesis in bivalves (e.g., Bayne et al. 1982; Fearman et al. 2009). Thus, maintaining populations depends on the balance between energy availability and the energetic cost of metabolism in individuals. When the difference between availability and cost is positive, excess energy can be used for growth, storage, and, ultimately, reproduction, but when the difference is negative, energy reserves are depleted (Bayne and Newell 1983; Widdows and Johnson 1988; Van Haren and Kooijman 1993). Changes in environmental salinity can affect energy storage in bivalves. Depletion of stored energy has been documented in marine bivalves exposed to low-salinity water (Kumar et al. 2015; Bertrand et al. 2017) and in the freshwater clam *Corbicula fluminea* exposed to high-salinity water (Bertrand et al. 2017). However, to our knowledge, changes in energy storage in freshwater mussels as a result of exposure to elevated salinity, particularly the mixture of major ions found in mining-impacted streams, have not been evaluated.

Evaluating energy storage in bivalves as a response to seasonal or environmental changes requires distinguishing between energy reserves in soft tissues and structural biomass (Van Haren and Kooijman 1993). Energy storage in soft tissues can be measured directly, as glycogen, lipid, or protein content (e.g., Bayne 1982; Kumar et al. 2015; Bertrand et al. 2017). Energy storage also can be measured indirectly using the entire animal. Energy reserves or “fatness” of oysters was first defined as a condition index (CI) in the early 1900s, and it was measured as the proportion of internal shell volume occupied by soft body tissue (Crosby and Gale 1990). Crosby and Gale (1990) reviewed the methodology for calculating CI in bivalves and found a lack of uniformity in applied

measurements and formulas, preventing comparisons across studies. After testing three commonly used formulas, they recommended a standardized method, calculated as  $\text{CI} = \text{dry soft tissue weight (g)} \times 1000 / \text{internal shell cavity capacity (g)}$ . However, review of recent literature for freshwater bivalves still shows wide variation in the methodology used to calculate CI, with soft tissue weight (wet or dry) divided by either shell length (Blaise et al. 2017), shell length <sup>3</sup> (Spooner and Vaughn 2009), shell weight (Payton et al. 2016; Bertucci et al. 2017; Zhao et al. 2017), shell cavity volume (Nobles and Zhang 2015; Otter et al. 2015), total dry weight (Ganser et al. 2015), or total wet weight (Michel et al. 2013), with or without the use of scaling factors ( $\times 10$ ,  $\times 100$ , etc.).

The primary objective of our study was to evaluate the effect of exposure to elevated concentrations of major ions found in the Powell River on energy storage, assessed as CI and glycogen content, in adult *Lampsilis fasciola*, a native freshwater mussel. A secondary objective was to assess potential effects of coal-contaminated sediment from the Powell River on metrics of energy storage in *L. fasciola*. Our final objective was to evaluate several methods of measuring CI for differences in sensitivity to treatment effects and precision. We included measurements on live mussels in this comparison for potential application to imperiled species of freshwater mussels, for which nondestructive measurement techniques are preferred.

## METHODS

We obtained *Lampsilis fasciola*, approximately 2 yr old, from the Aquatic Wildlife Conservation Center (AWCC; Virginia Department of Game and Inland Fisheries, Marion, Virginia, USA) in July 2013, which produced them from one gravid female collected from the Clinch River, Virginia, USA. Hatchery-reared host fish (*Micropterus salmoides*) were infested in May 2011, and excysted juvenile mussels were collected in late May through June 2011. Juveniles were held in downwelling bucket systems followed by outside troughs. The South Fork Holston River (Marion, Virginia, USA) was the water source for both systems. Mussels were transferred to the Freshwater Mollusk Conservation Center (FMCC; Virginia Tech, Blacksburg, Virginia) in coolers with aerated river water. Mussels were held at the FMCC in flow-through systems using water and sediment from an on-site pond until October 2016; they were approximately 5 yr old at the time of this study (mean length =  $40.68 \pm 2.37$  mm, mean weight =  $11.59 \pm 1.48$  g). They were sexually mature ( $>3$  yr old; Zale and Neves 1982) but smaller than wild 5-yr-old *L. fasciola* collected from the Clinch River (mean length  $\sim 50$  mm; Jones and Neves 2011).

We designed a full-factorial study to evaluate the effects of elevated concentrations of major ions and coal-contaminated sediment on freshwater mussels. The four treatments included (1) control water and control sediment (CWCS), (2) control water and Powell River sediment (CWPS), (3) simulated Powell River water and control sediment (PWCS), and (4) simulated Powell River water and Powell River sediment (PWPS).

### Treatment Preparation

Pond water from the FMCC pond was filtered through a 5- $\mu$ m polypropylene microfiber filter (Vortex Filter, Filter Specialists, Inc., Michigan City, IN, USA). A 50:50 mixture of filtered pond water:deionized water was used as the control water and as a base water to prepare the simulated Powell River water. A previous study demonstrated excellent mussel survival in the 50:50 mixture, compared to poor survival in 100% pond water when used in closed exposure systems (Ciparis et al. 2015). The target TDS concentration for simulated Powell River water was 950 mg/L, similar to the target concentration for simulated Powell River water (942 mg/L) derived in Ciparis et al. (2015). This represents recent TDS concentrations measured during low-flow conditions at a Virginia Department of Environmental Quality (VDEQ) long-term monitoring station on the Powell River, at Big Stone Gap, Virginia (6BPOW179.20; for location information see Fig. 1 in Ciparis et al. 2015). Nominal ion concentrations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ , and  $\text{HCO}_3^-$ ) for the Powell water treatments (Table 1) were based on recipes developed by Ciparis et al. (2015), adjusted for the diluted base water used in the current study. Control and simulated Powell River water were prepared weekly. We prepared treatments from base waters using certified American Chemical Society (ACS) reagent-grade salts. Potassium chloride (KCl), potassium bicarbonate ( $\text{KHCO}_3$ ), sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), sodium bicarbonate ( $\text{NaHCO}_3$ ), calcium carbonate ( $\text{CaCO}_3$ ), magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), and sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) were purchased from Fisher Chemical (Fair Lawn, NJ, USA). We purchased calcium sulfate ( $\text{CaSO}_4$ ) from Sigma-Aldrich (St. Louis, MO, USA). For simulated Powell River water, salts were mixed into 17 L of base water in 18-L buckets and held in a water bath (see below) for 24 h prior to water exchanges. Control water also was held in 18-L buckets in a water bath for 24 h.

Two types of sediment were used in the exposure. We obtained control sediment from the FMCC mussel culture system used for older juvenile mussels (grow-out phase); we collected Powell River sediment from the Powell River at Big Stone Gap, Virginia, USA (36.8635 N, -82.7855 W) using a stainless-steel shovel and plastic bucket. Sediment from this area of the Powell River has visible coal particles and previously documented elevated concentrations of nickel (mean = 26 mg/kg, max = 49 mg/kg,  $n = 9$ ) and naphthalene (1.2 mg/kg,  $n = 1$ ) (MapTech 2011). Control sediment and Powell River sediment were held for two weeks at 4°C to minimize activity of indigenous animals and microbes. After the holding period, we poured the Powell River sediment into a long, clear plastic bin and used forceps to remove large debris and *Corbicula fluminea* shells. We mixed the sediment thoroughly with a stainless-steel spoon and distributed it into 16 4-L glass jars, at a target volume of 750 mL, equivalent to a height of 5 cm (Hazelton et al. 2014). Control sediment also was distributed into 16 4-L jars at the same target volume. We placed water from the FMCC pond in each jar (approximately

3 L) and held the jars in the exposure system (see below) for 1 wk with constant aeration. Ammonia concentrations were measured on day -1 and determined to be negligible. The pond water was exchanged for treatment water in each jar on day 0.

Prior to distribution in the jars, six subsamples were collected from each sediment type for estimation of organic content based on loss-on-ignition (500°C for 12 h) from dried (60°C for 3 days) samples. Organic content was  $2.9 \pm 0.3\%$  in Powell River sediment and  $1.3 \pm 0.1\%$  in control sediment. To document presence of coal particles in the sediment, we determined a crude estimate of contribution to sediment dry weight. Coal particles were visually identified in the inorganic fraction of the Powell River sediment subsamples (postignition), separated using forceps, and weighed; we then determined the proportion of total sediment dry weight as coal. This method estimated  $1.5 \pm 0.6\%$  coal in the sediment (dry wt. basis), which is similar to previously documented amounts (Wolcott 1990).

### Mussel Exposure System

Experimental units consisted of 4-L glass jars with 3 L of water and 750 mL of sediment. Each jar had an airline affixed with a glass pipet to maintain oxygen near saturation. We placed each jar in an 18-L bucket containing approximately 3 L of water; we placed four buckets into each of four 757-L containers filled with water to serve as a temperature control bath (water bath). Temperature (target = 22°C) was maintained in the water baths using aquarium heaters. Each water bath contained one replicate (jar) of each treatment randomly arranged ( $n = 4$  for all treatments). We used a blocked design to account for any temperature differences between water baths.

On day 0 (October 24, 2016), we randomly selected a total of 32 mussels from the original cohort. At the time of selection, each mussel had a unique Hallprint shellfish tag (Hallprint Inc., Hindmarsh Valley, South Australia) affixed to the shell. Shell shape was used for initial determination of the sex of each mussel. Females were gravid at the time of the study, and sex was confirmed using the presence (F) or absence (M) of glochidia in marsupial gills observed after gently opening the shell. We randomly assigned two mussels, one male and one female, to each jar. Prior to placement in the jars, each mussel was weighed to the nearest 0.0001 g using a digital scale and measured to the nearest 0.1 mm using dial calipers; we measured the volume of the mussel to the nearest 1 mL using pond water displacement in a graduated cylinder.

During the exposure, mussels were fed daily by adding 0.9 mL of a 1:1 algal cell ratio from two premixed commercial micro-algae diets (Nanno 3600 and Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA) to each jar at a concentration of 20,000 cells/mL. This feeding regime was derived from a base mixture previously used for juvenile mussels (Carey et al. 2013; Ciparis et al. 2015), adjusted to a feeding rate



Table 1. Ions as nominal concentrations in control water (CW) and simulated Powell River water (PW) and mean measured concentrations ( $n = 6$  weekly measurements, standard deviation in parentheses) in four treatments, consisting of either CW or PW and control sediment (CS) or Powell River sediment (PS). Total dissolved solids (TDS) is the sum of all ion concentrations.

	CW Nominal	CWCS Mean	CWPS Mean	PW Nominal	PWCS Mean	PWPS Mean
Ca <sup>2+</sup>	15.6	22.1 (0.60)	23.4 (0.92)	86.0	30.2 (6.6)	34.5 (7.2)
K <sup>+</sup>	1.15	1.63 (0.09)	1.79 (0.12)	6.00	7.28 (0.85)	7.36 (0.70)
Mg <sup>+</sup>	15.7	16.2 (0.99)	16.0 (1.1)	49.0	53.0 (5.2)	52.3 (4.7)
Na <sup>+</sup>	2.70	5.88 (0.83)	6.06 (0.55)	114	179 (12)	179 (12)
SO <sub>4</sub> <sup>2-</sup>	7.75	18.1 (2.2)	18.6 (1.8)	452	472 (32)	477 (30)
HCO <sub>3</sub> <sup>-</sup>	110	114 (5.6)	133 (5.6)	229	163 (6.0)	179 (15)
<b>TDS<sup>a</sup></b>	<b>158</b>	<b>183</b>	<b>204</b>	<b>955</b>	<b>924</b>	<b>948</b>

<sup>a</sup>Includes the nominal concentration of Cl<sup>-</sup> of 5.1 for all CW treatments and 19.1 for all PW treatments; Cl<sup>-</sup> could not be measured due to a faulty probe.

(~10,000 cells/mL/mussel) used for similarly sized *L. fasciola* (Hazelton et al. 2014). For each jar, a 100% water exchange occurred weekly. Water was gently siphoned in and out of each jar in order to minimize disturbance of the sediment. We measured temperature (°C), specific conductance (µS/cm), dissolved oxygen (% saturation), and pH just prior to, and 24 h after, water exchanges using a YSI 556 Multi-Probe Sensor (YSI Inc., Yellow Springs, OH, USA). We sampled concentrations of NH<sub>3</sub>-N, alkalinity, and elements (Na, K, Mg, Ca, and S) just prior to weekly water exchanges. A sample (25 mL) from each jar was filtered (0.45-µm pore size) and aliquots were combined into a pooled sample for each treatment. Ammonia (NH<sub>3</sub>-N) was measured weekly using a HACH DR/2400 meter (Hach, Inc., Loveland, CO, USA) following the manufacturer's methods. Total alkalinity (mg/L CaCO<sub>3</sub>) was measured weekly using a standard titration method and was converted to HCO<sub>3</sub><sup>-</sup> using the equation mg/L HCO<sub>3</sub><sup>-</sup> = mg/L CaCO<sub>3</sub> × 1.22. Element concentrations were measured weekly. The Virginia Tech Soil Testing Laboratory measured elements in solution using Inductively Coupled Plasma Atomic Emission Spectrometry (Spectro ARCOS ICP, Spectro Analytical Instrumentation, Kleve, Germany) following standard methods (USEPA Method 200.7 [USEPA 1994] and APHA Method 3120 [APHA 2012]) and standard operating and quality assurance/quality control (QA/QC) as detailed in Ciparis et al. (2015). Sulfate concentration was calculated from measured total S; all S was assumed to be present as SO<sub>4</sub><sup>2-</sup>.

On day 40 (December 3, 2016), mussels were removed from each treatment; they were weighed and measured, and their volume recorded, as described above. All mussels were dissected. Wet tissue weight was measured to the nearest 0.0001 g using a digital scale. The viscera and a section of mantle tissue (target 0.25 g, mean 0.28 g ± 0.08 g SD) were removed from each mussel, weighed, placed individually in 1.5-mL microcentrifuge tubes, and immediately frozen. The remaining tissue was reweighed, and the tissues and mussel shells were dried at 60°C. We measured and recorded dry tissue weight and shell weight. The proportion of water in the tissue and total wet weight were used to determine the total dry

weight, prior to removal of digestive gland and mantle tissue. Finally, we determined the cavity volume by filling one of the valves of each mussel full of water, measuring the amount, and doubling it. We maintained consistent meniscus shape and height. We did not use the method of water displacement to determine shell cavity volume (Crosby and Gale 1990) due to the low density of the shells, which prevented accurate measurement of displacement.

### Glycogen Determination

Mantle tissue was homogenized in 300 µL sodium citrate buffer (0.1 M, pH 5), placed in a boiling water bath at 100 °C for 5 min, and centrifuged at 10,000 g for 5 min. We added duplicate 100 µL aliquots of the supernatant to a microplate and 5 µL of 1% amyloglucosidase to one replicate to hydrolyze glycogen to glucose (Carr and Neff 1984). The plate was incubated at 25°C for 12 h. We quantified glucose in treated and untreated supernatants using a glucose oxidase assay (Sigma Glucose [G-O] Assay Kit, Sigma-Aldrich). Glucose concentration was determined spectrophotometrically at 540 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and normalized to a glucose standard curve. Glycogen content was determined as the amount of glucose produced by treatment with amyloglucosidase. Glycogen content in each sample was normalized to wet weight of extracted mantle tissue. Analysis of a glycogen standard (*Mytilus edulis* [blue mussel] tissue; Sigma-Aldrich) demonstrated a mean recovery of 91% (±3% relative standard deviation [RSD]).

### Data Analysis

We calculated several metrics of mussel body condition. On live mussels, we calculated two metrics on day 0 (initial) and day 40 (end): (1) Fulton's K = (mussel W/L<sup>3</sup>) × 10, where W = weight of the entire mussel in g, L = length in cm, and 10 is a scaling factor (Heinke 1908; Nash et al. 2006) and (2) MW:MV = mussel W/mussel V, where W = weight of the entire mussel in g, and mussel V = volume in mL of water

displaced by the live mussel. On dissected mussels, we calculated two CI metrics using both wet and dry tissue weights: (1) TW:SV = tissue W/shell V, where tissue W = weight of tissue in g and shell V = shell cavity volume in mL, measured as the amount of water held by one valve of the shell  $\times 2$  and (2) TW:SC = tissue W/shell cavity capacity, where tissue W is tissue weight in g and shell cavity C is the weight of the shell cavity in g, determined by subtracting the weight of the dry shell from the weight of the whole mussel. A final metric, described as a body component index (Crosby and Gale 1990), was calculated as TW<sub>dry</sub>:SW, where SW is the weight of the dry shell.

We conducted statistical tests using SAS software (SAS 9.4, SAS Institute, Inc., Cary, NC, USA) with a significance level of  $\alpha = 0.05$ . We compared each metric of mussel condition and glycogen content of mantle tissue between treatments using a mixed model with a normal distribution (Proc GLIMMIX). Similarly, mussel lengths (initial and end) and growth (as change in shell length during the exposure) were compared between treatments. All measurements were normally distributed (Shapiro-Wilk W test,  $P > 0.05$ ), with the exception of glycogen content of mantle tissue, which was  $\log_{10}$  transformed prior to analysis in order to fit the normal distribution. The initial model contained three predictors—water type (CW or PW), sediment type (CS or PS), and sex—as well as all possible interactions (water $\times$ sediment, water $\times$ sex, sediment $\times$ sex, and water $\times$ sediment $\times$ sex). Water bath (block) was included as a random variable, and jar was the subject of measurement. Final models contained only significant predictors. We evaluated any significant interactions as pairwise comparisons of all relevant treatment combinations, using a Tukey post-hoc test with  $P$  values adjusted for multiple comparisons. We determined the relative standard deviation (RSD) as  $RSD = (\text{standard deviation}/\text{mean}) \times 100$  for each metric of condition calculated for dissected mussels, because one objective of the study was to evaluate methods of determining mussel condition.

## RESULTS

Total dissolved solids concentrations, as the sum of measured ions, were similar to nominal concentrations (Table 1). Simulated Powell River water TDS concentrations were within 3% of nominal concentrations. Control water TDS concentrations exceeded nominal concentrations by 16–28%, due to lack of rainfall influencing pond water composition at the time of the study. Differences in concentrations of individual ions in the simulated Powell River water compared to nominal concentrations were due to either elevated concentrations in the base water (e.g.,  $\text{SO}_4^{2-}$ ), incomplete solubility of Ca-containing salts, or the accidental replacement of  $\text{NaHCO}_3$  (recipe) with  $\text{Na}_2\text{CO}_3$  (used). Despite these minor differences between nominal and measured ion concentrations, a five-fold difference in both TDS concentrations and specific conductance between control water ( $\sim 250 \mu\text{S}/\text{cm}$ ) and simulated Powell River water ( $\sim 1,250 \mu\text{S}/\text{cm}$ ) was achieved

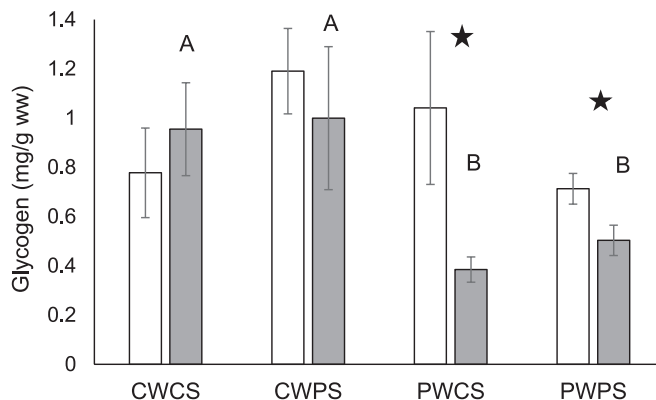


Figure 1. Mean glycogen content in mantle tissue of female (open) and male (gray) mussels in each treatment ( $n = 4$ ) at the end of the exposure (day 40). Error bars are standard errors of the mean. Each treatment consisted of one water type and one sediment type, where CW = control water, PW = simulated Powell River water, CS = control sediment, and PS = Powell River sediment. Letters indicate a statistically significant difference between water type for males only ( $P = 0.020$ ) and a  $\star$  indicates a statistically significant difference between males and females in the PW treatments ( $P = 0.038$ ).

(Tables 1 and 2). There was little variation in measured ion concentrations and specific conductance throughout the study (Tables 1 and 2).

Water quality measurements were consistent over time and were within acceptable ranges for toxicity tests with freshwater mussels. Dissolved oxygen was maintained at  $>95\%$  saturation throughout the study. Mean measured temperatures were similar between treatments; all were within  $0.1^\circ\text{C}$  of the  $22^\circ\text{C}$  target temperature (Table 2). Measured pH was stable within each treatment over the course of the study (Table 2). Ammonia-N concentrations during the 1-wk acclimation period for sediment within the jars were low, ranging from 0.01 to 0.09 mg/L on day  $-1$ . During the exposure,  $\text{NH}_3\text{-N}$  was below detection in all treatments from day 7 to day 40 (Table 2).

Survival in all treatments was 100% for the entire study. Mussel length (initial and end) and growth, as change in shell length over the course of the study, were similar between treatments, with no relationship to water type, sediment type, or significant interaction. Mean mussel growth was  $\leq 0.2$  mm in all treatments; negligible growth was expected given the ages of the mussels and the relatively short duration of the study.

For live mussels, initial weight:length ( $K_{\text{initial}}$ ) and weight:volume ( $\text{MW}:\text{MV}_{\text{initial}}$ ) metrics were similar between treatments, with no relationship to water type, sediment type, or significant interaction (Table 3). There was a significant effect of sex on  $K_{\text{initial}}$  (GLIMMIX,  $P < 0.0001$ ), with no significant interactions. Females had a higher  $K_{\text{initial}}$  ( $1.86 \pm 0.05$  SE) compared to males ( $1.58 \pm 0.01$  SE). This effect was maintained at the end of the exposure;  $K_{\text{end}}$  was significantly higher (GLIMMIX,  $P = 0.0006$ ) in females ( $1.81 \pm 0.04$  SE) compared to males ( $1.61 \pm 0.03$  SE), with no significant interactions between sex and other variables. There was no

Table 2. Mean concentrations of water quality parameters, with standard deviation in parentheses, for each treatment ( $n = 4$  replicates) measured just prior to each water change. Treatments: control water + control sediment (CWCS), control water + Powell sediment (CWPS), simulated Powell water + control sediment (PWCS), and simulated Powell water + Powell sediment (PWPS).

Parameter	CWCS	CWPS	PWCS	PWPS
Temperature (°C)	22.1 (0.79)	22.0 (0.79)	22.1 (0.78)	22.0 (0.82)
Specific conductivity ( $\mu\text{S}/\text{cm}$ )	250 (7.6)	256 (10)	1,263 (50)	1,274 (42)
pH	7.01 (0.14)	7.00 (0.12)	7.15 (0.14)	7.16 (0.12)
NH <sub>3</sub> -N (mg/L; days 7–40)	<0.01	<0.01	<0.01	<0.01

effect of sex (or interactions) on either  $\text{MW}:\text{MV}_{\text{initial}}$  or  $\text{MW}:\text{MV}_{\text{end}}$ . At the end of the exposure, there was no effect of water type, sediment type, or an interaction on either  $\text{K}_{\text{end}}$  or  $\text{MW}:\text{MV}_{\text{end}}$  (Table 3).

For dissected mussels, there was a statistically significant effect of water type on all metrics of body condition, regardless of whether they were determined using wet or dry tissue weights (Table 3). Mussels exposed to simulated Powell River water had significantly lower  $\text{TW}:\text{SV}$  and  $\text{TW}:\text{SC}$  compared to mussels exposed to control water (GLIMMIX,  $P < 0.030$ ), with no effect of sediment type or significant interaction (Table 3). There was no effect of sex nor significant interactions with sex. The body component index,  $\text{TW}_{\text{dry}}:\text{SW}$ , was also significantly lower for mussels exposed to simulated Powell River water compared to control water (GLIMMIX,  $P = 0.007$ ; Table 3), with no effect of sediment type, sex, or significant interactions.

We assessed measurement variability of the two CI metrics recommended by Crosby and Gale (1990). Within each treatment, relative standard deviation was lower for  $\text{TW}:\text{SC}$  compared to  $\text{TW}:\text{SV}$  when metrics were calculated using either wet or dry tissue (Table 4).

For glycogen content of mantle tissue, there was a

significant effect of water type (GLIMMIX,  $P = 0.014$ ) with no effect of sediment type or significant interaction. There was also a significant effect of sex on glycogen content in mantle tissue (GLIMMIX,  $P = 0.036$ ) and an interaction between water type and sex that was not statistically significant at  $\alpha = 0.05$  (GLIMMIX,  $P = 0.066$ ) but warranted further exploration (Fig. 1). Pairwise comparisons revealed that males had significantly lower mantle glycogen content compared to females in simulated Powell River water (adjusted  $P = 0.038$ ) and that males exposed to Powell River water had significantly lower mantle glycogen content than males exposed to control water (adjusted  $P = 0.020$ ) (Fig. 1). Females and males had similar glycogen content in control water (adjusted  $P = 0.99$ ), and females exposed to simulated Powell River water had similar glycogen content to females exposed to control water (adjusted  $P = 0.94$ ) (Fig. 1).

## DISCUSSION

Compared to control water, exposure to elevated major ion concentrations in the simulated Powell River caused a decrease in the proportion of the body cavity occupied by tissue (CI) and the body-component index for both male and

Table 3. Mean (standard error) of metrics of mussel condition measured in each treatment ( $n = 4$ ) and overall means of treatments within each water type. Each treatment consisted of one water type and one sediment type, where CW = control water, PW = simulated Powell River water, CS = control sediment, and PS = Powell River sediment. Asterisks indicate metrics were significantly lower in PW compared to CW treatments; there were no significant effects of sediment type or interactions between water and sediment type for any metric. For metrics,  $\text{K} = (\text{weight} [\text{g}]/\text{length} [\text{cm}]^3) \times 10$ , MW = weight of live mussel (g), MV = volume of live mussel (mL), TW = tissue weight (g), SV = shell cavity volume capacity (mL), SC = shell cavity capacity, determined as MW minus shell weight, and SW = shell weight. Subscripts indicate whether the measurement was performed on day 0 (initial) or day 40 (end) for live mussels, and whether TW was for wet or dry tissue of dissected mussels. The horizontal line separates measurements on live and dissected mussels.

	Treatment				Water Type	
	CWCS	CWPS	PWCS	PWPS	CW	PW
$\text{K}_{\text{initial}}$	1.69 (0.12)	1.72 (0.06)	1.81 (0.12)	1.66 (0.11)	1.70 (0.06)	1.74 (0.08)
$\text{K}_{\text{end}}$	1.67 (0.09)	1.72 (0.07)	1.76 (0.10)	1.69 (0.09)	1.70 (0.15)	1.72 (0.18)
$\text{MW}:\text{MV}_{\text{initial}}$	1.47 (0.06)	1.34 (0.05)	1.35 (0.07)	1.36 (0.09)	1.40 (0.04)	1.35 (0.05)
$\text{MW}:\text{MV}_{\text{end}}$	1.50 (0.09)	1.48 (0.07)	1.45 (0.09)	1.39 (0.03)	1.49 (0.06)	1.42 (0.04)
$\text{TW}:\text{SV}_{\text{wet}}$	0.41 (0.03)	0.45 (0.03)	0.39 (0.03)	0.37 (0.02)	0.43 (0.02)	0.38 (0.02)*
$\text{TW}:\text{SC}_{\text{wet}}$	0.34 (0.02)	0.37 (0.02)	0.32 (0.02)	0.32 (0.01)	0.35 (0.01)	0.32 (0.01)**
$\text{TW}:\text{SV}_{\text{dry}}$	0.044 (0.004)	0.052 (0.006)	0.037 (0.004)	0.039 (0.004)	0.048 (0.004)	0.038 (0.003)**
$\text{TW}:\text{SC}_{\text{dry}}$	0.036 (0.002)	0.042 (0.004)	0.030 (0.003)	0.033 (0.002)	0.039 (0.003)	0.031 (0.002)**
$\text{TW}:\text{SW}_{\text{dry}}$	0.045 (0.005)	0.052 (0.008)	0.035 (0.005)	0.038 (0.003)	0.048 (0.004)	0.037 (0.003)**

\* $P = 0.030$ .

\*\* $P \leq 0.0086$ .

Table 4. Relative standard deviation for each metric calculated for dissected mussels in each treatment ( $n = 4$ ). Each treatment consisted of one water type and one sediment type, where CW = control water, PW = simulated Powell River water, CS = control sediment, and PS = Powell River sediment. For metrics, TW = tissue weight (g), SV = shell cavity volume capacity (mL), and SC = shell cavity capacity, determined as MW (weight of live mussel in grams) minus shell weight. Subscripts indicate whether TW was for wet or dry tissue of dissected mussels.

	CWCS	CWPS	PWCS	PWPS
TW:SV <sub>wet</sub>	13.9	11.7	16.1	10.8
TW:SC <sub>wet</sub>	8.95	8.82	12.7	7.44
TW:SV <sub>dry</sub>	17.2	22.1	22.3	19.7
TW:SC <sub>dry</sub>	11.4	19.1	20.0	14.7

female *L. fasciola*. Differences between water types were comparable for indices calculated using wet and dry tissue weights, indicating that tissue mass was lower in mussels exposed to simulated Powell River water. These results demonstrate that the mixture of major ions present in the Powell River likely results in a reduction in total energy storage in adult freshwater mussels, producing a measureable reduction in CI. There was no effect of Powell River sediment on CI of *L. fasciola* compared to control sediment. We did not measure contaminant concentrations in the sediment used in the exposure, but previous sampling of sediment in the upper Powell River in the vicinity of the collection site showed elevated concentrations of naphthalene and nickel (MapTech 2011), and the estimated coal content of the sediment used in the study (1.5%) was similar to previous studies of the Powell River (Wolcott 1990). Exposure to coal fines in sand caused apparent energetic stress in female *Villosa iris*, measured as significantly higher proportion of resorbing oocytes compared to controls (Henley et al. 2015). However, unlike coal particles in the Powell River sediment, the coal fines evaluated in Henley et al. (2015) were not weathered and were suspended in the water column, which may have increased bioavailability of coal-associated contaminants. Results of our study suggest that the bioavailability of coal-associated contaminants in Powell River sediment may be limited, but the study design does not allow their exclusion as a potential stressor to freshwater mussels inhabiting the river. Instead, these results clearly demonstrate that the elevated concentrations of major ions in the simulated Powell River water reduces the condition index of adult freshwater mussels, a measurable adverse physiological effect.

When compared to mussels in control water, all mussels exposed to simulated Powell River water had lower tissue mass, but only males had lower glycogen content in mantle tissue. This indicates that in order to compensate for increased salinity stress, males were using energy stored as glycogen whereas females likely were using energy stored in another form. At the start of this study (October), female mussels had obviously inflated marsupial gills containing glochidia, which is consistent with the classification of *L. fasciola* as bradytic and indicates spawning and fertiliza-

tion occurred previously. In bivalves, oocyte resorption occurs after spawning as a normal part of the gametogenic cycle (Kennedy and Battle 1964; Dorange and Le Pennec 1989; Henley et al. 2015). Cyclic resorption of atretic oocytes provides an efficient mechanism of nutrient recycling, particularly for lipids and proteins (Pipe 1987). Untimely resorption of developing oocytes also has been observed in marine and freshwater bivalves exposed to contaminants, and it is likely related to an energetic deficit (Bayne et al. 1981; Henley et al. 2015). In our study, females exposed to simulated Powell River water potentially were using energy stored in resorbing oocytes to compensate for increased stress, which explains a reduction in CI similar to males but not a concurrent loss of glycogen reserves. This finding is consistent with observations of energy use in marine mussels; when subjected to starvation during the period of gametogenesis and spawning (winter), mussels used stored protein, followed by lipid and carbohydrate, and when subjected to starvation during gametogenic quiescence (summer), mussels used only stored carbohydrate (Bayne and Newell 1983). Limited study of gametogenesis in *L. fasciola* from one tributary in the Upper Tennessee River watershed found the presence of early-stage glochidia in gill marsupia in early September, suggesting spawning in late August (Zale and Neves 1982), which is consistent with observations of gravid females during this study. Residual gametes were present several months after spawning, and active gametogenesis occurred throughout the year (Zale and Neves 1982). Thus, *L. fasciola* exposed to simulated Powell River water potentially could have resorbed either atretic oocytes or developing oocytes to compensate for increased energetic demands. However, definitively determining the use of this pathway would require histological evaluation or direct measurement of energy substrates in the viscera, which were beyond the scope of this study.

The mechanism for increased energy use by adult *L. fasciola* when exposed to elevated concentrations of major ions in simulated Powell River water remains unclear. One possibility is that *L. fasciola* exposed to simulated Powell River water closed their valves to avoid exposure, inducing anaerobic catabolism of energy reserves. Shell closure is a common avoidance response in freshwater mussels exposed to high concentrations of toxicants (Cope et al. 2008). The salinity in the current study was <1 ppt, and *L. fasciola* remained buried in all treatments for the duration of the exposure. Limited observations indicated that the mussels were also actively siphoning in all treatments. Blakeslee et al. (2013) found that the freshwater mussel *Elliptio complanata* could acclimate to 1 ppt (psu) salinity within 7 days, maintaining oxygen consumption rates similar to controls, with no shell closure observed up to 4 ppt salinity. Although mussel behavior was not specifically documented in the current study, shell closure and resulting anaerobic catabolism do not appear to be the primary mechanism for a decrease in metrics related to energy storage in *L. fasciola* exposed to simulated Powell River water.



Increased energy expenditure for osmoregulation is another potential mechanism for the observed decrease in metrics related to energy storage in *L. fasciola* exposed to the elevated concentrations of major ions in simulated Powell River water. Freshwater bivalves are generally osmoconformers when exposed to water with moderately elevated salinity (Dietz et al. 2000; Ruiz and Souza 2008; Griffith 2017). As extracellular osmotic pressure increases, bivalves increase intracellular concentrations of inorganic and organic (amino acids) osmolytes to maintain cell volume (Jordan and Deaton 1999; Ruiz and Souza 2008). The intracellular amino acids are generated from both increased synthesis and protein catabolism, and the increased activity of the associated transaminases and proteolytic enzymes likely has a high energetic cost (Bishop et al. 1994). In addition to regulating cell volume, there is likely an energetic cost for maintaining individual ions at concentrations necessary to avoid ionoregulatory imbalance. Maintaining intracellular concentrations of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{H}^+$  depends at least in part on the activity of energy-dependent ATPases and is also affected by cotransport of other ions, including  $\text{HCO}_3^-$ ,  $\text{Cl}^-$ , and  $\text{Mg}^{2+}$  (Byrne and Dietz 2006; Griffith 2017). Thus, the ion concentrations in simulated Powell River water may have increased the energetic cost of osmoregulation for *L. fasciola* by necessitating increased intracellular concentrations of amino acids and increased transport of individual ions to maintain ionic homeostasis.

Sulfate concentrations were particularly high in the simulated Powell River water, reflecting conditions in the river and in other mining-impacted rivers in central Appalachia (e.g., Pond et al. 2008). The exact mechanism of  $\text{SO}_4^{2-}$  uptake and transport in freshwater invertebrates is unclear (Griffith 2017). When exposed to elevated  $\text{SO}_4^{2-}$  concentrations, mayflies take up  $\text{SO}_4^{2-}$  rapidly (Scheibener et al. 2017); freshwater mussels take up  $\text{SO}_4^{2-}$  more slowly, but the concentration in the hemolymph eventually becomes isoionic with the exposure water (Dietz et al. 2000). Mayflies exposed to elevated concentrations of  $\text{SO}_4^{2-}$  had reduced time to emergence, attributed to the energetic cost of active  $\text{SO}_4^{2-}$  excretion (Buchwalter et al. 2018). There also may be an energetic cost of  $\text{SO}_4^{2-}$  excretion in freshwater mussels, as both freshwater mussels and mayflies appear to transport  $\text{SO}_4^{2-}$  using anion exchange (Dietz et al. 2000; Buchwalter et al. 2018). In rainbow trout, the  $\text{SO}_4^{2-}$ /anion exchanger (SLC26A1) in the renal proximal tubule is colocalized with both  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and vacuolar-type  $\text{H}^+$ -ATPase (Kato et al. 2006), providing a potential mechanism for increasing energy expenditure with increasing  $\text{SO}_4^{2-}$  excretion. In our study, the apparent energetic stress associated with osmoregulation observed for mussels exposed to the simulated Powell River water, coupled with the elevated concentration of  $\text{SO}_4^{2-}$  in the exposure water, suggests that the mechanism of  $\text{SO}_4^{2-}$  transport in freshwater mussels warrants further study, because it potentially could be an energetically demanding process.

The elevated concentrations of major ions in simulated Powell River water caused a significant reduction in metrics related to energy storage in adult *L. fasciola*, in contrast to

results of a previous study in our laboratory, which showed no significant effect of this water on growth of juvenile *Villosa iris* (Ciparis et al. 2015). Food availability may affect the response of freshwater invertebrates to the energetic demands of salinity stress; individuals with optimal nutrition may be less sensitive compared to individuals with suboptimal nutrition (Buchwalter et al. 2018). The ratio of food availability to energy demands may have been greater for juvenile *V. iris*, because the feeding rate used in Ciparis et al. (2015) was optimized to promote mussel growth. In contrast, the feeding rate for adult *L. fasciola* was based on limited published information of feeding regimes for similarly sized individuals (e.g., Hazelton et al. 2014). The feeding rate was sufficient for maintenance, as indicated by 100% survival of *L. fasciola* in all treatments, but the food availability may not have been sufficient to meet additional energetic demands from exposure to the simulated Powell River water. Differences in responses to the elevated concentrations of major ions between the two studies also could be related to life stage; the juvenile mussels (age 3–5 mo) were undergoing rapid shell growth, which requires sequestering of ions, predominantly  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  with smaller amounts of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Cl}^-$ , and  $\text{SO}_4^{2-}$  (Marin et al. 2012). Sequestration could reduce extracellular concentrations of these ions and thus the energetic demands of osmoregulation upon exposure to water with elevated ion concentrations. Finally, CI and other measures of energy storage in mussel tissues may be more sensitive than measurements involving only the mussel shell (e.g., shell growth).

In general, toxicity tests with juvenile freshwater mussels are considered protective of adult mussels for acute effects (Cope et al. 2008). However, toxicological studies focusing only on survival and growth of juveniles fail to capture potential effects of nonlethal concentrations on the reproductive potential of freshwater mussels. There is a direct relationship between energy storage and reproductive potential, because bivalves use stored glycogen during gametogenesis (Bayne et al. 1982; Pipe 1985), and females under energetic stress will actively resorb oocytes (Bayne and Newell 1983; Henley et al. 2015). The finding of significantly lower CI, a metric of energy storage, in *L. fasciola* exposed to simulated Powell River water has direct implications for sustainability of mussel populations in the river. In the upper Powell River, a decline in mussel recruitment has been observed since 1980 (Wolcott 1990; Johnson et al. 2012), and reproductive failure of adults is one potential contributing factor. Thus, energy storage is an ecologically relevant endpoint for freshwater mussels, which are long-lived and generally reproduce annually.

Evaluation of metrics related to energy storage in freshwater mussels showed differences in sensitivity. Metrics calculated on live mussels were not significantly different between treatments, in contrast to measurements on dissected mussels. This is likely due to shell weight dominating total body weight measurements, overshadowing the relatively small changes in tissue weight between treatments. In addition,

the significant difference in Fulton's K between males and females highlights a potential pitfall of the use of weight-length ratios in sexually dimorphic mussel species, particularly if sex is not included as a covariate. Given the imperiled status of many species, measurements on live freshwater mussels often are preferred, but our results suggest that CI metrics on live mussels may not accurately assess the impacts of environmental stressors on energy storage. On dissected mussels, results were similar for metrics using wet and dry tissue weights, indicating both are suitable for measurement of condition index. Generally, dry tissue weight is preferred because it removes water in the tissue as a source of variability (Crosby and Gale 1990), but if further analysis of the tissue precludes drying, use of wet tissue weight appears to be an acceptable method for assessing CI. Both TW:SV and TW:SC had similar differences between treatments, but variability in TW:SC was lower, which supports the findings of Crosby and Gale (1990). Although shell cavity capacity (SC) is technically the weight of tissue and water held by the shell cavity, it provides a close approximation of the shell cavity volume due to water comprising the majority of the weight of a live mussel and water's specific gravity. Shell cavity capacity can be measured more precisely than shell cavity volume (SV), as demonstrated by this study and by Crosby and Gale (1990). Therefore, for future studies of energy storage in freshwater mussels, we recommend the use of TW<sub>dry</sub>:SC. A scaling factor of 1000 was recommended by Crosby and Gale (1990), but this was developed for oysters, which generally have heavier shells compared to freshwater mussels. A scaling factor of 100 appears more appropriate for freshwater mussels, to bring the calculated CI close to 1 (Nash et al. 2006). As we have demonstrated, the body component index (TW<sub>dry</sub>:SW) may show similar responses as CI to environmental stressors, but Crosby and Gale (1990) caution against its use for assessment of temporal changes in nutritive status, particularly for bivalves with active or variable shell growth.

## ACKNOWLEDGMENTS

We thank Martha Chapman for providing Virginia DEQ water monitoring data; William Hopkins for the provision of laboratory equipment; Athena Tilley for analysis of major ion concentrations; William Henley for thoughtful feedback and discussion; and the staff of both FMCC and AWCC for rearing and maintenance of mussels for their use in this study. This study was funded, in part, by U.S. Fish and Wildlife Service (USFWS). The views expressed in this article are the authors' and do not necessarily represent those of USFWS. Use of trade names is for identification purposes only and does not imply endorsement by the U.S. Government.

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