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Copper Uptake, Patterns of Bioaccumulation, and Effects in Glochidia (Larvae) of the Freshwater Mussel (*Lampsilis cardium*)

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Abstract: The early life stages of freshwater mussels are particularly sensitive to copper (Cu) contamination. We measured the acute toxicity, bioaccumulation, and sublethal effects of Cu in glochidia. In addition, we used radiolabeled Cu (^{64}Cu) to examine the time-dependent kinetics of uptake over 24 h. Uptake of ^{64}Cu by live and dead glochidia exposed to 0.11 μmol/L exhibited similar hyperbolic patterns over the first 40 min, indicating an adsorptive phase independent of larval metabolism. Thereafter, uptake was linear with time, with a 10-fold lower bioaccumulation rate in live than in dead animals, representing a close to steady state of Cu regulation. In contrast, dead glochidia exhibited a progressively increasing uptake, possibly attributable to the fact that metal-binding sites become more accessible. Mortality was strongly correlated with bioaccumulation (48 h); live glochidia exposed to Cu concentrations >0.27 μmol/L lost their regulatory ability and accumulated Cu to an even greater extent than dead animals. Exposure to Cu induced significant decreases in whole-body Na^+ and Mg^{2+} concentrations; increases in reactive oxygen species concentration, lipid peroxidation, and protein carbonylation; and a decrease in antioxidant capacity against peroxyl radicals. Overall, these results clarify the patterns of Cu uptake and regulation, emphasize the importance of distinguishing between live and dead larvae, and indicate that toxicity is associated with Cu bioaccumulation, involving both ionoregulatory disturbance and oxidative stress. *Environ Toxicol Chem* 2018;37:1092–1103. © 2017 SETAC

Keywords: Freshwater mussel; Ionoregulatory toxicity; Oxidative stress; Mechanism of copper toxic action; Glochidia

INTRODUCTION

Freshwater mussels are among the most imperiled faunal groups in North America, with approximately two-thirds of native unionids listed as endangered, threatened, or of special concern (Lydeard et al. 2004). Although this decline has been attributed to a range of factors from loss of fish hosts (Miller et al. 1989) to invasive species (Gillis and Mackie 1994), water quality plays a key role in the distribution and success or failure of freshwater mussels (Strayer et al. 2004). Recent studies have demonstrated that early life stages of freshwater mussels have heightened sensitivity to some ubiquitous waterborne contaminants such as ammonia (Augspurger et al. 2003), copper (Cu; Wang et al. 2007; Gillis et al. 2008), and chloride (Gillis 2011). However, the reason for these sensitivities, specifically the mechanisms of toxic action, remain mostly unknown.

Unionids have a unique and complex life history that includes marsupial brooding, where embryos develop into an obligate parasitic larval stage known as glochidia. Once mature, glochidia are released into the water column to make contact with a suitable host (Barnhart et al. 2008). This period when glochidia are free (i.e., released) lasts from seconds to days, depending on species-specific life-history strategies (Barnhart et al. 2008). For some waterborne toxicants, the free glochidia are the freshwater mussel's most sensitive life stage (Jacobson et al. 1997).

Copper and other aquatic contaminants can potentially limit survival of free glochidia (Jacobson et al. 1997; Wang et al. 2007; Gillis et al. 2008; Gillis 2011) or their ability to attach to a host, ultimately affecting recruitment (Jacobson et al. 1997). In fact, Canadian recovery strategies for endangered freshwater mussels indicate that ambient Cu <0.05 μmol/L (3 μg/L) is a necessary attribute of critical habitat for reproductive success (Fisheries and Oceans 2014). In aquatic organisms Cu is essential to various biological processes. However, at high concentrations this redox-active transition metal may enhance the formation of reactive oxygen species (ROS) and subsequently initiate

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oxidative damage. In addition, Cu can compete with other cations for binding and active uptake sites at the gills of aquatic animals (Niyogi and Wood 2004), exerting a range of toxic effects (Grosell et al. 2007; Grosell 2012). For example, in juvenile freshwater mussels Cu has been shown to bioaccumulate and to act as a "sodium antagonist," reducing Na^+ uptake rates, whole-body Na^+ stores, and Na^+,K^+-ATPase activity (Jorge et al. 2013; Giacomin et al. 2013). Furthermore, during chronic exposures, Cu also induced increased lipid peroxidation (LPO) in juvenile mussels, an indicator of oxidative stress (Jorge et al. 2013). However, little is known about the kinetics of Cu uptake and its effects on glochidia, which is potentially the most sensitive life stage.

With this background in mind, the present study used radiolabeled Cu (^{64}Cu) to examine the time-dependent kinetics of Cu uptake at environmentally relevant exposure levels and evaluate the associated sublethal effects in glochidia. Our goal was to understand the toxic mode of action of Cu in this understudied life stage. Our overall hypothesis was that the same mechanisms of Cu toxicity would occur in the glochidia as documented in juvenile mussels (Jorge et al. 2013; Giacomin et al. 2013). Therefore, we focused on bioaccumulation, ionoregulatory homeostasis (whole-body ion contents of Na^+, K^+, Ca^{2+}, and Mg^{2+}), and oxidative stress (ROS, antioxidant capacity against peroxyl radicals, LPO, and protein carbonylation) in short-term laboratory exposures to cast light on the mechanisms of Cu toxic action.

MATERIALS AND METHODS

Mussel collection and laboratory care

Gravid *Lampsilis cardium* (Rafinesque 1820) were collected from a reference site in the Maitland River, Ontario, Canada (43.71775, –81.12662). They were held in dechlorinated tap water in a flow-through environment (pH 8.2, dissolved oxygen = 8.7 mg/L, conductivity = 351 μS/cm, Ca = 39 mg/L, Cl = 30 mg/L, SO_4 = 28 mg/L, Na = 18 mg/L, and Mg = 9 mg/L) at Environment and Climate Change Canada's Aquatic Life Research Facility (Burlington, ON, Canada). Temperature was maintained at 12 ± 2 °C, a condition that prevents glochidia release. During this time, mussels were fed a commercial shellfish diet (Instant Algae Shellfish Diet 1800®), at a rate of approximately 1.2×10^{10} algae cells/mussel/d. Gravid females were held for up to 6 wk prior to testing.

Test organisms

All experiments were performed in reconstituted moderately hard water (experimental water, 80–100 mg/L $CaCO_3$) based on the US Environmental Protection Agency (USEPA) formulation (US Environmental Protection Agency 1994) (measured parameters: pH 7.5, Ca = 14.0 mg/L, Mg = 12.1 mg/L, Na = 26.3 mg/L, K = 2.1 mg/L, SO_4 = 81.4 mg/L, Cl = 1.90 mg/L, alkalinity = 65.0 mg/L, and S = 0.0003 mg/L) at 20 ± 1 °C. Room photoperiod was fixed at 16: 8-h light: dark. Exposure solutions were not renewed during the experiments, and the tests were conducted without aeration or food. Glochidia were collected by flushing the marsupium of a mussel (i.e., brooding chamber in the gill) with reconstituted moderately hard water, using a syringe. The viability (ability to close valves) of each mussel's glochidia was assessed prior to use, and only gravid mussels whose glochidia exhibited >90% viability were used for ^{64}Cu uptake, Cu accumulation, and Cu toxicity assays, as well as physiological and biochemical assays. Glochidia were pooled from at least 3 mussels for each experiment.

A practical problem in working with glochidia is that they are extremely small (*L. cardium* glochidia 240 μm; Tremblay et al. 2015), and as such, measurements were made on groups of glochidia, rather than individuals. However, this is complicated by the fact that some individuals in the group may not be viable (i.e., dead) as a result of toxicant (e.g., Cu) exposure. Therefore, in the present study all endpoints were also measured in 100% dead (i.e., killed) glochidia and used as background correction for live glochidia control and Cu concentration–exposed samples.

The viability of glochidia was assessed on subsamples of the test populations using a saturated salt solution (240 g/L) as described in ASTM International (2006). Valve closure is an ecologically relevant endpoint that is critical for glochidia to successfully transform on the host (ASTM International 2006). The animals used to determine viability (a destructive method) were not used in the assays.

Because glochidia are obligatory parasites, for practical purposes nonviable glochidia are considered functionally dead because they would be unable to attach to a host and complete their life cycle. Therefore, glochidia that closed their valves in response to NaCl were categorized as alive (or viable), and glochidia that had their valves closed before the addition of NaCl or that remained with their valves open after the addition of NaCl were categorized as dead (nonviable). Survival (viability) of glochidia was calculated as

$$V = 100 \times (G1 - G2)/GT \quad (1)$$

where V is the viability in percentage, G1 is the number of glochidia with their valves closed after adding NaCl solution, G2 is the number of glochidia with their valves closed before adding NaCl solution, and GT is the total number of glochidia with their valves open and those with their valves closed after adding NaCl solution. Survival was based on the mean viability of 3 subsamples of glochidia, each containing 100 to 200 glochidia.

^{64}Cu uptake assay

Short-term (24 h) Cu uptake assays were performed using ^{64}Cu in reconstituted moderately hard water (incubation medium) at a final nominal concentration of 0.16 μmol Cu/L (6 μCi/L). Dried Cu (1 mg) in the form of copper nitrate was irradiated in the Nuclear Research Reactor at McMaster University (Hamilton, ON, Canada) to achieve a radioactivity level of 0.6 mCi (^{64}Cu, half-life = 12.7 h). After irradiation, a stock solution (final concentration of 7.86 mmol Cu/L) was prepared by

resuspending ^{64}Cu in 400 μL of HNO_3 (0.1 mmol/L), 400 μL of $NaHCO_3$ (0.01 mmol/L), and 1.2 mL of reconstituted moderately hard water (total volume 2 mL). The stock solution was then diluted 1000 times to a final concentration of 7.86 μmol Cu/L (0.3 μCi/mL) and then diluted to the final incubation medium.

To investigate the involvement of energy in the Cu uptake and binding processes during the exposure, both live and dead glochidia larvae were used in this experiment and in the Cu accumulation assays, as described in *Cu accumulation and toxicity assay*. Our initial reasoning was that if Cu uptake were dependent on metabolism, Cu concentrations in live larvae would be regulated and therefore different from those in dead larvae. If it were not metabolism-dependent, Cu concentrations in live and dead larvae should be approximately the same. Larvae were killed with saturated NaCl solution (240 g/L) and then thoroughly washed with reconstituted moderately hard water.

The ^{64}Cu uptake experiments were conducted in 1.5-mL Eppendorf™ microcentrifuge tubes (Thermo Fisher) with 400 μL of incubation medium and 100 μL of experimental water (reconstituted moderately hard water without Cu) with 100 glochidia (previously separated under a dissecting microscope using a 12-well, 2-mL cell culture dish). Room photoperiod and temperature were 16: 8-h light: dark and 20 ± 1 °C. Radioactivity of the ^{64}Cu solution (i.e., hot Cu) and dead and live glochidia samples was determined in 5 replicate Eppendorf microcentrifuge tubes that had been exposed for either 1, 5, 10, 15, 20, and 40 min or 1, 2, 4, 6, 12, and 24 h. At the end of each time period, the (100 glochidia) content of each replicate was gently filtered (30 s) using polycarbonate membrane filters (0.45 μm, GE Polycarbonate; GE Water and Process Technologies) and then rinsed with 4 mL of a nonradioactive $Cu(NO_3)_2$ solution (i.e., cold Cu) containing a 10-fold higher concentration than the radioactive solution. This procedure was performed to displace any radioisotope that had adsorbed to the larvae. The polycarbonate membranes with the filtered glochidia were each transferred to a 20-mL plastic vial for subsequent radioactivity measurement. To correct for any residual isotopic interferences by the presence of the polycarbonate membrane in the vial, a blank was performed with a filter membrane without glochidia following the procedure described above.

Radioactivity from ^{64}Cu was determined using a gamma-counter (Canberra Packard Minaxi Auto gamma 5000 series gamma-counter; Meriden). The ^{64}Cu radioactivity was measured immediately after the experiments because of its short half-life (12.7 h) and automatically corrected for decay by an on-board program for decay correction of ^{64}Cu. The total dissolved (i.e., <0.45 μm) Cu concentration was measured by atomic absorption spectroscopy as described in *Cu accumulation and whole-body ion concentration analyses*. Mean measured ^{64}Cu-specific activities in the incubation medium over the appropriate periods in the experiments could then be calculated and were used for Cu uptake rate calculations. Specific activity (SA) expresses the counts per minute of ^{64}Cu per micromole of the total Cu and was calculated as

$$SA = {}^{64}Cu/\text{Total Cu} \quad (2)$$

where ^{64}Cu is radioactivity in counts per minute per liter and the total Cu concentration is in micromoles per liter in the incubation medium.

Measurements of Cu uptake from water per dead or live glochidia (micromoles per glochidia) were calculated based on the formula

$$\text{Cu uptake} = (H1 - H2) \times (1/SA) \times (1/G) \quad (3)$$

where H1 and H2 are the radioactivity values of the ^{64}Cu (counts per minute) in glochidia and blank samples, respectively; SA is the mean specific activity (counts per minute per micromole) of the radioisotope during the experimental period; and G is the number of glochidia per sample (each with 100 glochidia). Uptake of Cu in dead (i.e., killed) glochidia was also used as background correction for live glochidia samples, as described in *Corrections for dead glochidia*.

Cu accumulation and toxicity assay

To determine Cu accumulation, the lethal Cu accumulation for 50% of glochidia tested (LA50) and the concentrations of Cu that affect 20 and 50% of the exposed population (EC20 and EC50) in Cu accumulation and toxicity assays were tested. Acute Cu exposures (48 h) were performed using both dead and live glochidia under constant conditions. The 100% dead samples for bioaccumulation measurements or for background correction of the control group and Cu concentration exposures (see *Corrections for dead glochidia*) were obtained by killing glochidia as previously described in ^{64}Cu uptake assay. Exposures were conducted with a control group (0 μmol Cu/L) and 4 (nominal) Cu concentrations (0.16, 0.31, 0.62, and 1.10 μmol Cu/L, corresponding to 10, 20, 40, and 70 μg Cu/L, respectively) in 250-mL glass beakers ($n = 10$ exposure beakers for each exposure concentration) containing 200 mL of exposure medium and approximately 500 glochidia (viability >90%). Because Cu accumulation measurements require a large number of glochidia, the 10 initial beakers (per exposure concentration) were collapsed into 5 final samples (~ 1000 glochidia) for each concentration.

Exposure solutions were prepared from a stock solution made from certified American Chemical Society grade cupric sulfate ($CuSO_4 \times 5H_2O$; Fisher Scientific). All glassware was acid-washed with 10% (v/v) nitric acid (reagent grade; Fisher Scientific) prior to use, and all solutions were prepared with reconstituted moderately hard water. Preliminary tests showed that Cu loss by adsorption to the beaker surfaces was negligible. The percentage viability (i.e., survival) of a subsample of the glochidia exposed to each Cu concentration was determined as described previously in *Test organisms*. The remainder of the exposed glochidia (known proportions of dead and live glochidia) was gently filtered using polycarbonate membrane filters (0.45 μm; GE Water and Process Technologies) and then rinsed with 50 mL of Cu-free reconstituted moderately hard water.

Physiological and biochemical assays

The acute exposures destined for physiological and biochemical analyses were conducted with live and dead glochidia

exposed to 3 treatments chosen from the previous Cu accumulation and toxicity assay. Exposures were conducted with a control group (0 μmol Cu/L) and 2 (nominal) Cu concentrations (0.16 and 0.31 μmol Cu/L, corresponding to 0, 10, and 20 μg Cu/L, respectively) in 250-mL glass beakers ($n = 40$ exposure beakers for each exposure concentration) containing 200 mL of exposure medium and approximately 500 glochidia (viability >90%). Because physiological and biochemical analyses require a large number of glochidia, although the exposures were conducted in 40 individual beakers per exposure concentration, these were collapsed into 10 final samples (~2000 glochidia) for each concentration. That way, there were 5 samples for each Cu exposure concentration (either dead or alive in a known proportion) available for physiological (whole-body ion content: Na^+, K^+, Ca^{2+}, and Mg^{2+}) and for biochemical (ROS, antioxidant capacity against peroxyl radicals, LPO, and protein carbonylation) analyses, as described in *Cu accumulation and whole-body ion concentration analyses* and *Biochemical determinations*. The exposed glochidia were also collected and rinsed in Cu-free water as described in *Cu accumulation and toxicity assay*.

Cu accumulation and whole-body ion concentration analyses

Pooled glochidia collected on filters were dried (60 °C for 48 h), weighed, digested in 250 μL of 65% (v/v) HNO_3 (Suprapur®; Merck) for 48 h, and diluted up to 1500 μL with Milli-Q water. Total Cu content in the glochidia and in the experimental medium was measured by graphite furnace atomic absorption spectroscopy (Varian Spectra AA-220 with a Spectra AA GTA-110 furnace). Accumulation of Cu is expressed as micromoles per gram dry weight. Whole-body ion concentrations (Na^+, K^+, Mg^{2+}, and Ca^{2+}) were also measured in the digested samples using a flame atomic absorption spectrophotometer (Varian AA 220FS) and are expressed as micromoles per gram dry weight. Internal Cu standards were made using Cu reference solution (Fisher Scientific).

Biochemical determinations

For biochemical analyses, pooled glochidia collected on filters were placed in 1.5-mL microcentrifuge tubes containing a buffer solution (Tris-HCl 100 mmol/L, EDTA 2 mmol/L, $MgCl_2 \times 6H_2O$ 5 mmol/L, pH 7.75, 1:10 weight/volume, 4 °C), sonicated on ice (~1 min), and centrifuged (10 000 *g*, 20 min, 4 °C). Aliquots of the supernatant were collected for biomarker measurements. One aliquot was immediately used to quantify ROS, and the others aliquots were frozen (–80 °C) for measurements of antioxidant capacity against peroxyl radicals, LPO, and protein carbonylation.

The ROS and antioxidant capacity against peroxyl radicals assays followed the protocol of Amado et al. (2009) and were described in detail by Jorge et al. (2013). Briefly, total ROS was determined by the quantification of a fluorescent compound generated by the cleavage of 2′,7′-dichlorodihydrofluorescein diacetate (40 mmol/L, ~90%; Sigma-Aldrich). Fluorescence readings (excitation 488 nm, emission 525 nm) were performed using a fluorometer (Biotek FLx800; Biotek Instrument). Total fluorescence production was calculated after 45 min, with readings every 5 min. Data were fitted to a second-order polynomial function by integrating the fluorescence units over time. Measurement of ROS was expressed in relative units as fluorescence area and normalized by the total protein content in the sample homogenate (relative area per milligram of protein).

We evaluated antioxidant capacity against peroxyl radicals by the thermal decomposition of 2,2′-azobis 2 methylpropionamidine dihydrochloride (4 mmol/L; Sigma-Aldrich) and ROS formation. The resulting fluorescent compound was quantified for 30 min, as described for ROS measurement. The relative difference between ROS area in the presence and absence of 2,2′-azobis 2 methylpropionamidine dihydrochloride was considered as a measure of the tissue antioxidant capacity. Values of antioxidant capacity against peroxyl radicals were expressed as 1/relative fluorescence area and normalized to the total protein content in the sample homogenate (1/relative ROS area/mg protein).

We analyzed LPO using a commercial assay kit (no. 10009055; Cayman Chemicals), based on the quantification of malondialdehyde (MDA) production attributable to peroxidative damage in lipids induced by ROS. The MDA formed reacts with thiobarbituric acid, generating a fluorescent chromogen (excitation 515 nm, emission 553 nm). Values of LPO were expressed as micromoles of MDA per gram of wet weight, using an external standard curve built with 1,1,3,3-tetramethoxypropane standard solutions (Sigma-Aldrich).

The concentration of protein carbonylation was quantified using a commercial assay kit (no. 10005020; Cayman Chemicals) based on the 2,4-dinitrophenylhydrazine reaction. The amount of protein-hydrozone produced was quantified spectrophotometrically, after 3 washing steps, by measuring the sample absorbance (405 nm) using a microplate reader (ELx800; Biotek). Results were normalized by the total protein concentration in the homogenate supernatant. Values of protein carbonylation were expressed as nanomoles per milligram of protein. Total protein content in the sample homogenate or supernatant for data normalization was determined using the Bradford reagent (B6916; Sigma-Aldrich). The total protein content of glochidia was not statistically different ($p > 0.05$) among treatments (0, 0.16, and 0.31 μmol Cu/L).

Corrections for dead glochidia

For Cu uptake, Cu accumulation, and physiological and biochemical determinations, dead (i.e., killed) glochidia were used as background correction for live glochidia samples in the control group and the Cu exposures. In all cases, the correction was done proportionately based on determination of the relative fractions of viable and nonviable glochidia present in each sample

$$VL = [VLD - (VD \times DR)]/LR \quad (4)$$

where VL is the corrected value for live glochidia for a given parameter, VLD is the value determined for live glochidia with a

known proportion of dead glochidia, VD is the value determined for dead glochidia, DR is the fractional proportion of nonviable glochidia, and LR is the fractional proportion of viable glochidia present in the VLD determination.

Water chemistry analyses

The pH (Accumet Basic AB15; Fisher Scientific) and dissolved oxygen concentration (YSI Meter, Model 8510) were measured in the exposure medium daily. At the beginning ($t = 0$ h) and end ($t = 48$ h) of each exposure, filtered (0.45-μm syringe-tip filters, Acrodisc Supor membrane; Pall Life Sciences) samples (10 mL) of the test medium were also collected and acidified (0.5% [v/v] HNO_3; Suprapur) for Cu and water chemistry analyses, as described. The concentrations of total (nonfiltered samples) and dissolved (filtered samples) Cu in the exposure solutions were determined using graphite furnace atomic absorption spectroscopy. Internal Cu standards were made using a Cu reference solution (Fisher Scientific). Calibration was accepted if the Cu recovery was 90 to 110% based on certified reference materials TM24 and TM25 (National Water Research Institute, Environmental and Climate Change Canada). Concentrations of DOC in the exposure medium were determined in filtered water samples, collected prior to (0 h) and after (48 h) the introduction of glochidia using a total organic carbon (TOC) analyzer (Shimadzu TOC-VCPH/CPN-5050A). Reported DOC concentrations are a mean of the $t = 0$ and $t = 48$ h values. The reproducibility of measurements performed by the TOC analyzer was checked using carbon standard solutions (5 and 10 mg/L TOC), prepared from potassium hydrogen phthalate (BioXtra, ≥99.95%; Sigma-Aldrich).

Statistical analyses

The EC20 and EC50 values were derived by linear regression ($y = 6.48 + 3.04x$, $R^2 = 0.95$) between the probit transformation of the corrected mortality curve (y) and the logarithm of measured dissolved Cu concentrations (x) obtained from the toxicity assay (48 h) data presented in Figure 1A (corrected mortality [y] and measured dissolved Cu concentrations [x]). Corrected observed mortality (percentage) was calculated using Abbott's formula (Abbott 1925): ([observed mortality – control mortality]/[100–control mortality]) × 100. The LA50 value was derived by the linear regression ($y = 4.56 + 1.38x$; $R^2 = 0.97$) between the probit transformation of the corrected mortality curve (y) and the logarithm of corrected Cu accumulation (x; Figure 1B). Corrected Cu accumulation was calculated by subtracting the measured control background Cu burden (already corrected for dead larvae present) from the measured Cu bioaccumulation for each Cu exposure concentration (already corrected for dead larvae present), obtained from the accumulation assay. Values of EC20, EC50, and LA50 and their corresponding 95% confidence intervals are presented.

Dissolved Cu concentrations are expressed as mean ± standard deviation ($n = 5$). Data for Cu accumulation and biomarkers are expressed as mean ± standard deviation ($n = 5$). Differences among treatments were assessed by one-way (whole-body ion content, ROS, antioxidant capacity against peroxyl radicals, LPO, and protein carbonylation) and 2-way (Cu accumulation in live [A factor] and dead [B factor] glochidia) analysis of variance (ANOVA), followed by Tukey's test using the SigmaPlot software. When necessary, data were mathematically (log) transformed to meet ANOVA assumptions (data normality and homogeneity of variances).

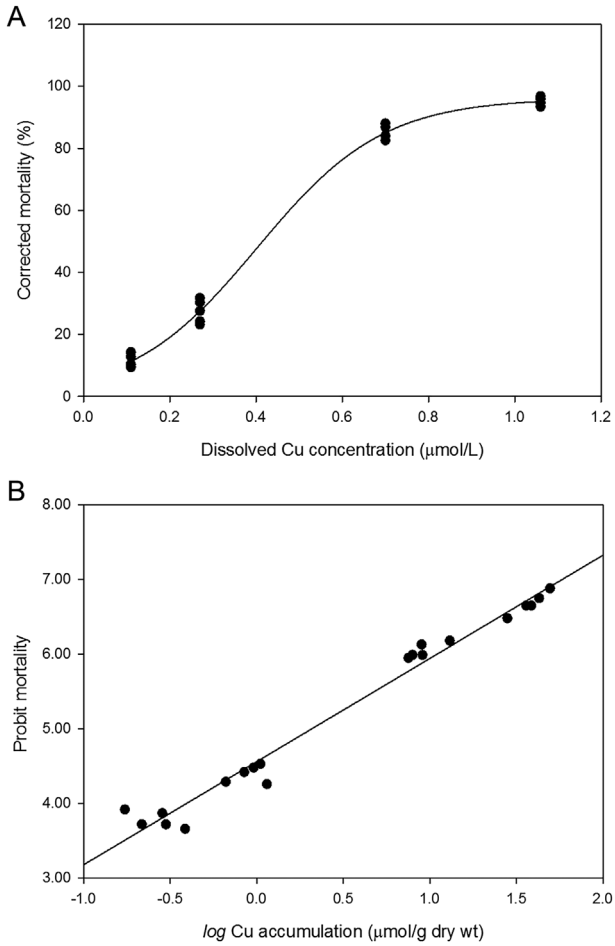

FIGURE 1: (**A**) Sigmoidal relationship ($y = 96.16/[1 + EXP(–[x – 0.40]/0.14)]$, $R^2 = 0.99$) between corrected mortality curve (y) and measured dissolved Cu concentrations (x) obtained from a 48-h exposure with Cu. (**B**) Regression analysis ($y = 4.56 + 1.38x$; $R^2 = 0.97$) between mortality (probit corrected mortality [y]) and corrected Cu accumulation (log of Cu accumulation [x]) in live *Lampsilis cardium* glochidia exposed to Cu for 48 h. Corrected mortality was calculated using the formula (observed mortality – control mortality)/(100–control mortality) × 100 (Abbott 1925).

Regression curves, both linear ($y = mx + b$, where y represents glochidia Cu uptake [micromoles per larva], x the time of exposure [hours], and m the slope of bioaccumulation against time [micromoles per larva per hour]) and hyperbolic ($y = B_{max}x/T50 + x$, where y is the Cu uptake [micromoles per larva], x is the time of exposure [hours], B_{max} is the maximum number of binding sites [micromoles per glochidia], and $T50$ is the time to 50% saturation of these binding sites [hours]), were fitted to the data from Cu uptake using SigmaPlot software. Differences between dead and live glochidia for the B_{max} and T50 parameters were assessed by Student's t test using SigmaPlot.

In all cases, the significance level adopted was 95% ($\alpha = 0.05$). Across all experiments conducted, the mean percentage of viability in the control treatments declined by 6.25 ± 0.95% over the 48-h experimental period, thus meeting the ASTM International (2006) criterion of <10% decline in control viability.

RESULTS

Water chemistry analyses

Measured dissolved Cu concentrations in the exposure medium corresponded to 0.11 ± 0.05, 0.27 ± 0.04, 0.70 ± 0.03, and 1.06 ± 0.04 μmol Cu/L for the nominal concentrations of 0.16, 0.31, 0.62, and 1.10 μmol Cu/L, respectively. The Cu concentration did not vary significantly over the duration of the acute experiments (48 h). No Cu was detected in the control medium (detection limit = 0.003 μmol/L). Because biological responses in freshwater are elicited by dissolved Cu, all results are expressed in terms of the dissolved Cu concentration. Major ion composition and other water chemistry parameters (i.e., hardness, alkalinity) were within the range described by Jorge et al. (2013) and expected for moderately hard reconstituted water (US Environmental Protection Agency 1994). Across the various experiments ($n = 5$), pH, dissolved oxygen, and DOC were 7.68 ± 0.17, 7.23 ± 0.20 mg/L, and 0.36 ± 0.13 mg C/L, respectively.

Acute Cu toxicity

Figure 1A shows the sigmoidal relationship between Cu exposure concentration and mortality after 48 h: $y = 96.16/(1 + EXP[-(x-0.40)/0.14])$, $R^2 = 0.99$. The dissolved EC50 and EC20 derived by the linear regression analysis of probit mortality ($y = 6.48 + 3.04x$, $R^2 = 0.95$) and the corresponding 95% confidence intervals were 0.33 (0.32–0.34) and 0.17 (0.15–0.19) μmol Cu/L, respectively. These correspond to 21.0 and 10.8 μg/L, respectively. Based on the linear regression analysis of probit mortality against Cu body burden (Figure 1B), the LA50 value was 2.08 (1.39–3.63) μmol/g dry weight.

Cu^{64} uptake and Cu accumulation

Uptake of Cu by live and dead glochidia (Figure 2A) exposed to a measured dissolved Cu concentration of

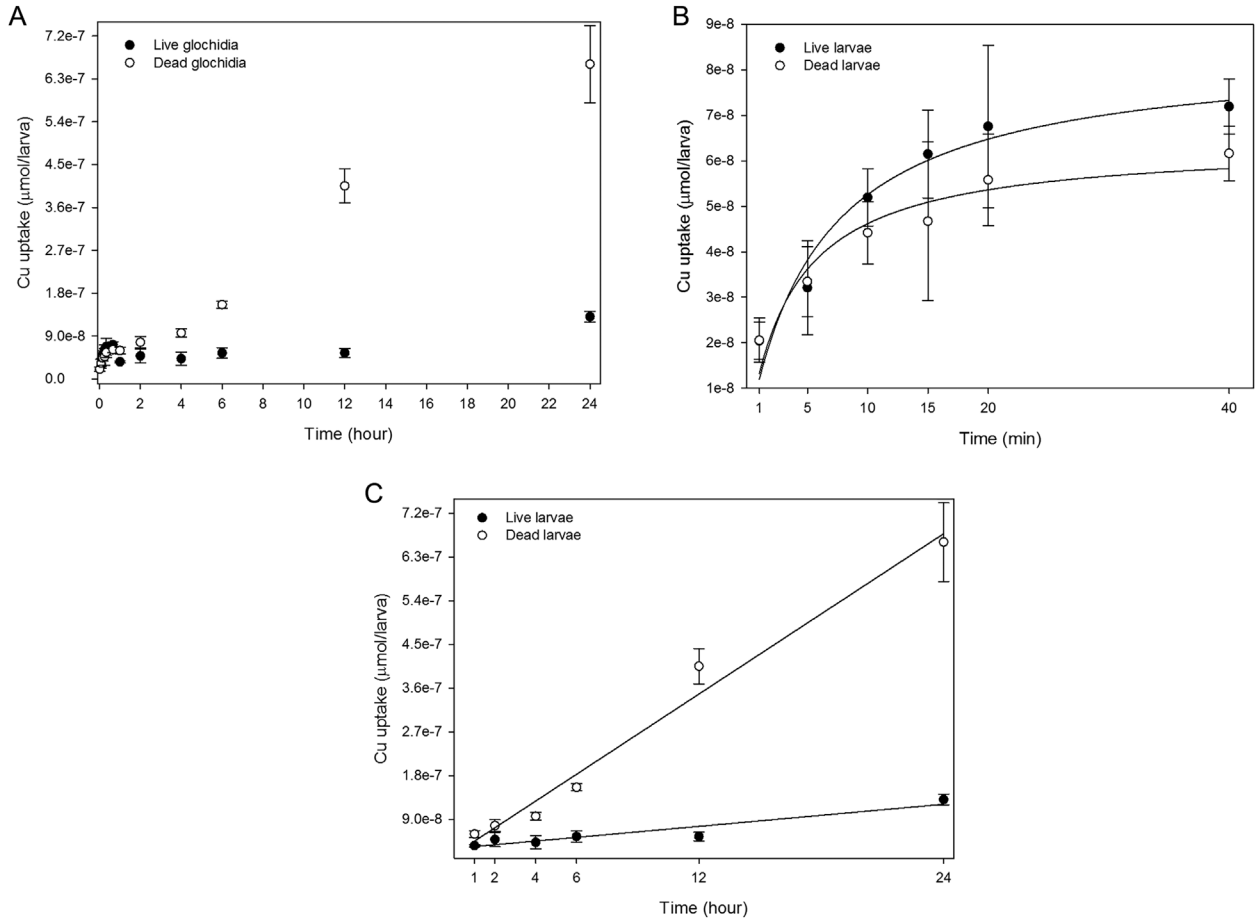

FIGURE 2: Time-dependent kinetics of Cu uptake, as determined by ^{64}Cu accumulation, in live and dead *Lampsilis cardium* glochidia exposed to a dissolved Cu concentration of 0.11 μmol Cu/L. (**A**) Uptake of Cu over the entire 24-h exposure. (**B**) Uptake of Cu over the first 40 min of exposure. (**C**) Uptake of Cu between 1 and 24 h of exposure. Data are expressed as mean ± standard deviation ($n = 5$), and accumulation is expressed as micromoles per larva. Solid lines in **B** and **C** represent the regression curves for live ($y = 8.43e\text{-}8x/[6.04 + x]$, $R^2 = 0.94$, and $y = 3.01e\text{-}8 + 3.79e\text{-}9x$, $R^2 = 0.89$, respectively) and dead ($y = 6.39e\text{-}8x/[3.83 + x]$, $R^2 = 0.91$, and $y = 1.76\ e\text{-}8 + 2.75e\text{-}8x$; $R^2 = 0.98$, respectively) glochidia.

0.11 μmol Cu/L (nominal = 0.16 μmol Cu/L) radiolabeled with ^{64}Cu showed different patterns over time, which appear to be composed of 2 components, a hyperbolic phase (Figure 2B) and a linear phase (Figure 2C). During the first 40 min of ^{64}Cu exposure (Figure 2B), both live and dead glochidia showed a time-dependent pattern of uptake represented by the regression curve $y = B_{max}x/T50 + x$. This pattern of Cu uptake revealed that live glochidia exhibit a significantly greater number of binding sites, but the time to 50% saturation is the same (B_{max} = 8.43e-8 ± 7.89e-9 μmol/glochidia; $T50$ = 6.04 ± 1.90 min; R^2 = 0.94) as for the dead ones (B_{max} = 6.39e-8 ± 5.50e-9 μmol/glochidia, $T50$ = 3.83 ± 1.36 min; R^2 = 0.91). After 40 min, the R^2 values of the hyperbolic curve fitting for live glochidia (R^2 = 0.96) progressively declined, eventually reaching R^2 = 0.25. Therefore, a linear fit became more appropriate for both live and dead animals after 40 min, with R^2 values of 0.89 and 0.98, respectively.

After 1 h of exposure (Figure 2C), net accumulation appeared to plateau in the live glochidia and the relationships crossed over, resulting in a significant difference in total accumulation between live and dead animals (3.57e-8 ± 2.14e-9 and 5.96e-8 ± 7.06e-9 μmol/glochidia, respectively). These linear accumulation patterns in both live (m = 3.79e-9 ± 6.61e-10 μmol/larvae/h; b = 3.01e-8 ± 7.54e-9 μmol/larvae; R^2 = 0.89) and dead (m = 2.73 e-8 ± 1.91e-9 μmol/larvae/h; b = 1.76e-8 ± 2.17e-8 μmol/larvae; R^2 = 0.98) glochidia continued for the remainder of the 24-h exposure. Regression analyses revealed that the slope of bioaccumulation against time (m) was significantly higher for dead glochidia. Indeed, Cu uptake by dead glochidia continued to increase steadily for the remaining time of exposure (24 h), whereas there was clear evidence of regulation in live glochidia, and no further appreciable increase in uptake occurred. After 24 h of exposure, the Cu accumulation of dead glochidia (6.60 e-7 ± 8.12e-8 μmol/larvae) was 5.06-fold higher than that in live ones (1.30e-7 ± 1.11e-8 μmol/larvae).

This pattern of differential temporal accumulation of Cu by live and dead glochidia was confirmed at low Cu concentrations by the differential concentration-dependent Cu accumulation measured in the "cold" Cu exposures (Figure 3). Live glochidia showed lower Cu accumulation than dead glochidia at the first 2 exposure concentrations (0.11 and 0.27 μmol Cu/L), but the pattern changed to higher Cu accumulation in live glochidia at the higher exposures (0.70 and 1.06 μmol Cu/L). It is important to note that Cu accumulation for live glochidia was based on the percentage of viable glochidia (94, 83, 68, 14, and 5%) at each exposure concentration (0.00, 0.11, 0.27, 0.70, and 1.06 μmol Cu/L, respectively).

Whole-body ion concentrations

Whole-body Na^+ and Mg^{2+} concentrations decreased with exposure to increasing measured dissolved Cu concentrations of 0.11 and 0.27 μmol Cu/L for 48 h, with losses of 30 to 40% (Figure 4A and C). There were no significant changes in K^+ and Ca^{2+} concentrations for either of the Cu exposures (Figure 4B and D).

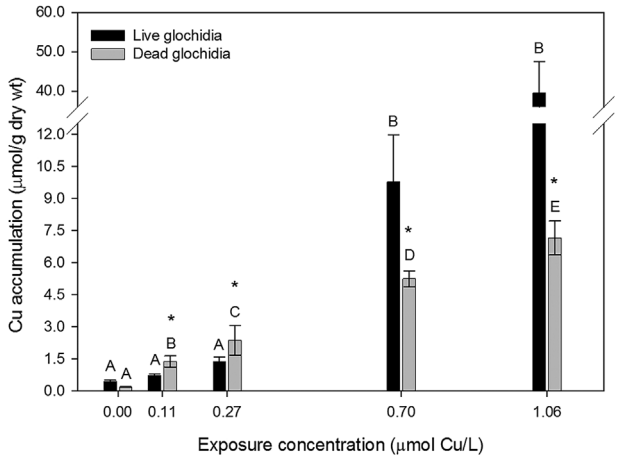

FIGURE 3: Accumulation of Cu in live and dead *Lampsilis cardium* glochidia exposed to different dissolved Cu concentrations (0, 0.11, 0.27, 0.70, and 1.06 μmol Cu/L) after a 48-h exposure. Data are expressed in micromoles per gram dry weight as mean ± standard deviation (n = 5). Different capital letters indicate significant differences over exposure concentration for the same glochidia condition (live or dead), and asterisks indicate significant differences between glochidia condition (live and dead) for the same exposure concentration.

Biochemical determinations

Because of the redox properties of Cu, we examined whether this metal would cause oxidative stress in glochidia. Larvae exhibited elevated levels of ROS after 48 h of Cu exposure, a difference which was significant (90% increase) only at 0.27 μmol Cu/L, the highest exposure concentration (Figure 5A). An indicator of the tissue capacity to scavenge ROS, antioxidant capacity against peroxyl radicals was significantly lower in glochidia exposed to 0.11 and 0.27 μmol Cu/L than in control glochidia (Figure 5B). Indicators of oxidative damage, LPO and protein carbonylation were significantly higher in glochidia larvae exposed to 0.27 μmol Cu/L (Figure 6A and B) than in control glochidia. Besides antioxidant capacity against peroxyl radicals, there were no significant changes in other oxidative stress parameters at 0.11 μmol Cu/L.

DISCUSSION

Responses of live and dead glochidia

In the present study, dead and live glochidia were assessed to investigate the involvement of active processes in Cu uptake and accumulation. The present results clearly show that Cu accumulation patterns differ between live and dead glochidia, revealing the importance of active processes in Cu regulation. The significantly elevated Cu burdens in dead (nonviable) glochidia and the observed depression in whole-body Na^+ and Mg^{2+} concentrations illustrate the importance of distinguishing between live and dead glochidia when quantifying the effects of Cu exposure on ion regulation and oxidative status. However, whether a glochidia is alive (i.e., viable) or not is grossly evident only after application of an acutely toxic salt solution that triggers the valve closure response. Because this assessment method is destructive, viable glochidia cannot be physically separated

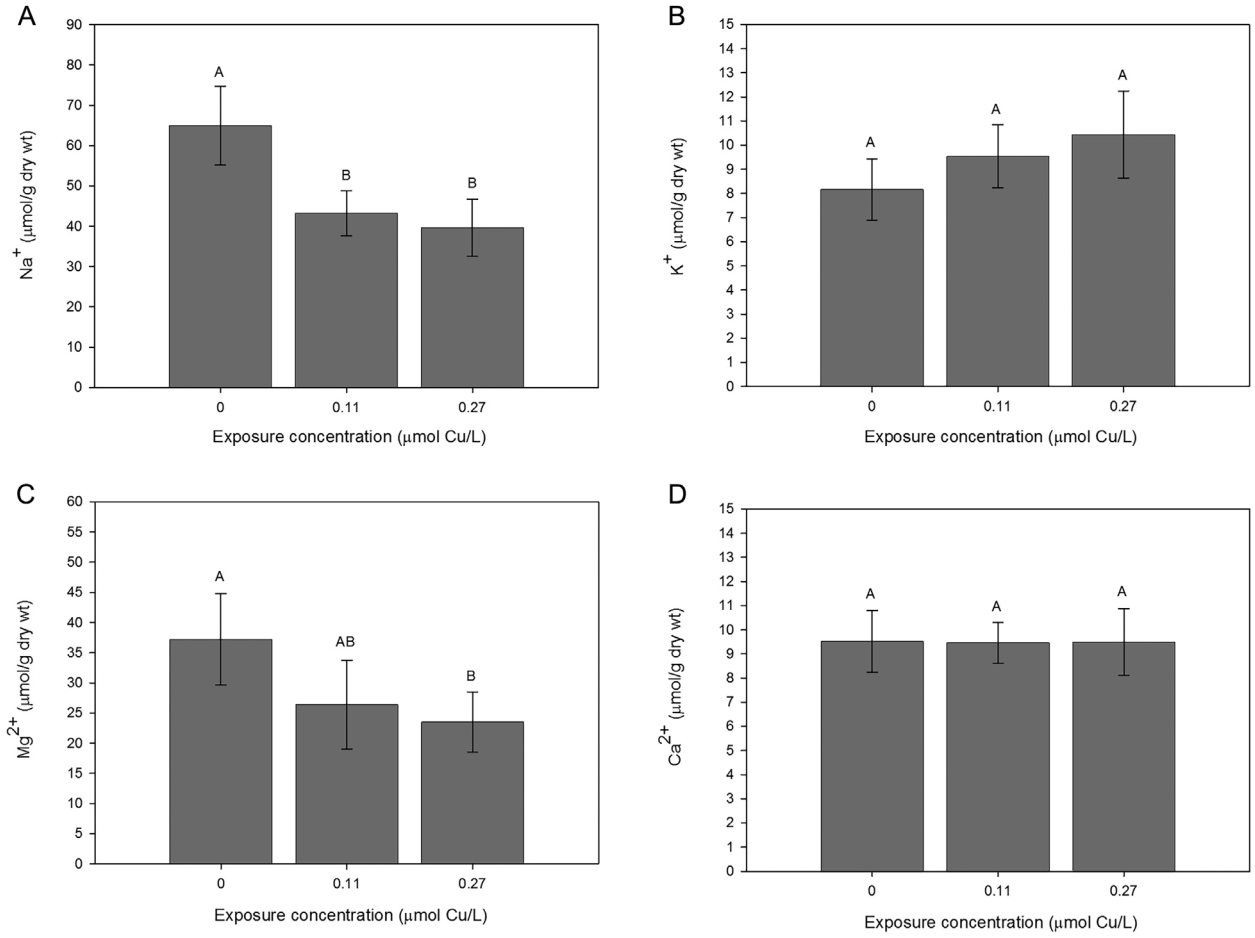

FIGURE 4: Whole-body ion content in live *Lampsilis cardium* glochidia exposed to either 0, 0.11, or 0.27 µmol Cu/L (nominal Cu concentrations) for 48 h. (**A**) Sodium (Na^+), (**B**) potassium (K^+), (**C**) magnesium (Mg^{2+}), and (**D**) calcium (Ca^{2+}) concentrations are all expressed in micromoles per gram dry weight. Data are expressed as mean ± standard deviation ($n = 5$). Different capital letters indicate significant differences across exposure concentrations.

from the nonviable glochidia, so the separation in physiological and toxicological parameters must be done mathematically. Therefore, the present results demonstrate that endpoints of interest should be assessed in both 100% live and 100% dead glochidia to reveal whether the inclusion of dead glochidia would introduce an artifact that may affect the interpretation of results.

Glochidia sensitivity

Several aspects of Cu uptake and effects on glochidia were assessed in the present study. During acute Cu exposures (48 h), *L. cardium* glochidia were sensitive to Cu in the low micrograms per liter range (48-h EC50 = 0.33 µmol Cu/L = 21.0 µg/L), which is reasonably similar to the glochidia 48-h EC50 (0.07–0.34 µmol Cu/L) reported for 7 other Ontario freshwater mussel species (Gillis et al. 2008). A number of other studies have also demonstrated the heightened sensitivity of glochidia to Cu (Jacobson et al. 1997; Wang et al. 2007). Indeed, the low EC20 (0.17 µmol Cu/L = 10.8 µg/L) observed in the present study for *L. cardium* glochidia suggests that they may not be adequately protected by the USEPA hardness-derived water quality criteria (WQC) of 0.19 µmol Cu/L (Jorge et al. 2013), though they would be protected by the more recent USEPA biotic ligand model–derived WQC of 0.034 µmol Cu/L (Jorge et al. 2013) as well as by the Canadian water quality guideline of 0.031 µmol Cu/L for waters with hardness of <120 mg $CaCO_3$/L (Canadian Council of Ministers of the Environment 2005). In fact, Wang et al. (2007) reported that glochidia were more sensitive to Cu than many of the commonly employed aquatic test organisms, such as *Daphnia magna*, *Hyalella azteca*, and *Oncorhynchus mykiss* (EC50 >0.91 µmol Cu/L). According to Gillis et al. (2008) and Jacobson et al. (1997), conglutinate and encysted glochidia, respectively, are more resistant to acute Cu exposure than released (i.e., free) glochidia. Therefore, it is important to understand the mechanism driving the sensitivity of free glochidia to ubiquitous waterborne contaminants such as Cu because mortality of this life stage can affect the reproductive success of this imperiled group.

Freshwater mussels are a large and diverse group (300 North American species) with varying life-history strategies. The size of glochidia can vary significantly across species (e.g., Ontario species lengths 56–360 µm; Tremblay et al. 2015), and the length of time they remain in the water column before making

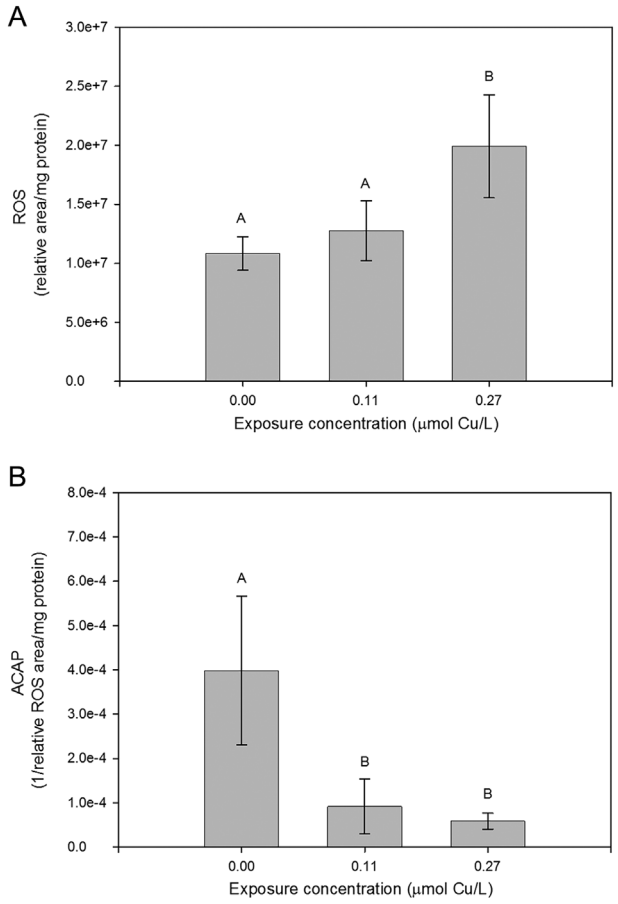

FIGURE 5: (A) Reactive oxygen species and (B) antioxidant capacity against peroxyl radicals in live *Lampsilis cardium* glochidia exposed to different nominal Cu concentrations (0, 0.11, and 0.27 µmol Cu/L) for 48 h. Data are expressed as mean ± standard deviation ($n = 5$). Different capital letters indicate significant differences across exposure concentrations. ACAP = antioxidant capacity against peroxyl radicals; ROS = reactive oxygen species.

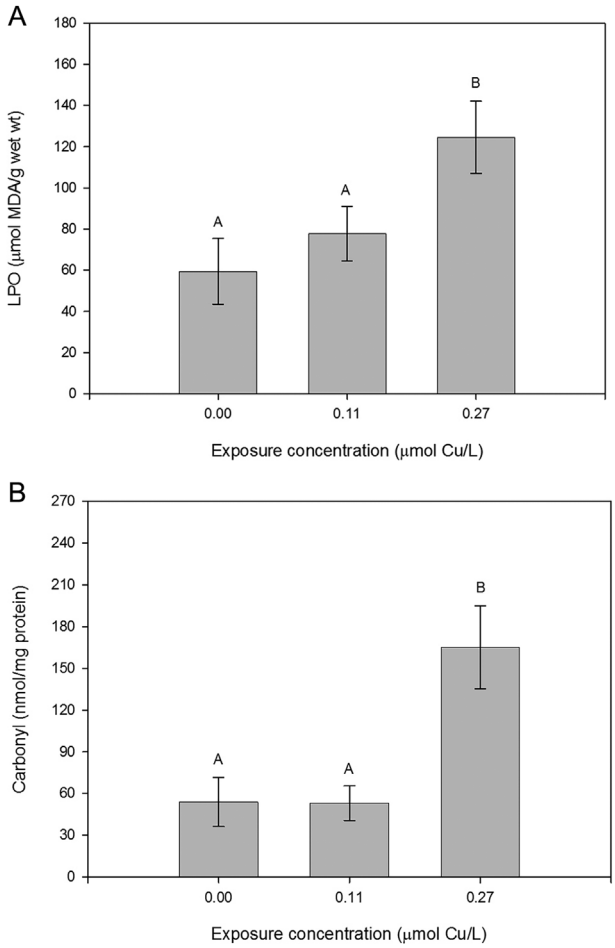

FIGURE 6: (A) Lipid peroxidation and (B) protein carbonylation in live *Lampsilis cardium* glochidia exposed to different nominal Cu concentrations (0, 0.11, and 0.27 µmol Cu/L) after 48 h. Data are expressed as mean ± standard deviation ($n = 5$). Different capital letters indicate significant differences across exposure concentrations. LPO = lipid peroxidation; MDA = malondialdehyde.

contact with a host can vary from seconds to minutes for luring species and from days to a week for species that broadcast glochidia (Cope et al. 2008). Although the present study revealed ionoregulatory disturbance as the mechanism of toxic action for one species of glochidia (*L. cardium*) after acute (48 h) Cu exposure, the same mechanism was reported in juvenile (6–8 mo old) *Lampsilis siliquoidea* after chronic (28 d) exposure (Jorge et al. 2013), indicating that the mechanism of toxic action may be conserved across species, life stage, and exposure time. However, because *L. cardium* and *L. siliquoidea* glochidia are somewhat less sensitive (EC50s ~ 20 µg/L) to Cu than other freshwater mussel species (EC50s 5–16 µg/L; Gillis et al. 2008), to fully understand this group's heightened Cu sensitivity, further research is needed to confirm whether more sensitive species and those of varying reproductive strategies have the same physiological response to Cu.

Cu uptake and accumulation

In general terms, metal uptake and accumulation may occur by 2 recognized mechanisms: one carrier-mediated, often active, and dependent on cellular metabolism, the other passive (e.g., diffusive and adsorptive processes) and not dependent on cellular metabolism. Adsorptive processes, characterized by a physicochemical interaction between metal and functional groups of the cells, can occur in both live and dead animals because such processes are not dependent on cellular metabolism. For example, metals can adhere to surface molecules such as proteins on cell membrane mucopolysaccharides (Veglio and Beolchini 1997). According to Kaduková and Virčková (2005), metal binding by adsorption is very rapid and mostly reversible. A similar but independent process is metal complexation by organic ligands, such as histidine-, cysteine- and methionine-containing proteins, with high metal affinities (Leal and Van Den Berg 1997). This type of binding is slower, more stable, and characterized by an interaction through a covalent bond between metal and ligand.

The time course of ^{64}Cu uptake into glochidia (Figure 2A) revealed different patterns for the live and dead glochidia during the exposure (24 h), indicating different uptake and binding processes. For both live and dead glochidia, Cu uptake was well

described by similar hyperbolic relationships with time, although short-term B_{max} at 40 min was greater in the live larvae (Figure 2B). However, thereafter (1–24 h), live and dead glochidia exhibited linear ^{64}Cu uptakes with very different trajectories (Figure 2C), indicative of 2 different processes. The hyperbolic patterns demonstrated that Cu uptake from water by glochidia was similar and rapid for both live and dead glochidia, suggesting an initial adsorptive phase (passive mechanism) as the first process of Cu accumulation in this life stage. After this time (1 h), the results suggest that the presence of homeostatic regulatory mechanisms in live glochidia operate to bring Cu uptake into a more or less constant equilibrium. In contrast, dead glochidia exhibited a steady increase in Cu uptake over time, probably because metal-binding sites become more accessible in dead larvae because of the loss of structural integrity and exposure of cell membranes (e.g., S-containing proteins) during the degradation process of dead larvae (Timmermans et al. 1992) after 40 min of exposure. The linear regression relationships observed from 1 to 24 h of exposure showed that the slope value (*m*) for Cu accumulation against time was over 10 times faster for dead glochidia, which supports our theory that dead glochidia expose additional binding sites which complex Cu during disintegration.

Accumulation of Cu at 48 h increased with exposure concentration for both live and dead glochidia (Figure 3). As with ^{64}Cu uptake (Figure 2C), Cu concentrations in the glochidia were higher for the dead animals at the lower Cu exposures (0.11 and 0.27 μmol Cu/L). However, at the higher Cu exposure concentrations (0.70 and 1.06 μmol Cu/L), the pattern reversed, and the live glochidia had accumulated more Cu than the dead ones by 48 h. These complex patterns emphasize the importance of distinguishing live from dead glochidia in these experiments. The regression analysis between live glochidia Cu accumulation and mortality (Figure 1B) indicates that after 48 h of exposure to elevated Cu concentrations (>0.27 μmol Cu/L), the homeostatic regulatory mechanisms observed up to the 24-h point in exposure (Figure 2C) can no longer maintain Cu uptake at a constant equilibrium. Based on these results, we conclude that the mechanisms of Cu bioaccumulation observed in live glochidia are metabolism-dependent and that Cu uptake can disturb homeostatic regulation to such an extent that death ensues. Disturbances associated with ionoregulatory homeostasis (in whole-body concentrations of Na^+ and Mg^{2+}) and oxidative stress (ROS, antioxidant capacity against peroxyl radicals, LPO, and protein carbonylation) were also observed and are discussed in *Oxidative status* as indicators of Cu toxicity.

Whole-body ion concentrations

As documented in juvenile freshwater mussels (Jorge et al. 2013; Giacomin et al. 2013), Cu bioaccumulates in glochidia and seems to act as a "sodium antagonist," reducing whole-body Na^+ stores at both Cu exposures examined (0.11 and 0.27 μmol Cu/L) (Figure 4A). The higher Cu concentration (0.27 μmol Cu/L) also reduced whole-body concentrations of Mg^{2+} to a similar degree (Figure 4C). In many other organisms, Cu has been recognized as an ionoregulatory toxicant because it reduces the ability to regulate the ionic concentration of the internal medium (Bianchini et al. 2004). Jorge et al. (2013) demonstrated that juvenile freshwater mussels (*L. siliquoidea*) chronically exposed (28 d) to 0.03 and 0.18 μmol Cu/L exhibited Na^+ losses up to 50 and 70%, paralleled by 21 and 70% mortality, respectively. Studies with freshwater fish have shown that a reduction of approximately 30% in the fish plasma or whole-body Na^+ concentration is associated with incipient mortality (Wood 2001; Paquin et al. 2002). In the present study, we observed reductions of 30 and 40% in the Na^+ and Mg^{2+} concentrations of *L. cardium* glochidia exposed to 0.11 and 0.27 μmol Cu/L, paralleled by 20 and 50% mortality, respectively. We conclude that Cu accumulation in live glochidia reduces the ability to regulate the ionic concentration of the internal medium, resulting in ionoregulatory toxicity.

Oxidative status

In addition to the ionoregulatory disturbances, the redox nature of Cu can induce rapid oxidative stress by the stimulation of ROS production or by the inhibition of antioxidant defense capacity (Harris and Gitlin 1996), resulting in oxidative damage to key molecules such as DNA, proteins, and lipids (Livingstone 2001). Jorge et al. (2013) assessed oxidative stress–related parameters (ROS, antioxidant capacity against peroxyl radicals, glutathione-*S*-transferase activity, glutathione concentration, and LPO) in whole soft tissues of juvenile freshwater mussels chronically exposed (28 d) to 0.03 and 0.18 μmol Cu/L and did not observe a clear relationship between Cu exposure and oxidative stress effects. However, that study did detect significant increases in LPO for both Cu concentrations after long-term exposure (28 d) compared with the control mussels. Gillis et al. (2014) monitored wild freshwater mussels exposed to complex mixtures of waterborne contaminants of urban origin which were rich in metals and showed significant increases in LPO and decreases in antioxidant capacity against peroxyl radicals values in mussels living downstream of the inputs. Other studies have also reported increased gill LPO in freshwater (Giguere et al. 2003) and marine (Geret et al. 2003) bivalves exposed to contamination gradients. Reduced antioxidant capacity, and thus higher susceptibility to oxidative stress, has also been reported in marine bivalves living in contaminated areas (Regoli et al. 2000; Bebianno et al. 2005).

In the present study, Cu exposure induced ROS formation in glochidia exposed to 0.27 μmol Cu/L (Figure 5A) and decreased antioxidant capacity against peroxyl radicals (Figure 5B) in glochidia exposed to 0.11 and 0.27 μmol Cu/L for 48 h, and this presumably led to oxidative damage to proteins (Figure 6A) and membrane lipids (Figure 6B). The interaction of ROS with biological membranes results in a variety of functional alterations attributable to direct interaction with the molecular cell machinery and/or oxidative modification of the biological macromolecules (Stark 2005). Increased ROS modifies the lipid composition, thus altering the phospholipid fatty acid profile of the cell membrane, which leads to loss of cellular functions through the inactivation of membrane enzymes, whose activity depends on its interaction with phospholipids (Vijayavel et al.

2007). According to Rikans and Hornbrook (1997), LPO is considered the major mechanism by which oxy-radicals can cause tissue damage, leading to impaired cellular function and alterations in physicochemical properties of cell membranes, which in turn disrupt vital functions. The present data clearly show that oxidative damage is also an important mechanism in Cu toxicity to glochidia.

CONCLUSIONS

The present results indicate that Cu bioaccumulation patterns, ionoregulatory disturbance, and oxidative stress indicators are sensitive parameters for monitoring Cu toxicity in freshwater mussel glochidia. Furthermore, these data are the first to demonstrate the causal relationship between Cu uptake and effects in this sensitive life stage. Overall, glochidia mortality showed a strong correlation with Cu bioaccumulation, though the patterns of Cu uptake differed over time, with an initial adsorptive phase independent of larval metabolism, followed by a regulatory phase that was dependent on metabolism. After 48 h of exposure, at Cu concentrations at or above 0.27 μmol/L (17.2 μg/L), glochidia lost their regulatory ability and accumulated Cu at a high rate. The elevated whole-body Cu burdens corresponded to increased glochidia mortality. The underlying mechanisms of this Cu sensitivity were related to the observed ionoregulatory disturbance and oxidative stress.

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Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (mb.jorge@ufma.br).

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