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ABSTRACT

Atmospheric carbon dioxide is rising at an accelerated rated due to increased anthropogenic activities. Metals have also been a noted problem; however, little research has addressed combined exposure of both pollutants to sensitive, calcifying organisms in freshwater habitats. This study examined copper accumulation (over 7 d) and activity of the antioxidant enzymes, catalase and glutathione peroxidase (over 2 d), in the freshwater common pond snail, *Lymnaea stagnalis,* after exposure to ambient and increased (2000 µatm) CO_2 and copper (control, 5, and 20 µg/L). Results demonstrated increased copper accumulation in soft tissue of snails exposed to copper; however, exposure to increased CO_2 did not increase the magnitude of copper accumulation. After 2 d, increased glutathione peroxidase activity was observed in snails exposed to increased CO_2 or copper individually; however, synergistic effects from exposure to both parameters were not observed. A greater response in glutathione peroxidase activity was observed in elevated CO_2-exposed snails as compared to those exposed to copper. This study provides new insight into exposure to multiple contaminants, which elicit a similar compensatory response in *L. stagnalis*.

Keywords*:* copper, acidification, toxicity, accumulation, snails

INTRODUCTION

Atmospheric carbon dioxide is rising at a rapid pace, with a reported increase of 30% over pre-industrial levels (Anthony et al. 2011; Hoegh-Guldberg 1999; Caldeira and Wickett 2003; Siegenthaler et al. 2005; IPCC 2007, 2014). Levels of CO_2 are predicted to continue increasing up to 1000 µatm by the end of this century and over 1900 µatm by the year 2300 (Caldeira and Wickett 2003). An estimated 25% of the CO_2 being emitted into the air is dissolving into the water resulting in acidification (IPCC 2007, 2014; Feely et al. 2009) and, in comparison to the oceans, freshwater systems have less buffering capacity. Water and CO_2 react to form carbonic acid which decreases the *p*H; carbonic acid can dissociate to bicarbonate, and further dissociate to carbonate. Shifting the balance between these different carbon species may have dramatic effects on aquatic life,

depending on which carbon species dominates (Fabry 2008; Fabry et al. 2008; Muehllehner et al. 2016; Wanninkhof et al. 2018; Lord et al. 2019).

Calcifying organisms are particularly threatened due to the decreased calcium carbonate saturation state caused by acidification (Orr et al. 2005; Fabry 2008; Guinotte and Fabry 2008; Lord et al. 2019). Calcification involves precipitation of dissolved ions into solid calcium carbonate structures, which can be later dissolved if the water body does not contain saturating carbonate ion concentrations (Caldeira and Wickett 2003; Feely et al. 2009). When calcium carbonate is not sufficiently available in the environment, calcifying organisms can be affected with slower rates of calcification and growth, shell deformations, altered ion balance and acid-base regulation, altered behavior, as well as other physiological effects (Fabry et al. 2008; Atli and Grosell 2016; Hannan et al. 2016; Jeffrey et al. 2017a, 2017b, 2018a, 2018b; Bielmyer-Fraser et al. 2018; Lord et al. 2019). Jeffrey et al. (2017a) reported a decrease in gene expression, associated with shell deformation, and an increased oxygen consumption and stress response in freshwater mussels after chronic exposure to elevated CO_2. The authors suggested the increased energy demand for the stress response decreased the energy diverted to shell formation (Jeffrey et al. 2017a). Additionally, effects of increased CO_2 on crustaceans and gastropods, such as reduced shelter-seeking behavior in crayfish and decreased predator-induced defenses in *Daphnia* and snails, have also been noted (Weiss et al. 2018; Robertson et al. 2018; Lord et al. 2019). The problems of elevated CO_2 may be exacerbated by increased pollution, such as that caused by metals.

Metal pollution is a common occurrence in many aquatic environments; especially in heavily populated areas (Klein 1979; Bielmyer-Fraser et al. 2017). Anthropogenic sources of copper include mining and refining, electrical manufacturing, pesticides, fertilizers, antifouling paints, and others; which can enter aquatic systems via industrial discharges, agricultural run-off, and domestic stormwater runoff (Pratt et al. 1981; Guzman and Jimenez 1992; Alva et al. 1995; Gonzalez et al. 1999; Hoang et al. 2008a; Echols et al. 2009). Although trace amounts of copper are necessary for carbohydrate metabolism and the functioning of enzymes, excess copper in aquatic systems can accumulate in aquatic organisms and cause adverse effects (Bielmyer et al. 2002, 2005, 2006). It is important to understand how and to what extent copper accumulates in sensitive calcifying organisms and the threshold concentrations causing adverse effects, particularly in conjunction with global stressors such as increased acidification (Ekstrom et al. 2015). Additionally, speciation and bioavailability of copper in aquatic systems is substantially influenced by *p*H, with a lower *p*H favoring the more bioavailable and toxic ionic form (Campbell 1995; DiToro et al. 2001).

Copper exposure can cause the generation of reactive oxygen species via Haber-Weiss and Fenton reactions in aquatic organisms, which can lead to degradation of macromolecules like proteins, lipids, and DNA (Chang et al. 1996; Nagalakshmi and Prasad 1998; Abele and Puntarulo 2004; Luschak 2011). Oxidative reactions that can protect the cell from hydrogen peroxide and mitigate the effects of reactive oxygen species are catalyzed by the enzymes catalase and glutathione peroxidase (Stauber and Florence 1987; Srivastava et al. 2006; Main et al. 2010; Brock and Bielmyer 2013; Patel and Bielmyer-Fraser 2015; Siddiqui and Bielmyer-Fraser 2015; Siddiqui et al. 2015; Bielmyer-Fraser et al. 2018). Both enzymes can catalyze the conversion of hydrogen peroxide to water and oxygen via different reactions (Halliwell and Glutteridge 1999). Upregulation of genes associated with oxidative stress has been reported in the freshwater common

pond snail, *Lymnaea stagnalis*, as a consequence of pollutant exposure (Bouetard et al. 2013). Early life stages of *L. stagnalis* have been shown to be very sensitive to metals (Grosell and Brix 2009; Brix et al. 2011; 2012; Tania et al. 2011) and altered activity of antioxidant enzymes was a reported effect of copper exposure in adult *L. stagnalis* (Atli and Grosell 2016). Quantifying the level of reactive oxygen species in *L. stagnalis* may give insight to the overall health of the snail, particularly concerning DNA, protein, or lipid damage (Ali et al. 2015). Little research has examined the effects caused by both global changes in acidification and local changes in water quality, such as metal pollution, in sensitive freshwater calcifying organisms (Lefcort et al. 2015). The goal of this project was to characterize copper accumulation and the antioxidant enzyme response in juvenile *L. stagnalis* exposed to increased CO_2-driven acidification and copper.

MATERIALS & METHODS

Testing Organisms

Lymnaea stagnalis egg masses were shipped from McMaster University, Canada, and have been cultured in the Jacksonville University laboratory since May 2017, in 38-L tanks filled with moderately hard water and constant aeration. Temperature was maintained at 24 ºC using a water bath and recirculating chiller/heater (Model MC-1/4HP; AquaEuro Systems; Los Angeles, California), and the light cycle was maintained at 12 h light, 12 h darkness. The snails were fed daily romaine lettuce and sweet potatoes ad libitum. Temperature and dissolved oxygen were measured daily using a YSI meter (Model YSI-85; Pentair Aquatic Ecosystems, Apopka, Florida), and *p*H was measured weekly using a calibrated *p*H meter (WTW, Xylem Inc.). Hardness, alkalinity, nitrite, nitrate ammonia, and chloride were measured weekly using a LaMotte colorimetric kit (Pentair Aquatic Ecosystems, Apopka, Florida).

Testing Solutions

Synthetic moderately hard water was made following U.S. EPA guidelines (USEPA 2002). Briefly, 96 mg/L $NaHCO_3$, 90 mg/L $CaSO_4$, 60 mg/L $MgSO_4$, and 4 mg/L KCl were added to ultrapure 18 mΩ Milli-Q water and vigorously mixed for 24 h. At the start of the experiments, hardness and alkalinity of the base water were measured using standard titrimetric methods according to American Public Health Association guidelines (method 2340 and 2320, respectively; APHA 1985); and concentrations of Ca^{2+}, Mg^{2+}, K^+, and Na^+ were measured using atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800; Table I).

For each experiment, two sets (one for each CO_2 level, ambient [~400 µatm; IPCC 2014] and 2000 µatm [near the predicted level by the year 2300; Caldeira and Wickett 2003]) of three copper concentrations (control [0], 5 µg/L [near the U.S. Environmental Protection Agency Water Quality Criterion; USEPA 2007], and 15 µg/L [the concentration commonly found in polluted water bodies; Pyati et al. 2012]) were made by mixing moderately hard water and a certified 1 mg/L copper, as $Cu(NO_3)_2$ stock solution (in varying amounts). Metal solutions (8 L per tank) were added to six 38-L tanks, covered with plexiglass, and equilibrated for 24 h prior to testing.

Table I. Water chemistry values in the moderately hard testing water at two CO_2 levels from both experiments measured at the beginning of each experiment

Measured parameter	Experiment 1	Experiment 2
Calcium (mg/L)	18.1	17.8
Magnesium (mg/L)	2.65	10.8
Sodium (mg/L)	36.8	26.3
Potassium (mg/L)	1.42	20.5
Chloride (mg/L)	20	53
Alkalinity (mg $CaCO_3$/L)	85	85
Hardness (mg $CaCO_3$/L)	110	89

Experimental Design

A *p*H/*p*CO_2 DAQ-M system with CapCTRL software (Loligo Systems, Viborg, Denmark) was used in the experiments. In each experiment, three of the tanks were attached to the *p*H/*p*CO_2 system to maintain increased CO_2 level (2000 µatm) and three were maintained at ambient CO_2 (~400 µatm; IPCC 2014). This system has been used previously (Siddiqui and Bielmyer-Fraser 2015; Duckworth et al. 2017). A standard curve for the calibration was prepared using a known *p*CO_2/*p*O_2 gas mixture of 2250 ppm, 100% CO_2, and ambient *p*CO_2. The *p*H of each tank was continuously monitored and regulated using an automated negative feedback system, which dispensed pure CO_2 gas into the water when needed. Each tank was also continuously aerated.

Two sequential experiments were performed. Mortality was assessed daily in both experiments. In the first experiment, 21-day old snails were exposed to increased copper and CO_2 for 7 d and tissue copper concentration was measured at 0 (at the start of the experiment), 4, and 7 d. At the start of the experiment, four snails were obtained directly from the holding tanks and dissected (soft tissue was removed from the shell) under a dissection scope. The soft tissue from two snails was pooled in two replicate pre-weighed weight boats, dried in an oven for 24 h at 70 ºC, and digested in concentrated, trace-metal grade nitric acid for metal analysis. At 4 and 7 d, four snails (two replicates each with two snails) from each control group (400 and 2000 µatm CO_2), and six snails (three replicates each with two snails) from all other experimental groups were dissected, dried in an oven, and digested (as specified above).

In the second experiment 18-day old pond snails were exposed to increased copper and CO_2 for 2 d, and activity of the enzymes catalase and glutathione peroxidase were measured at the start and end. At the start of the experiment, 12 snails (three replicates of four pooled snails) were obtained directly from the holding tanks, dissected (soft tissue was removed from the shell) under a dissection scope, rinsed with 18 mΩ ultrapure water, and immediately frozen at -80 °C for later enzyme analysis. A total of 12 snails were added to each tank and, at 48 h, all the snails from each tank were dissected. The soft tissue from four snails was pooled in three replicate microcentrifuge tubes and immediately frozen at -80 °C. We assumed that the 18- and 21-d old snails would respond similarly, given that there was no appreciable size difference, and the snails were at the same developmental stage.

Water Chemistry

During both experiments, temperature and dissolved oxygen were measured using a YSI meter (Model YSI-85; Pentair Aquatic Ecosystems, Apopka, Florida), and *p*H was measured using a calibrated *p*H meter (WTW, Xylem Inc., USA) and probe, daily in every tank. At the beginning and end of the experiments, nitrate, nitrite, and ammonia were measured using a LaMote colorimetric testing kit (Pentair Aquatic Ecosystems, Apopka, Florida). All water chemistry data, including ion concentrations from the base moderately hard water, are presented in Tables I and II.

Table II. Water chemistry (mean ± standard deviation) in the experimental solutions from both experiments

	Experiment 1		Experiment 2	
Measured parameter	Ambient CO_2	2000 µatm CO_2	Ambient CO_2	2000 µatm CO_2
Temperature (°C)	23.5 ± 0.11	23.6 ± 0.11	21.6 ± 0.04	22.1 ± 0.13
DO[a] (mg/L)	8.66 ± 0.05	8.51 ± 0.07	8.48 ± 0.70	8.18 ± 0.70
*p*H	8.19 ± 0.04	6.67 ± 0.05	7.99 ± 0.16	6.70 ± 0.18
CO_2 (µatm)	Ambient	1838 ± 320	Ambient	2576 ± 94.8

[a]Dissolved oxygen

Copper Analysis

Water samples were collected, filtered (with a syringe and 0.45 µm filter) in 15-mL centrifuge tubes, and acidified with trace metal grade nitric acid on each sampling day in both experiments. Filtered water samples and digested tissue samples were measured for copper using atomic absorption spectrophotometry with graphite furnace detection (detection limit = 1 µg/L). Certified copper standards were used for each calibration, and recalibration was performed after every 30 samples, with periodic quality controls. Copper concentrations in the experimental solutions are presented in Table III. Copper accumulation in the tissue is presented as micrograms of copper per gram of dry weight. Copper speciation was modeled using Visual MINTEQ (Table IV).

Table III. Measured copper concentrations (micrograms per liter; mean ± standard deviation) in testing solutions at two CO_2 levels from both experiments

	Experiment 1		Experiment 2	
Nominal [Cu]	Ambient CO_2	2000 µatm CO_2	Ambient CO_2	2000 µatm CO_2
Control	0.87±0.33	1.19±0.53	1.02±0.72	0.73±0.34
5 µg/L	4.64±0.31	5.56±0.08	3.85±0.20	5.53±0.27
15 µg/L	12.61±1.59	12.11±0.26	13.22±0.51	15.45±0.69

Protein and Enzyme Assays

Frozen snail samples were homogenized using 50 mM KH_2PO_4/K_2HPO_4 buffer and a mortar and pestle on ice. Homogenate was transferred to polypropylene centrifuge tubes and centrifuged at 5000 rpm for 10 min at 4 °C. Supernatant was collected and preserved at -80 °C for protein quantification. A Bradford assay (Bradford 1976) was performed to quantify total soluble protein using the Bio-Rad quick start kit (Bio-Rad, California)

according to the manufacturer's instructions. Bovine serum albumin (BSA) standards (0, 0.02, 0.04, 0.08, 0.12, and 0.24 mg/mL) were prepared from a 2 mg/ml BSA stock solution and 50 mM KH_2PO_4/K_2HPO_4 buffer. Absorbance was recorded at a wavelength of 595 nm using a Perkin Elmer Lamda 35 UV/Vis spectrometer. Catalase and glutathione peroxidase activities were also measured using UV/VIS spectrometric methods. Catalase activity was measured using the rate of H_2O_2 decomposition at a wavelength of 240 nm, following a Sigma method (Sigma 1994a; EC 1.11.1.6). Glutathione peroxidase activity was determined following a Sigma protocol with which we measured the decrease in NADPH absorbance at 340 nm for 5 min (glutathione peroxidase, EC 1. 11. 1. 9; Sigma 1994b). Activity of the enzymes was normalized for protein content of the sample (Patel and Bielmyer-Fraser 2015; Siddiqui et al. 2015; Duckworth et al. 2017).

Data Analysis

Treatments were statistically compared using SigmaStat. Data were analyzed for normality and equal variance using Shapiro-Wilks and Brown-Forsythe tests, respectively; followed by a one-way ANOVA. Copper accumulation data were normally distributed and significant differences between treatments ($p \leq 0.05$) were determined using a Student's *t* test or Welch's *t* test, depending on whether the data had equal variances or did not have equal variances, respectively. Glutathione peroxidase and catalase data were normally distributed with equal variances and significant differences were determined using Holm-Sidak method ($p \leq 0.05$).

RESULTS

Copper Concentrations and Speciation

Measured copper concentrations in the exposure media were comparable between the two experiments and close to nominal values ranging from 77–111% (Table III). There was a similar distribution of copper species at a particular CO_2 level between the experiments (Table IV). In both experiments, ionic copper concentration was greater at 2000 µatm CO_2 than at ambient CO_2. Copper ion concentrations were similar between the two experiments at 2000 µatm CO_2 (6.22E-08 M versus 6.12E-08 M) and less than a one-fold difference at ambient CO_2 (2.53E-09 M versus 4.23E-09 M; Table IV).

Tissue Copper Accumulation

At both CO_2 levels, *L. stagnalis* exposed to 5 µg/L copper ($p = 0.0335$ at ambient CO_2; $p = 0.0200$ at 2000 µatm CO_2) and 15 µg/L copper ($p = 0.007$ at ambient CO_2; $p = 0.0146$ at 2000 µatm CO_2) had significantly increased copper in their soft tissue at 4 d, as compared to controls; however, soft tissue copper decreased by 7 d and was not significantly different from control values (Figure 1). Significant differences in soft tissue copper were not observed between the 5 and 15 µg/L copper treatments at 4 d at both CO_2 levels, and at 7 d at 2000 µatm CO_2. At 7 d of exposure to 15 µg/L copper at ambient CO_2, the snails were too small to accurately measure the tissue copper (Figure 1). Mean tissue copper over the 7 d was 1.1 µg/g dry weight in all control groups and reached values of 13.8 and 12.7 µg/g dry weight in the 5 and 15 µg/L copper treatments (from both CO_2 levels), respectively (Figure 1). No significant differences were observed in *L. stagnalis*

Table IV. Inorganic copper speciation (concentrations in moles per liter) in the moderately hard testing water at two CO_2 levels from both experiments[a]

	Experiment 1		Experiment 2	
Species	Ambient CO_2	2000 µatm CO_2	Ambient CO_2	2000 µatm CO_2
$Cu(CO_3)_2^{2-}$ (aq)	1.32E-08	3.1E-10	8.71E-09	3.42E-10
$Cu(OH)_2$ (aq)	2.28E-09	5.1E-11	1.46E-09	5.57E-11
$Cu(OH)_3^-$	1.77E-11	1.2E-14	7.25E-12	1.41E-14
$Cu(OH)_4^{-2}$	1.89E-16	3.85E-21	5E-17	5E-21
Cu^{+2}	2.53E-09	6.22E-08	4.23E-09	6.12E-08
$Cu_2(OH)_2^{+2}$	3.28E-12	1.8E-12	3.52E-12	1.93E-12
Cu_2OH^{+3}	1.96E-16	3.57E-15	3.48E-16	3.73E-15
$Cu_3(OH)_4^{+2}$	7.3E-15	8.98E-17	5.03E-15	1.05E-16
$CuCl^+$	2.16E-12	5.29E-11	9.22E-12	1.33E-10
$CuCl_2$ (aq)	2.73E-16	6.69E-15	3.03E-15	4.38E-14
$CuCl_3^-$	1.4E-21	3.43E-20	4.11E-20	5.94E-19
$CuCl_4^{-2}$	4.72E-27	1.16E-25	3.74E-25	5.41E-24
$CuCO_3$ (aq)	2.08E-07	1.58E-07	2.11E-07	1.59E-07
$CuHCO_3^+$	3.28E-10	8.25E-09	5.33E-10	7.83E-09
$CuOH^+$	9.56E-09	7.08E-09	9.82E-09	7.28E-09

[a]Inorganic speciation calculations were performed by inputting measured water chemistry and copper concentrations in the highest exposure treatment (15 µg/L Cu) into the metal speciation program MINTEQ.

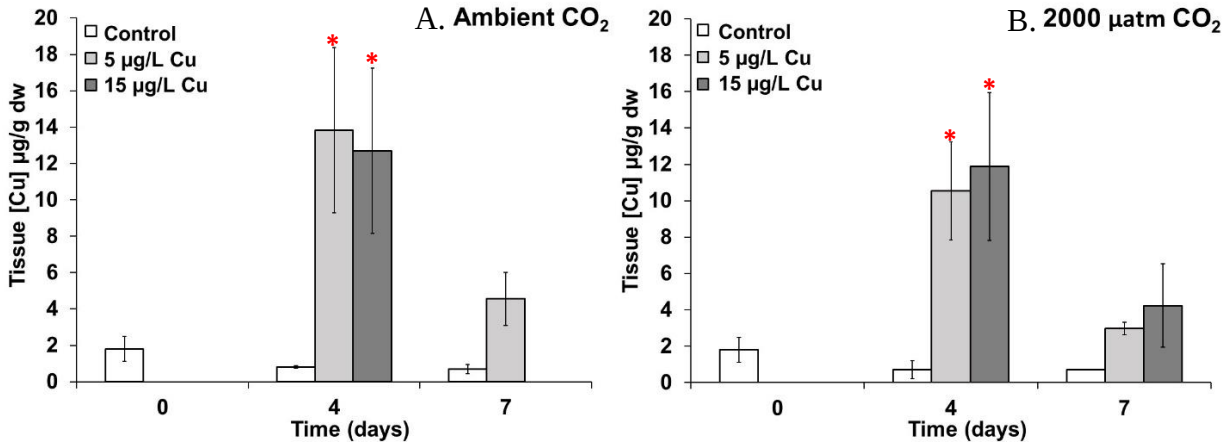

Figure. 1. Mean soft tissue copper concentrations in *L. stagnalis* exposed to control (white bars), 5 (light gray bars), and 15 µg/L Cu (dark gray bars) at A) ambient CO_2 (~400 µatm) and B) 2000 µatm CO_2 over 7 d. Error bars represent standard error of the mean (n = 2, each with two pooled snails in the controls; n = 3, each with two pooled snails in all copper-exposed treatments). Asterisks indicate significant differences from controls at a specific time ($p \leq 0.05$). At 7 d of exposure to 15 µg/L copper at ambient CO_2, the snails visually decreased in size and were too small to accurately measure their tissue copper.

soft tissue copper as a consequence of increased CO_2 at a specific copper concentration (Figure 1). No mortality was observed in any of the treatments.

Antioxidant Enzyme Activity

At ambient CO_2, glutathione peroxidase activity appeared to increase, although not significantly, in *L. stagnalis* exposed to 5 µg/L copper (p = 0.057); however a significant increase was observed in the highest copper concentration of 15 µg/L copper (p = 0.009), as compared to controls (Figure 2A). The glutathione peroxidase activity was also significantly increased due to CO_2 exposure (p = 0.0400) alone; however, synergistic effects from exposure to both parameters were not observed (Figure 2A). The glutathione peroxidase activity in snails exposed to both copper (5 and 15 µg/L) and 2000 µatm CO_2 were more variable and not significantly different from the controls (p = 0.095). Additionally, none of the copper exposed groups, regardless of CO_2 level, were significantly different from each other. The increase in glutathione peroxidase activity observed in *L. stagnalis* exposed to 2000 µatm CO_2 alone was greater (approximately double) than the increase in glutathione peroxidase activity from exposure to 5 and 15 µg/L copper (Figure 2A). Contrary to glutathione peroxidase activity, no significant differences in catalase activity in *L. stagnalis* were observed due to increased copper or CO_2 level (Figure 2B).

DISCUSSION

Copper Accumulation and Toxicity

The mean tissue copper (13 µg/g dry weight) accumulating in 21-d old, copper-exposed *L. stagnalis* in the present study is similar to that reported in snails in other studies. Hoang et al. (2008b) reported whole body soft tissue copper concentrations of 29.57, 48.41, and 61.87 µg/g dry weight in juvenile apple snails (*Pomacea paludosa*) exposed to 6.0, 8.2, and 12.2 µg/L copper for 14 d. Rogevich et al. (2008) reported a lethal whole body (including shell) copper concentration of 30 µg/g dry weight in dead 120-d old apple snails (*P. paludosa*) after exposure to 172 µg/L copper for 4 d; whereas, surviving apple snails accumulated 13.3 µg/g dry weight after exposure to 51.4 µg/L copper for 7 d. Adult *L. stagnalis* exposed to 3 mg/L copper for 2 h had tissue copper concentrations of 13.4, 8.9, and 17.0 µg/g dry weigth in head-foot, intestinal mass, and mantle, respectively (Spronk et al. 1973).

Copper uptake occurs through protein channels in the skin (body surface), gills, or gill-like structures of many freshwater aquatic animals, generally as a function of sodium turnover rate (Grosell et al. 2002). In the present study, copper tissue concentration increased from 0 to 4 d in copper-exposed groups, and then decreased from 4 to 7 d; possibly due to detoxification, excretion, or potentially copper transfer to the shell. The latter is less likely because generally less than 5 % of accumulated metal has been shown to partition in the shell of snails (Laskowski and Hopkin 1996; Pyatt et al. 2003; Desouky 2006; Hoang et al. 2008b; Rogevich et al. 2009). However, Pyatt et al. (2003) reported a difference in copper tissue and organ distribution in the adult snail, *Lymnaea peregra*, exposed to different copper concentrations. This change in tissue copper distribution due to copper exposure concentration was also noted in adult apple snails (Rogevich 2009). Spronk et al. (1973) reported a levelling of copper uptake rate in the shell rim, mantle rim,

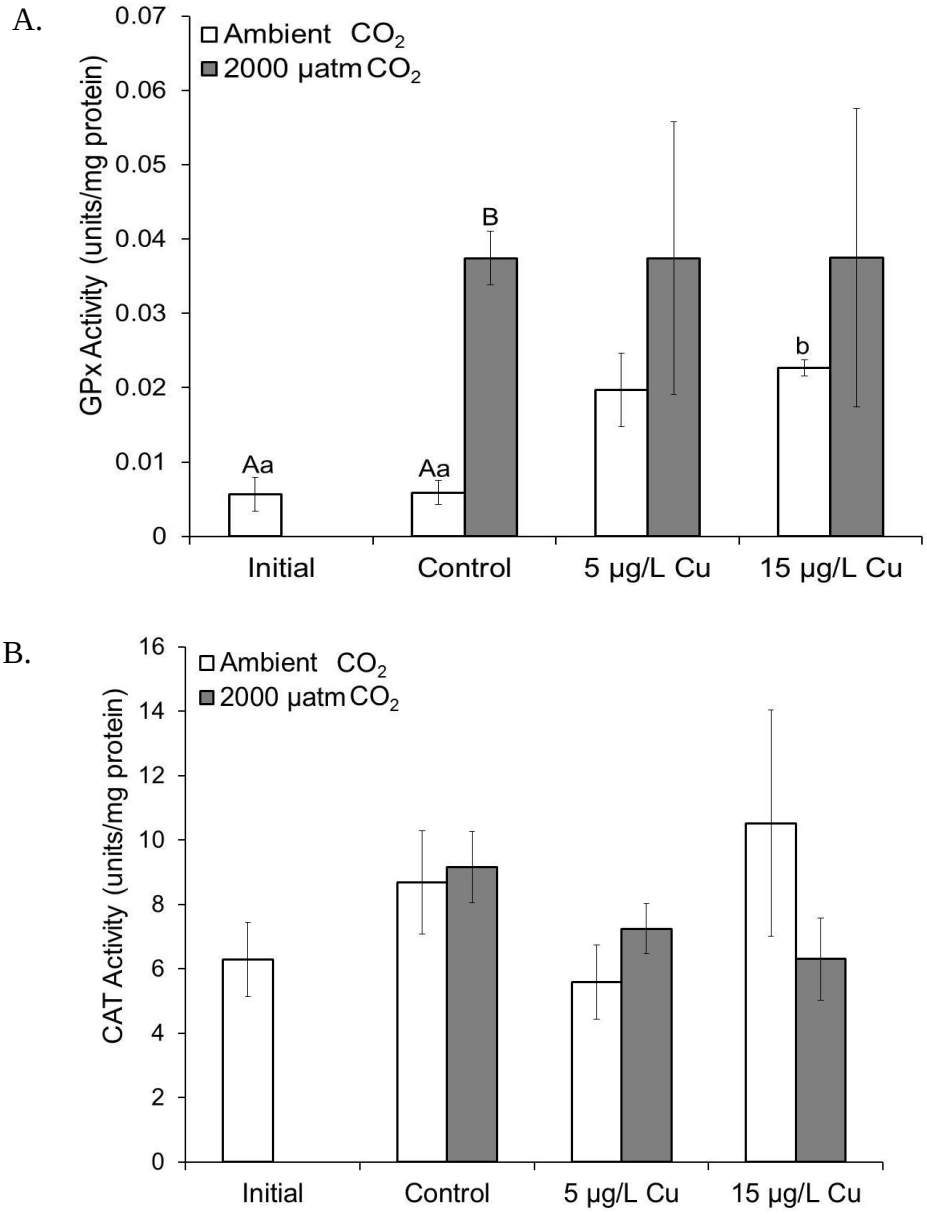

Figure. 2. A. Mean glutathione peroxidase and B. mean catalase activity in *L. stagnalis* exposed to control, 5, and 15 µg/L copper at ambient CO_2 (~400 µatm; open bars) and 2000 µatm CO_2 (gray-filled bars) for 2 d. Error bars represent standard error of the mean (n = 3, each with four pooled snails). Different upper-case letters indicate a significant difference ($p < 0.05$) between groups due to CO_2 concentration at a specific copper concentration. Different lower-case letters indicate a significant difference ($p < 0.05$) between groups due to copper exposure at a specific CO_2 concentration.

and intestinal mass of *L. stagnalis* over 4-h exposure to 3 mg/L copper. Spronk et al. (1973) proposed that copper intake equilibrated over time and copper was transferred to slime or other tissues and perhaps secreted. The higher metabolic rate of the smaller, early-life stage snails may have enabled a faster homeostatic regulation of the sublethal copper concentrations used in this study.

Tissue copper in *L. stagnalis* was not changed as a consequence of increased CO_2 at a specific copper concentration. This result was unexpected given that ionic copper is generally known to be the most bioavailable form (DiToro et al. 2001). However, it is also possible that copper carbonate complexes are bioavailable to the snails in addition to the ionic copper species, which has been suggested in other studies (Rogevich et al. 2008). In our study, $CuHCO_3^+$ concentration was greater at the highest CO_2 level, like the Cu^{2+} ion concentration; $CuCO_{3(aq)}$ concentration was similar between the two CO_2 levels; but $Cu(CO_3)_2^{2-}{}_{(aq)}$ concentration was an order of magnitude higher in the ambient CO_2 treatments. Bioavailability of $Cu(CO_3)_2^{2-}{}_{(aq)}$ to *L. stagnalis* could have been one reason for the similar concentrations of tissue copper at both CO_2 levels. An additional explanation is that exposure to increased CO_2 could have influenced copper detoxification and homeostasis capabilities in the snails. The response in glutathione peroxidase activity (discussed below) supports this reasoning.

Copper concentrations in this experiment were chosen to enable measurement of sublethal responses, using published toxicity values as a reference. Rogevich et al. (2008) reported 96-h concentrations causing lethality to 50% of the organisms (LC_{50}) values of 30.7, 43 and 45 µg/L copper in less than 4-d, 14-d and 30-d old apple snails (*P. paludosa*), respectively. Similarly, a 96-h LC_{50} value of 31 µg/L copper was reported for 7-d old *L. stagnalis* (Brix et al. 2011) and 96-h LC_{50} and LC_{20} values of 24.9 and 18.0 µg/L, respectively, were reported for juvenile *L. stagnalis* (Tania et al. 2011). The response of snails in the present experiment exhibited a similar degree of toxicity, as no mortality occurred up to the highest concentration tested of 15 µg/L copper for 7 d. Although not quantified, it should be noted that qualitative observations of shell thinning and small size were observed in the copper-exposed and 2000 µatm CO_2-exposed groups. Several studies have reported shell thinning in gastropods and mussels exposed to increased CO_2 (Crim et al. 2011; Feely et al. 2012; Jeffrey et al. 2017a), i.e., an effect like that of copper.

Copper has been shown to inhibit calcium uptake and displace calcium in aquatic organisms. Brix et al. (2011) reported a significant decrease in calcium uptake in young adult *L. stagnalis* exposed to 48 µg/L copper for 7 d. Additionally, an EC_{20} of 1.8 µg/L copper was reported for growth of juvenile *L. stagnalis* after 30 d of copper exposure (Brix et al. 2011). Likewise, Tania et al. (2011) reported reduced tissue sodium and calcium in juvenile *L. stagnalis* after acute copper exposure; and decreased growth after 28 d of chronic copper exposure. Decreased growth and reproduction were also reported in adult apple snails exposed to copper (Rogevich et al. 2009). Changes in calcium (induced by increased copper) and carbonate (induced by increased CO_2) may both lead to shell thinning and decreased growth in juvenile *L. stagnalis*. However, more research is needed.

Antioxidant Enzyme Activity

In this study, clear differences were observed in glutathione peroxidase activity between juvenile unshelled whole snails exposed to copper or increased CO_2 and those in the control groups. Similarly, in adult *L. stagnalis* an increase in glutathione peroxidase activity in foot muscle after exposure to 90 µg/L copper, an increase glutathione peroxidase activity in mantle after exposure to 38 and 90 µg/L copper, and a decrease in hepatopancreas glutathione peroxidase activity after exposure to 2–90 µg/L copper was reported (Atli and Grosell 2016). Metal bioaccumulation and increased glutathione peroxidase and catalase activity were reported in another freshwater snail, *Lymnaea*

natalensis, collected from a metal contaminated site, as compared to those collected from a reference site (Siwela et al. 2010).

In the present study, no significant differences in catalase activity in *L. stagnalis* were observed. This could be due to variable responses in catalase activity among different tissues. Atli and Grosell (2016) reported an increase in hepatopancreas catalase activity after exposure to 2 μg/L copper with no change in catalase activity after exposure to 5–90 μg/L copper, a decrease in foot muscle catalase activity after exposure to 2–90 μg/L copper, and an increase in mantle catalase activity after exposure to 90 μg/L copper in adult *L. stagnalis*. Because of the small size of *L. stagnalis* in this study, all the soft tissue was used, and snails were pooled to enable the analyses. Different responses of tissues and individuals could have contributed variability. The catalase response may also be affected by copper concentration and exposure duration. Other studies have reported variable catalase activity responses in the sea anemone, *Exaiptasia pallida*, exposed to copper and CO_2, as a consequence of exposure duration and concentration (Main et al. 2010; Siddiqui and Bielmyer-Fraser 2015). An additional explanation is that the detoxification reaction catalyzed by catalase may not be as important as the one catalyzed by glutathione peroxidase in 18-d old *L. stagnalis*.

Contrary to our hypothesis, synergistic effects on glutathione peroxidase activity from exposure to both increased copper and CO_2 in *L. stagnalis* were not observed; however, each parameter individually induced glutathione peroxidase activity, with CO_2 inducing the greatest response. Lefcort et al. (2015) reported that snails (*Physella columbiana*) collected from a metal (lead and zinc) polluted site were more tolerant to acidic conditions (increased CO_2) than those collected from a reference site. The observations of Lefcort et al. (2015) can be partially explained by the detoxification strategies observed in the present study. Glutathione peroxidase plays a key role in an organism by protecting it from oxidative damage. The increased activity of glutathione peroxidase in this study was likely a compensatory response or acclimatization strategy of *L. stagnalis* from exposure to increased acidification or copper. Therefore, if the snails are exposed to one of the stressors, they may be partially protected (at least acutely) from subsequent exposure of the other contaminant.

Results of the present study also suggest that CO_2-based acidification may cause different organismal responses (particularly in conjunction with metal exposure) than nitric and sulfuric acid-driven acidification. Rogevich et al. (2008) reported 96-h LC_{50} values of 31 μg/L copper at a *p*H of 7.76 and 20 μg/L copper at a *p*H of 6.79 in 4-d old apple snails. Differences in toxicity values were attributed to increased availability of Cu^{2+} ion in the lower *p*H water; however, the *p*H was adjusted by addition of nitric acid in that study (Rogevich et al. 2008), rather than CO_2 like the present study. In past decades, nitric and sulfuric acids were the main components of acidic precipitation and, therefore, these compounds were used in many studies to assess effects of acidification. In addition to causing changes in copper speciation, CO_2 exposure had a direct effect on the organism, as demonstrated with increased glutathione peroxidase activity in *L. stagnalis*.

In conclusion, increased tissue copper accumulation (after 4 d) and increased glutathione peroxidase activity (after 2 d) were both observed in unshelled *L. stagnalis* exposed to copper. Glutathione peroxidase activity was a good bioindicator for exposure to copper and CO_2 individually. Because these sensitive snails share a similar defense response for both stressors it may be possible that exposure to one of the contaminants could ameliorate the effects of the other contaminant. More research is needed assessing

CO_2 and copper exposure in varying combinations over a variety of exposure times to early-life stages of *L. stagnalis*.

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