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Copper toxicity does not affect low tide emersion tolerance of *Mytilus galloprovincialis*

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ABSTRACT

Intertidal mussels are well adapted to withstand emersion from water during low tide, but they may be intermittently exposed to waterborne toxicants such as copper, which targets physiological processes including metabolism, ammonia excretion, and osmoregulation. To determine if copper exposure damages intertidal organisms' ability to tolerate tidal emersion, Mediterranean mussels (*Mytilus galloprovincialis*) were exposed to copper for 96 h followed by 6 h of emersion. Oxygen uptake increased after copper exposure which suggests that copper accumulation caused moderate stress in the mussels, but ammonia excretion and anaerobic metabolism were unaffected by mixed copper and emersion exposures. Shell composition analyses indicate that cycles of copper exposure and tidal emersion may affect bivalve shell growth, but copper deposition into shells may decrease the metal's overall toxicity. Results suggest that copper does not damage *M. galloprovincialis*'s tolerance to tidal emersion, and insight is provided into the mussel's ability to overcome mixed stressor exposures.

1. Introduction

Intertidal zones of coastal regions contain valuable ecosystems like salt marshes, estuaries, and rocky shores. Salt marshes provide services such as coastal protection from flooding and erosion, nutrient filtration and delivery, pollutant interception, and carbon sequestration (Blake and Olin, 2022; Kirwan et al., 2016; Nelson and Zavaleta, 2012). They also provide habitat for many aquatic and terrestrial animals despite the highly variable environmental conditions caused by the tide cycle. Salt marshes are resilient ecosystems, but they may be sensitive to the effects of climate change (i.e., rising sea levels, increasing temperatures), and their frequent proximity to heavily populated areas mean the organisms that inhabit salt marshes are also vulnerable to anthropogenic toxicant exposure (Blake and Olin, 2022; Kirwan et al., 2016; Nelson and Zavaleta, 2012; Cao et al., 2006).

In California, it is estimated that at least 91 % of the historic wetland area has been fragmented or destroyed by human activities (Nelson and Zavaleta, 2012; Zedler, 1996). Carpinteria Salt Marsh is one of the few remaining salt marshes, located east of Santa Barbara in southern California. Channels of this marsh are regularly flooded and drained throughout the tidal cycle (Cao et al., 2006). During low tide, portions of the channels are entirely exposed to air for several hours, and isolated tide pools can reach high temperatures, high salinities, and low dissolved oxygen (DO) concentrations (Helmuth et al., 2006; Truchot and Duhamel-Jouve, 1980; Heard, n.d.). Such conditions present several physiological challenges to aquatic animals living in these channels. For example, tidal air exposure can cause desiccation of soft-bodied marine invertebrates or force water-breathing animals to make a facultative switch from aerobic to anaerobic metabolism due to low oxygen conditions (Crowe et al., 2000; de Vooys, 1979; Fields et al., 2014). Along with being exposed to these extreme conditions, animals in Carpinteria Salt Marsh must contend with input of anthropogenic pollutants. The marsh receives runoff from agricultural zones and is located near a busy freeway, both of which are potential sources of a variety of contaminants (Cao et al., 2006; Hwang et al., 2006; Melwani et al., 2013). One common contaminant in this area, and around the world, is the trace metal copper (Cu).

Cu is essential for life at low concentrations due to its role as a cofactor in several key enzymes (such as cytochrome *c* oxidase which is vital for oxidative phosphorylation), but high environmental doses

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cause toxicity (Copper, 2011). Anthropogenic sources of Cu in marine environments include agricultural pesticides, sewage, industrial and mining effluent, anti-fouling paint on boats, and run-off from roadways due to brake pad wear (Crowe et al., 2000; Melwani et al., 2013). In Carpinteria Salt Marsh, sediment samples have been found to contain up to 67.5 ppm of Cu, and levels reach upwards of 200 ppm in sediments of other California salt marshes (Cao et al., 2006; Hwang et al., 2006). Cu tends to partition into sediment to three to five-fold higher concentrations than in the water column, so typical Cu concentrations in these marsh waters are likely below about 70 μg/L. (Luoma, 1989) In the water, some Cu is present as the dissolved ion form (Cu^{2+}) which is most bioavailable to organisms, but much of it complexes with dissolved organic carbon (DOC) which lowers the toxicity (Copper, 2011; Rader et al., 2019). In seawater, Cu may also complex with anions to form Cu carbonates, which are likely nontoxic (Blanchard and Grosell, 2006; Erickson et al., 1996). Aquatic animals are extremely vulnerable to Cu contamination because all soft surfaces exposed to the water, particularly the skin and gills, are potential routes of uptake of dissolved Cu (Copper, 2011). Cu crosses epithelial membranes (e.g., gills) by mimicking transport routes for essential ions like sodium (Na) and calcium (Ca), and fish gills may have divalent metal transporters (DMT1) and Cu transporters (CTR1) that may also be used for Cu uptake (Copper, 2011). After crossing epithelial surfaces, excess Cu undergoes oxidation-reduction (redox) cycling, producing reactive oxygen species (ROS) which cause DNA damage and lipid peroxidation (Copper, 2011; Piscopo et al., 2018). Cu can also disrupt protein structure and function by binding to cysteine, methionine, or histidine residues; for example, Cu binds and inhibits Na/K-ATPase, an enzyme essential for maintenance of ion gradients across cell membranes (Copper, 2011; Harris and Gitlin, 1996). Cu toxicity results in damage to several physiological processes including respiration, metabolism, ammonia excretion, osmoregulation, and acid-base balance (Copper, 2011; Grosell et al., 2007). Depending on the concentration and duration of Cu exposure, Cu can affect reproductive output, growth, development, and survival - potentially leading to population and whole ecosystem effects (Copper, 2011; Lettieri et al., 2019). Thus, Cu contamination poses a threat to coastal ecosystems like Carpinteria Salt Marsh.

Since intertidal animals are regularly exposed to both natural stressors caused by the tidal cycle and anthropogenic stressors like toxic chemicals, interactions between multiple stressors need to be studied to further understand potential effects of human activity on these animals and their ecosystems (Blake and Olin, 2022; Crain et al., 2008; Gunderson et al., 2016). In a meta-analysis of studies that manipulated multiple stressors on coastal ecosystems, it was found that negative effects on organisms were mainly additive or synergistic, especially when more than two stressors were applied (Crain et al., 2008). This is of particular concern for intertidal salt marsh animals because these environments typically present several simultaneous stressors. However, mixed stressor exposures can have varying effects based on the ecology and physiology of the study species, so further research on intertidal animals spanning a diverse range of traits is necessary to gain a full picture of how stressors interact to impact sensitive ecosystems.

The Mediterranean mussel (*Mytilus galloprovincialis*) is an invasive marine bivalve mollusc that has origins in the Mediterranean but now inhabits intertidal regions around the world, including flood channels in Carpinteria Salt Marsh (Braby and Somero, 2006). This species is successfully able to invade new habitats due to its high tolerance to a range of environmental conditions (Han and Dong, 2020). These mussels are important members of coastal food webs and are commonly used in aquaculture (Inoue et al., 2021; Romero-Freire et al., 2020). They are sessile filter-feeders, producing strong byssal threads that adhere to hard substrates (Inoue et al., 2021). Because of their sessile nature and filter-feeding behaviour, mussels like *M. galloprovincialis* tend to bioaccumulate the contaminants present in their habitat and are thus used in water quality monitoring programs (Melwani et al., 2013). Intertidal *M. galloprovincialis* experience multiple stressors, including tidal emersion from water and Cu exposure. Since they are usually sessile and thus cannot move to escape exposure to such stressors, mussels have other strategies to survive adverse conditions; for example, *Mytilus* spp. close their valves in response to air exposure, decreasing metabolic rates and creating internal anoxic conditions that they tolerate by switching to anaerobic metabolism (Andrade et al., 2019; Shick et al., 1986; Wang and Widdows, 1993). Mussels may also utilize valve closure as a stress response to toxicant exposure, but anaerobiosis is not sustainable long-term, and contaminants like Cu may lower anoxia tolerance of the organisms (de Zwaan and Eertman, 1996). Cu is lethal to *Mytilus* spp. at high concentrations, and at sublethal levels it has been found to decrease metabolic rates, disrupt ammonia excretion, lower byssal thread production (byssogenesis), impair Ca homeostasis, and damage immune function (Brown and Newell, 1972; Scott and Major, 1972; Sunila, 1981; Torres-Duarte et al., 2019; Viarengo et al., 1996; Wilson-McNeal et al., 2020). Interestingly, tidal emersion impacts many of the same processes as Cu exposure, so it is possible that exposure to both stressors may exacerbate negative effects on mussels or other intertidal invertebrates. According to our previous research, exposure to Cu did not damage the ability of the orange sea cucumber *Cucumaria miniata* to withstand tidal emersion (Lowes et al., 2023), but mussels may have different physiological responses to mixed stressors. Studies of mixed stressors on *Mytilus* mussels have included air exposure/temperature (Andrade et al., 2019; Petes et al., 2007), temperature/pH (Lesser, 2016), Cu exposure/temperature/CO_2 (Romero-Freire et al., 2020), and Cu exposure/pH (Wilson-McNeal et al., 2020), but the combination of Cu and air exposure has not yet been studied in intertidal mussels.

Due to gaps in knowledge about how mixed stressors (Cu and air exposure) might impact the physiology and survival of *M. galloprovincialis*, the goal of this study was to elucidate the relationship between the two stressors on these mussels, and to apply the results to the context of Carpinteria Salt Marsh's highly variable conditions. Thus, we exposed mussels from the salt marsh to environmentally relevant concentrations of Cu followed by a period of air exposure and measured tissue-specific Cu accumulation, oxygen uptake and ammonia excretion rates, and levels of succinate in the gills and foot as a marker of anaerobic metabolism to investigate the relationship between the two stressors. Byssogenesis was quantified to determine if the chosen Cu concentrations in this study were high enough to alter this process which is essential for individual survival and formation of mussel beds, which provide habitat for many other species (Borthagaray and Carranza, 2007). We also examined major ions sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) in *M. galloprovincialis* hemolymph to determine if osmoregulation of the fluid was affected by Cu exposure, emersion, or both. Cu exposure and tidal emersion from water both affect major processes in mussels including aerobic metabolism and ammonia excretion, so it was expected that the high tolerance to air exposure of *M. galloprovincialis* would be sensitive to Cu exposure (Grosell et al., 2007; Andrade et al., 2019; Durand and Regnault, 1998), and that this would be apparent in our chosen measures. In particular we anticipated that the combination of Cu toxicity and emersion would result in decreased oxygen uptake and ammonia excretion, increased succinate production due to induction of anaerobic pathways, and altered concentrations of major ions Na, Ca, Mg, and K in the hemolymph due to osmoregulatory damage, compared to either of the stressors on their own.

Results of this study advance knowledge of how multiple stressors that alter similar physiological processes in intertidal animals may interact to decrease their fitness and survival. Toxicant-induced alteration of strategies to tolerate tidal stressors has the potential to affect the distribution of species like *M. galloprovincialis*, particularly in the context of a changing climate because varying conditions throughout the tide cycle (i.e., air exposure, temperature, salinity, pH) are predicted to become more extreme (Finke et al., 2007). Broadly, improved understanding of the harm caused by anthropogenic toxicants on intertidal communities is vital to inform regulations regarding toxicant release

and may provide a basis for predictions of future survival and distribution of species such as *M. galloprovincialis*. In particular, our results add to current knowledge of the overall ecosystem health of Carpinteria Salt Marsh which may be extrapolated to other salt marsh communities around the world.

2. Methods

2.1. Animal care

All protocols followed the Guidelines of the Canadian Council on Animal Care at the University of Alberta and the Institutional Animal Care and Use Committee at the University of California, Santa Barbara (UCSB). Mediterranean mussels (*M. galloprovincialis*) with mean whole-body mass ± standard error of the mean (S.E.M.) 22.05 ± 0.76 g were collected by hand from Carpinteria Salt Marsh Reserve (CSMR), California, United States in early April 2022. Collection was approved by California Department of Fish and Wildlife (CDFW; permit S-213430003-21,346-001). The mussels were transported to UCSB in aerated water coolers and their valves were gently scrubbed to remove barnacles, algae, and other debris. Mussels were acclimated for 7 days in 40 L aerated tanks with flow-through sea water with a mean temperature of 13.7 ± 0.13 °C and a salinity of 35 ppt. Animals were held under a natural light cycle (~14 h light: 10 h dark) and were not fed for 7 days prior to the experiments. UCSB facility water chemistry was measured (in mM) as 441 ± 7.3 Na, 11 ± 0.2 K, 11 ± 0.8 Ca, 52 ± 0.9 Mg.

2.2. Experimental Cu and emersion exposures

Experimental design and procedures were based on previous experiments (Lowes et al., 2023), with some adjustments. 1 g/L Cu stock solution was prepared in filtered UCSB seawater using Cu (II) sulfate pentahydrate (Sigma Aldrich, St. Louis, MO, USA) and acidified with 0.1 % nitric acid (HNO_3). The Cu stock was then diluted with seawater in large containers to make 20 L each of 35 μg/L and 160 μg/L Cu ("low" and "high" Cu, respectively). These concentrations were chosen to represent potential Cu contamination in CSMR under normal conditions and following a pulsed higher-level exposure (e.g., agricultural runoff following heavy seasonal rain) (Hwang et al., 2006). The control water was a container of 20 L of seawater without Cu added. All 3 stock containers (control, low Cu, and high Cu) were aerated and held in a flow-through sea table to maintain temperature at 14.2 ± 0.12 °C, and samples were collected to confirm nominal stock concentrations, with results presented in Table 1.

M. galloprovincialis were placed in 400 mL plastic containers (one mussel per container) and randomly assigned to control, low Cu, or high Cu seawater for 96 h. As in Lowes et al., 6 treatments (control, low Cu, high Cu, control + emersion, low Cu + emersion, and high Cu + emersion) with 10 replicates per treatment were used for the experiment (Lowes et al., 2023). All containers were constantly aerated and held in a flow-through water table where temperature was maintained at 14.2 ± 0.10 °C. A complete water change was conducted 48 h into the exposure. After the 96 h exposure, the non-emersed mussels were immediately removed for respirometry. The mussels in the emersion group were exposed to air for 6 h in their empty original containers floating in the same water table to maintain temperature before performing respirometry. Byssogenesis was quantified by counting the number of new byssal thread attachments each mussel made to the plastic container at the 48 h water change (after which all byssi were detached) and again at the end of the 96 h exposure period.

Table 1
Measured Cu concentrations in experimental and stock water, sampled at the start (t = 0 h) and finish (t = 96 h) of the Cu exposure period. Values reported are the means ± standard deviation (S.D.) of each group, with 9 to 10 replicates per group. Concentrations below the detection limit of 7 μg/L are reported as B.D.L.

Group	Treatment	Nominal [Cu] (μg/L)	[Cu] (μg/L) at t = 0 h	[Cu] (μg/L) at t = 96 h
Non-emersion	Control	0	9.2 ± 1.1	B.D.L.
	Low Cu	35	33.4 ± 1.0	19.4 ± 7.8
	High Cu	150	107.0 ± 3.5	20.1 ± 5.6
Emersion	Control	0	9.0 ± 1.5	B.D.L.
	Low Cu	35	32.9 ± 0.9	15.9 ± 1.2
	High Cu	150	108.5 ± 3.5	19.5 ± 3.8
Stock	Control	0	B.D.L.	B.D.L.
	Low Cu	35	32.8 ± 0.5	32.2 ± 0.7
	High Cu	150	109.3 ± 3.2	104.4 ± 1.2

2.3. Water chemistry

10 mL water samples were collected from each stock (control, low Cu, and high Cu) and from each experimental mussel container at the beginning and end of the Cu exposure experiments for inorganic analysis. Samples were filtered with 0.45 μM mixed cellulose ester membranes (Millipore Millex-GS, Merck) and acidified with 12 μL of trace metal grade HNO_3 per 10 mL of water. The filtered and acidified samples were then diluted with a solution of 2 % HNO_3 and 0.5 % HCl and concentrations of Cu and other inorganics (Table S1) were measured using inductively coupled plasma-mass spectrometry (ICP-MS/MS; Agilent 8800). For ICP-MS/MS analyses, standards were prepared in a matrix of 2 % HNO_3 and 0.5 % HCl and covered a range of 0.0005–50 ppm in two tiers to accommodate varying concentration levels within samples. Measurements were made using collision/reaction gases (He, H_2, O_2) to eliminate isobaric interferences, and internal standards (Sc, Ge, In, Lu, Bi) were used to account for instrumentation drift.

2.4. Cu accumulation and inorganic analysis

After respirometry, the valves of each mussel were gently pried open about 0.5 cm and hemolymph was collected from the posterior adductor muscle using a 27 G needle and 1 mL syringe, similar to methods used by Eggermont et al. (2020) Hemolymph, gill, and foot samples were digested in 2 N HNO_3, and shell samples were digested in 8.85 N HNO_3. All samples were incubated for 48 h at 65 °C and vortexed 24 h into the incubation period. The digested samples were filtered with 0.45 μM mixed cellulose ester membranes (Millipore Millex-GS, Merck) and diluted with 2 % HNO_3 and 0.5 % HCl to <2300 ppm total dissolved solids (TDS). Concentrations of Cu and other inorganics (Na, K, Ca, Mg) in the hemolymph, shells, and tissues were measured using ICP-MS/MS as described in Section 2.3 and using DOLT-5 reference standards.

2.5. Oxygen uptake rates

Immediately after exposure to Cu and/or emersion, the 10 individual mussels from each treatment were placed inside one of four 160 mL sealed glass chambers placed within two flow-through tanks containing fresh seawater for a 1 h closed respirometry protocol. Each chamber was equipped with a constant water recirculation loop of about 1 L/min, created using 5 L/min Eheim Universal 300 pumps (Eheim, Germany) slowed by the small diameter of the tubes entering the chambers. This flow rate was used to roughly mimic the flow of incoming and outgoing tides that intertidal *M. galloprovincialis* experience, and to encourage respiration as these animals depend on water flow across their gills for oxygen uptake (Tuffnail et al., 2009). Oxygen concentration (% saturation) over a 1 h period was continuously measured using robust fibre-optic oxygen probe with a 4-channel FireSting optical oxygen meter (Pyroscience, Germany) which was placed in the recirculation loop. Background respiration was measured at the beginning and end of each experimental day and was accounted for following the protocol and equations described by Rosewarne et al. (2016) Empty mussel shells after dissections of 2 replicates of each treatment were also measured to ensure that oxygen uptake rates were from the mussels themselves and

not bacteria or other organisms living on the shells. The rates of oxygen uptake by each mussel were extracted from the last 0.8 h of measurement, and only replicates with $R^2 > 0.9$ were included for analysis. Routine oxygen uptake rates ($\dot{M}O_2$, μmol O_2/kg/h) as proxies for routine metabolic rates, defined for this study as the average metabolism during normal/unstressed behaviours, were calculated using methods outlined by Rosewarne et al. and the following equation:

$$\dot{M}O2 = (K_1V_1 - K_2V_2)\cdot M^{-1}$$

where K_1 and K_2 are the rates of oxygen decline (μmol O_2/L/h) in the respirometer during the measurement periods with and without a mussel, respectively, V_1 is the respirometer volume (L) corrected for the mussel volume, V_2 is the total respirometer volume (L) without a mussel, and M is the wet mass (kg) of the soft tissue of the mussel (Rosewarne et al., 2016). Values were mass-scaled using the allometric metabolic mass exponent of 0.715 found by Arranz et al. (2016) Mussels that spawned in the respirometry chambers were not included in $\dot{M}O_2$ analysis, as this is not a resting behaviour but is likely a stress response which may affect metabolism (Petes et al., 2007; Sokolova et al., 2012). Five of the mussels exposed to high Cu without air exposure spawned during the respirometry period, along with one of the emersed high Cu and one non-emersed low Cu, all of which were removed from $\dot{M}O_2$ and AER analysis.

2.6. Ammonia excretion rates

Water samples were collected from each respirometry chamber before and after the 1 h measurement period and immediately frozen at −20 °C for analysis. Ammonia concentrations were measured following the sodium salicylate-hypochlorite method of Verdouw et al. with some adjustments (Verdouw et al., 1978). Briefly, each sample was plated with 40 % sodium salicylate, 0.76 mM sodium nitroprusside, and a 1:1 ratio of 6 % sodium hypochlorite and 1.2 M sodium citrate made in 1 N sodium hydroxide (Sigma Aldrich). Samples were incubated in the dark at room temperature for 1 h and then the absorbances were read in triplicate at 595 nm in a 96-well clear bottom plate using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany) and SoftMax Pro (v. 7.0.3). Ammonia concentrations were evaluated against an ammonium chloride standard curve, and ammonia excretion rates (AER, μmol/kg/h) were calculated using the following equation:

$$AER = \frac{\left(\left[Amm_f\right] \times V\right) - \left(\left[Amm_i\right] \times V\right)}{(m \times t)}$$

where, Amm_f and Amm_i are the final and initial ammonia concentrations (μM), respectively, V is the respirometer volume (L) corrected for mussel volume, m is the wet mass (kg) of the mussel soft tissue and t represents flux time period (h). AER values were mass-scaled using the allometric scaling exponent for ammonia excretion in *M. galloprovincialis* of 0.616 described by Arranz et al. (2016) Gametes in the water interfered with absorbance readings, so samples collected from spawned mussels were not included in analysis. The ammonia quotient (moles of ammonia excreted to oxygen consumed) was also calculated using unscaled $\dot{M}O_2$ and AER values, again excluding all spawned mussels. This resulted in n = 9 (non-emersion control), n = 9 (non-emersion low Cu), n = 4 (non-emersion high Cu), n = 10 (emersion control), n = 9 (emersion low Cu), and n = 8 (emersion high Cu) used for AER/AQ analysis.

2.7. Tissue succinate analysis

Succinate is a major end product of anaerobic metabolism in bivalves (de Vooys, 1979; Fields et al., 2014; Bacchiocchi and Principato, 2000; de Zwaan and Wijsman, 1976; Zurburg and RHM, 1980), so it was measured as a marker of anaerobiosis in this study. Succinate levels in the gills and foot of 6 replicates of *M. galloprovincialis* were measured according to manufacturer instructions of a succinate assay kit (Sigma Aldrich). Samples were ground using a mortar and pestle on dry ice, briefly homogenized in the kit-provided buffer with 0.5 mm zirconia/silica beads using a bead shaker, and centrifuged for 5 min at 10,000 ×*g*. The supernatant was collected and used for analysis. Sample absorbances were read in duplicate at 450 nm in a 96-well clear bottom plate using a FLUOstar Omega microplate reader (BMG Labtech) and SoftMax Pro (v. 7.0.3). Succinate concentrations were calculated from a succinate standard curve.

2.8. Data presentation and analyses

All values are presented as mean ± standard error of the mean (S.E.M.) unless otherwise indicated, and for all analyses $p < 0.05$ was considered significant. Statistical analyses and graphs were produced using R 4.2.0 (R Core Team, 2022). For all datasets, assumption checks and statistical tests were performed using R packages 'rstatix' and 'multcompView' (Graves et al., 2019; Kassambara, 2021). ICP-MS/MS measurements below limits of detection (LODs) were included as LOD/2 for analyses when significant effects did not differ from substituting 0 for values below LODs. Cu accumulation, hemolymph ion concentration, and shell calcium datasets did not meet assumptions for parametric analysis, so all data were log-transformed and modelled as functions of group (non-emersion or emersion) and treatment (control, low Cu, or high Cu) for two-way analyses of variance (ANOVA) tests. The $\dot{M}O_2$, AER, AQ, and gill succinate data met the assumptions for parametric analysis, so two-way ANOVAs were performed on each dataset using group and treatment as factors in the analyses. Foot succinate data failed the normality assumption, so a cube root transformation was applied to meet the assumptions of parametric analysis and perform a two-way ANOVA. Tukey *post-hoc* tests were run on those two-way ANOVAs which revealed significant effects of group, treatment, or both. The byssal attachment data was modelled as a function of treatment only (control, low Cu, or high Cu) since the emersion period was not involved in the collection of this data. A Kruskal-Wallis test was conducted on this dataset because the assumptions for parametric analysis were not met, and this was followed by Dunn's test to illuminate significant differences between treatments.

3. Results

3.1. Water chemistry

Measured Cu concentrations in control and low Cu stocks were roughly equivalent to nominal values, but the high Cu stock had less Cu than expected (just over 100 μg/L measured vs 150 μg/L nominal; Table 1). Stock Cu concentrations remained steady over 96 h. Cu levels just above the detection limit of 7 μg/L were initially measured in the experimental controls which is representative of background levels, but the 96-h control water samples were below the Cu detection limit. After the 96-h exposure period, Cu concentrations in both low Cu and high Cu experimental samples decreased compared to initial Cu concentrations (Table 1).

3.2. Cu bioaccumulation and shell Ca concentrations

Cu accumulated to the highest concentrations in *M. galloprovincialis* gills, followed by the hemolymph and foot (Fig. 1). Bioaccumulation of Cu in the gills was significantly affected by Cu treatment ($F_{(2,52)} = 94.511$, $p < 0.0001$), but independent of emersion group ($F_{(1,52)} = 0.227$, $p = 0.636$) and interactions of Cu treatment and emersion ($F_{(2,52)} = 0.582$, $p = 0.563$). The gills showed a significant increase in Cu bioaccumulation from control concentrations (non-emersion 1.14 ± 0.05 μg/g and emersion 0.53 ± 0.14 μg/g) to low Cu (12.5 ± 5.92 and 11.4 ± 3.08 μg/g) to high Cu (39.9 ± 9.02 and 41.6 ± 12.5 μg/g), but the emersed mussels did not accumulate significantly different concentrations of Cu between the low Cu and high Cu treatments (Fig. 1A).

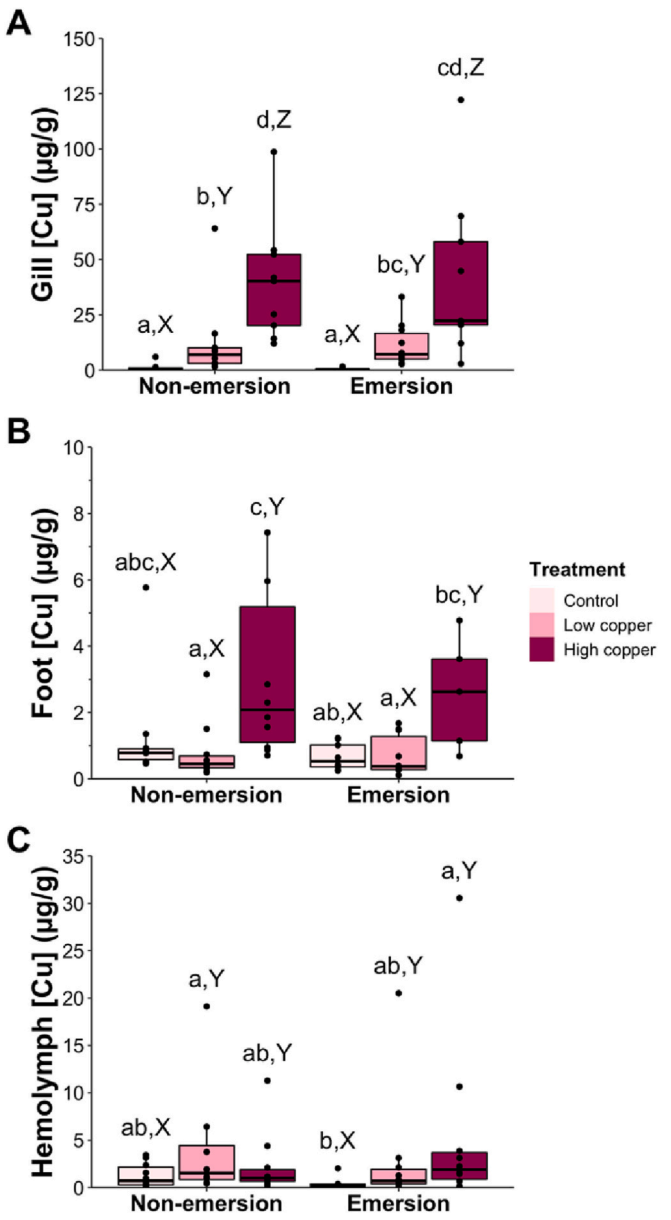

Fig. 1. Copper (Cu) accumulation in the (A) gill, (B) foot, and (C) hemolymph of *Mytilus galloprovincialis* after 96 h of control, low Cu, or high Cu exposure, with or without 6 h of emersion following the exposures. Individual datapoints represent Cu bioaccumulated by each individual mussel (n = 5 to 10). Horizontal lines within boxplots represent the median, upper and lower hinges represent the 25th and 75th percentiles (Q1 and Q3), respectively, and whiskers extend to the most extreme datapoints within Q1 − 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR. Outliers are denoted by individual datapoints outside the whiskers. Lowercase letters denote significant differences across all 6 group/treatment combinations, and uppercase letters denote differences between the 3 Cu treatments.

In the mussel foot, Cu accumulation was also dependent on Cu treatment ($F_{(2,49)} = 13.467$, $p < 0.0001$) but independent of emersion group ($F_{(1,49)} = 1.147$, $p = 0.289$) and interactions of the two factors ($F_{(2,49)} = 0.302$, $p = 0.741$). On average the foot accumulated about twice the Cu concentration after high Cu treatment for both non-emersed and emersed mussels (3.19 ± 0.85 and 2.57 ± 0.76 µg/g, respectively) than after control (1.27 ± 0.51 and 0.68 ± 0.13 µg/g) or low Cu (0.80 ± 0.29 and 0.70 ± 0.19 µg/g) conditions, though the high Cu mussels did not differ from the controls and showed high variability between individuals (Fig. 1B).

Accumulation of Cu in the hemolymph was also significantly affected by Cu treatment ($F_{(2,52)} = 6.114$, $p = 0.004$) but not by emersion group ($F_{(1,52)} = 2.001$, $p = 0.163$) or interactions ($F_{(2,52)} = 1.704$, $p = 0.192$). Emersed mussels exposed to high Cu accumulated on average about five-fold higher Cu (5.45 ± 2.95 µg/g) than the emersed controls (0.42 ± 0.21 µg/g), but no other treatments differed, and all showed high variation between individuals (Fig. 1C).

Finally, the shells of mussels exposed to both Cu treatments showed higher Cu concentrations (non-emersed and emersed low Cu, 19.3 ± 2.55 and 6.2 ± 0.76 µg/g, and non-emersed and emersed high Cu, 16.0 ± 1.91 and 13.2 ± 2.49 µg/g) than in both non-emersed and emersed controls (2.97 ± 0.46 and 2.66 ± 0.56 µg/g, respectively; Fig. 2A). Emersion group ($F_{(1,54)} = 14.785$, $p < 0.001$), Cu treatment ($F_{(2,54)} = 59.740$, $p < 0.0001$), and interactions ($F_{(2,54)} = 4.559$, $p = 0.015$) all had significant effects on shell Cu accumulation. Ca concentrations in *M. galloprovincialis* shells were dependent on emersion group ($F_{(1,54)} = 4.790$, $p = 0.033$) and Cu treatment ($F_{(2,54)} = 9.907$, $p < 0.001$), but not on interactions of group and treatment ($F_{(2,54)} = 1.936$, $p = 0.154$). Of note, average shell Ca concentrations in emersed mussels were about two-fold lower after low Cu (290 ± 25.4 mg/g) and high Cu treatment (261 ± 49.4 mg/g) than the controls (524 ± 80.8 mg/g; Fig. 2B).

3.3. Byssogenesis

M. galloprovincialis exposed to high Cu produced 12.5 ± 3.02 new byssal attachments during the 96-h exposure period, which is about a third the mean number produced by either control or low Cu treated mussels (32.9 ± 5.72 and 30.3 ± 5.79 byssus attachments, respectively) (Fig. 3). Cu treatment had a significant effect on the new number of byssal attachments formed ($H_{(2)} = 8.46$, $p = 0.015$). The byssus thread production in the high Cu treatment was significantly lower than both the control ($z = -2.71$, $p = 0.020$) and low Cu ($z = -2.28$, $p = 0.046$) treatments. The control and low Cu treatments did not produce a different number of byssal thread attachments ($z = -0.429$, $p = 0.668$).

3.4. Oxygen uptake and ammonia excretion

M. galloprovincialis $\dot{M}O_2$ was dependent on Cu treatment ($F_{(2,46)} = 7.077$, $p = 0.002$), but independent of emersion ($F_{(1,46)} = 0.948$, $p = 0.335$) or interactions of emersion and Cu treatment ($F_{(2,46)} = 0.428$, $p = 0.654$). In general, all Cu-exposed mussels had higher $\dot{M}O_2$ than all controls, but when the 6 group/treatment combinations were compared, the emersed mussels exposed to high Cu only exhibited higher $\dot{M}O_2$ than the non-emersed controls (1689 ± 183.3 and 1007 ± 127.9 µmol/kg$^{0.715}$/h, respectively; Fig. 4A). Though differences were not significant, the emersion group showed a slight trend toward increasing $\dot{M}O_2$ from control to low Cu to high Cu conditions.

AER in this study was independent of emersion group ($F_{(1,43)} = 0.640$, $p = 0.428$), copper treatment ($F_{(2,43)} = 0.593$, $p = 0.557$), and interactions ($F_{(2,43)} = 0.046$, $p = 0.955$). Mussel AER did not differ significantly across the 6 group/treatment combinations, but the emersed high Cu treatment shows slightly higher variability in AER measurements (ranging from 127.1 to 941.6 µmol/kg$^{0.616}$/h) than the non-emersed high Cu which ranged from 235.3 to 559.2 µmol/kg$^{0.616}$/h (Fig. 4B).

AQ analysis also did not reveal any effects of emersion group ($F_{(1,43)} = 0.072$, $p = 0.790$), Cu treatment ($F_{(2,43)} = 1.287$, $p = 0.287$), or interactions of the two stressors ($F_{(2,43)} = 0.360$, $p = 0.700$). Though there were no significant differences between the group/treatment combinations, the non-emersed and emersed controls and the emersed low Cu treatment had slightly higher AQs (0.398 ± 0.052, 0.382 ± 0.070, and 0.387 ± 0.055, respectively) than the non-emersed low Cu and high Cu treatments (0.317 ± 0.070 and 0.304 ± 0.068; Fig. 4C). The emersed high Cu mussels had the lowest AQ at 0.275 ± 0.048, though again this was insignificant (Fig. 4C).

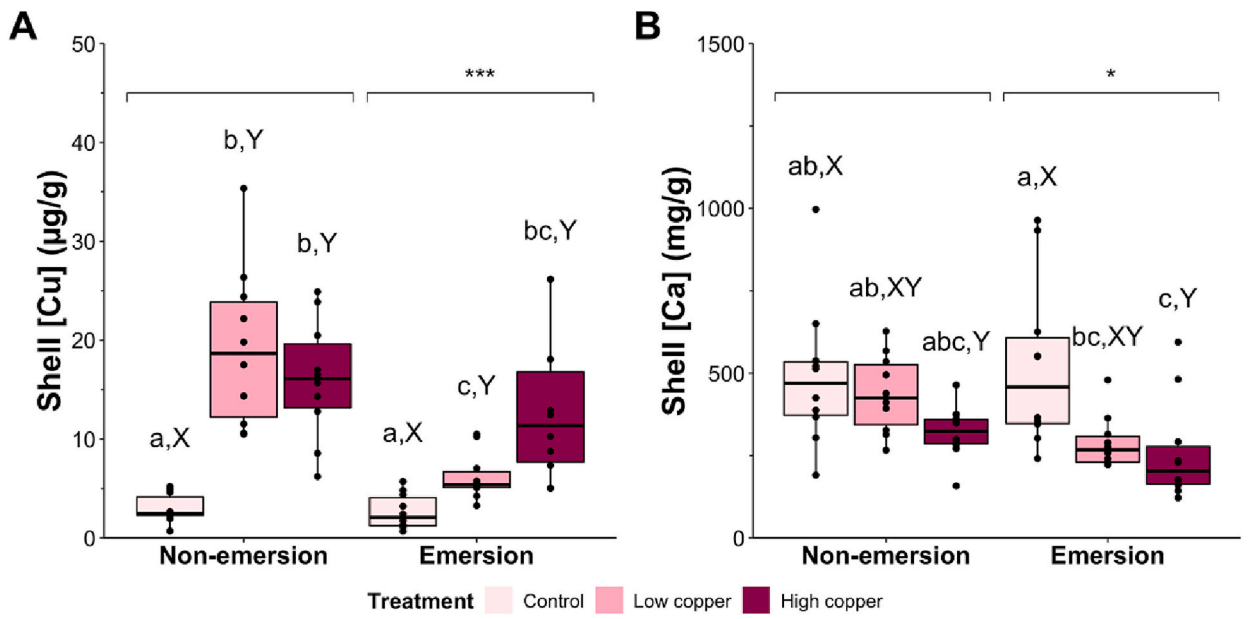

Fig. 2. Concentrations of (A) copper (Cu) and (B) calcium (Ca) in the shells of *Mytilus galloprovincialis* exposed to 96 h of control, low Cu, or high Cu conditions and with or without 6 h of emersion. Individual datapoints represent ion content of each individual mussel's shell (n = 10). Horizontal lines within boxplots represent the median, upper and lower hinges represent the 25th and 75th percentiles (Q1 and Q3), respectively, and whiskers extend to the most extreme datapoints within Q1 − 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR. Outliers are denoted by individual datapoints outside the whiskers. Lowercase letters represent significant differences across all 6 group/treatment combinations, uppercase letters represent differences between the 3 Cu treatments, and stars denote significance between the non-emersed and emersed groups as determined with a two-way ANOVA (* denoting $p < 0.05$, *** denoting $p < 0.001$).

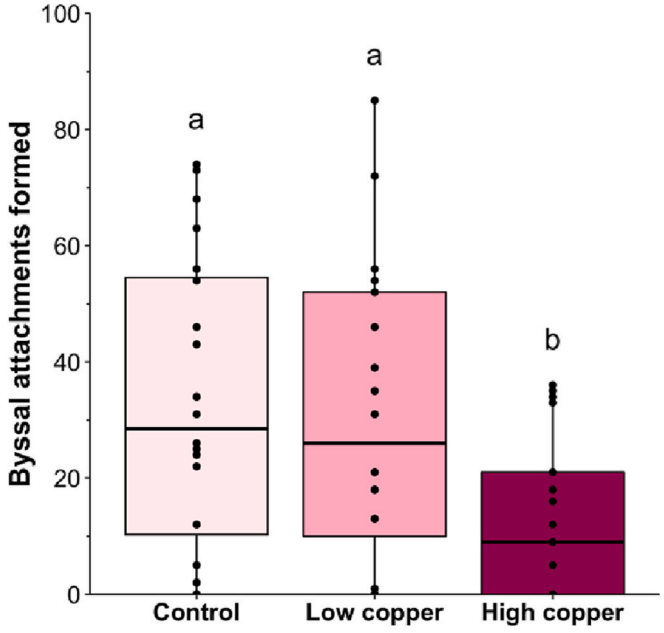

Fig. 3. The number of new byssal threads produced and adhered to experimental containers by *Mytilus galloprovincialis* during 96 h of control, low copper (Cu), or high Cu exposure. Individual datapoints represent byssi produced by each individual mussel (n = 20). Horizontal lines within boxplots represent the median, upper and lower hinges represent the 25th and 75th percentiles (Q1 and Q3), respectively, and whiskers extend to the most extreme datapoints within Q1 − 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR. Outliers are denoted by individual datapoints outside the whiskers. Letters denote significant differences between Cu treatments.

3.5. Succinate

Cu treatment did not have a significant effect on succinate production in the foot of *M. galloprovincialis* in this study ($F_{(2,30)} = 2.968$, $p = 0.067$). Emersion also did not have an effect on succinate production in the foot ($F_{(1,30)} = 0.002$, $p = 0.968$), nor did interactions of Cu and emersion ($F_{(2,30)} = 0.472$, $p = 0.628$). Succinate concentrations appeared to be higher and more variable between individuals in both high Cu treatments, though this escaped significance (Fig. 4A). A few outliers produced high succinate in the controls (Fig. 5A).

Emersion did have a significant effect on succinate production in *M. galloprovincialis* gills ($F_{(1,30)} = 13.078$, $p = 0.001$), and it was observed that succinate concentrations were higher in emersed mussel gills than in those of the non-emersed treatments (Fig. 5B). However, gill succinate levels were independent of Cu treatment ($F_{(2,30)} = 0.343$, $p = 0.712$) and interactions of Cu and emersion ($F_{(2,30)} = 0.858$, $p = 0.434$). When all 6 group/treatment combinations were compared, the only significant difference was that the emersed controls had about triple the gill succinate concentration than the non-emersed controls (Fig. 5B).

3.6. Hemolymph ion concentrations

M. galloprovincialis hemolymph ion concentrations were roughly equivalent to those measured in UCSB facility seawater (Fig. 6; Section 2.1). The mussels showed high individual variation in Na, K, Ca, and Mg concentrations. Na concentrations were unaffected by emersion group ($F_{(1,52)} = 0.500$, $p = 0.483$), Cu treatment ($F_{(2,52)} = 0.772$, $p = 0.467$), or interactions ($F_{(2,52)} = 0.132$, $p = 0.876$). Hemolymph [K] was independent of group ($F_{(1,52)} = 0.714$, $p = 0.402$), treatment ($F_{(2,52)} = 0.185$, $p = 0.832$), and interactions ($F_{(2,52)} = 0.503$, $p = 0.608$). Group ($F_{(1,52)} = 0.932$, $p = 0.339$), treatment ($F_{(2,52)} = 0.795$, $p = 0.457$), and interactions ($F_{(2,52)} = 0.365$, $p = 0.696$) also had no effect on [Ca]. Mg followed the same trend as the other ions, and no effects of group ($F_{(1,52)} = 0.372$, $p = 0.544$), treatment ($F_{(2,52)} = 0.242$, $p = 0.786$), or interactions ($F_{(2,52)} = 0.240$, $p = 0.788$) were found.

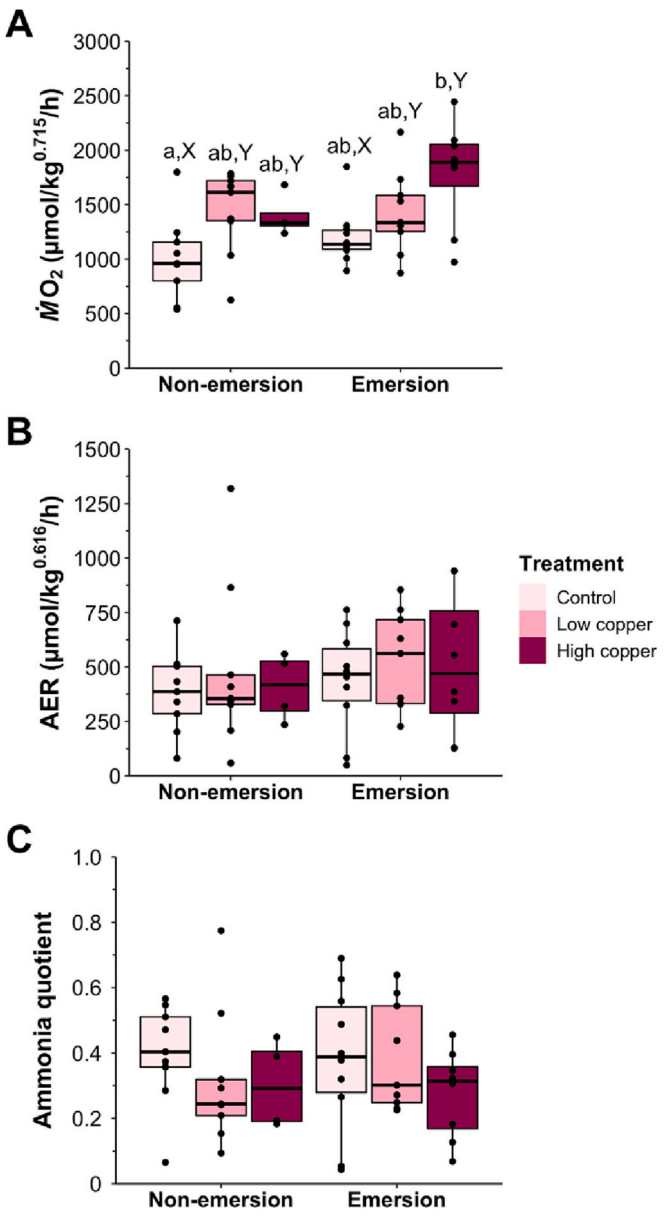

Fig. 4. (A) Mass-scaled oxygen uptake rate ($\dot{M}O_2$), (B) mass-scaled ammonia excretion rate (AER), and (C) ammonia quotient of *Mytilus galloprovincialis* after 96 h of exposure to control, low copper (Cu), or high Cu either immediately measured (non-emersion) or measured following 6 h of air exposure (emersion). Individual datapoints represent measures of each mussel (n = 4 to 10). Horizontal lines within boxplots represent the median, upper and lower hinges represent the 25th and 75th percentiles (Q1 and Q3), respectively, and whiskers extend to the most extreme datapoints within Q1 − 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR. Outliers are denoted by individual datapoints outside the whiskers. Lowercase letters denote significance across all 6 group/treatment combinations, and uppercase letters denote significant differences between the 3 Cu treatments as determined with a two-way ANOVA ($p < 0.05$).

4. Discussion

Overall, Cu accumulation was observed in *M. galloprovincialis*'s shell and internal tissues, but Cu and tidal emersion did not interact to cause physiological damage to this species. No additive or synergistic effects of the two stressors were observed, and results point toward the high tolerance of the mussel to multiple stressor exposures.

4.1. Water chemistry

Adsorption of Cu to surfaces of containers or air stones under the salinity and pH conditions of seawater (John and Leventhal, 1995) may have caused the high Cu stock to have slightly lower Cu than expected (Table 1). Both low and high Cu experimental exposures had lower Cu levels in the water after the 96-h exposure period, reflecting uptake of Cu by the mussels. Indeed, significant bioaccumulation of Cu was observed in *M. galloprovincialis* exposed to Cu (Fig. 1).

4.2. Cu bioaccumulation and shell Ca concentrations

M. galloprovincialis gills accumulated the highest concentrations of Cu, followed by hemolymph and foot (Fig. 1), which reflects the fact that Cu tends to bioaccumulate to the highest concentrations in tissues responsible for Cu uptake, and to a lesser extent in the circulatory fluid and sites of detoxification (Deb and Fukushima, 1999). Thus, it is generally observed that waterborne Cu accumulates most in the gills, hemolymph, liver (or equivalent organ), and to lower concentrations in the muscle (Copper, 2011; Deb and Fukushima, 1999). Fish gills have many ion channels and exchangers which Cu may use to cross epithelial barriers by mimicking ions like Na and Ca, and apical metal transporters like the divalent metal transporter DMT1 may also allow Cu to enter gill epithelia (Copper, 2011; Bury et al., 2003). Similar transporters may exist in mussel gills and allow high Cu bioaccumulation in this tissue. Gills also function in detoxification and excretion of excess metals by incorporating them into lysosomes and granules to be exocytosed (George and Pirie, 1980; Viarengo and Nott, 1993). Indeed, mussels have been observed to excrete Cu via mucus production at the gills (Scott and Major, 1972; Sze and Lee, 1995). It may be expected that most excess Cu would remain in the gills and lower amounts would be transported via hemolymph to other organs, which was observed in the current study (Fig. 1). It was previously found that significant but low Cu bioaccumulation occurred in the foot of the abalone *Haliotis rufescens* (Viant et al., 2002), similar to this study. However, high Cu concentrations after Cu exposure are typically measured in mussel hemolymph due to the metal's tendency to bind to hemocytes (Torres-Duarte et al., 2019; Deb and Fukushima, 1999), and while there was some significant accumulation of Cu in hemolymph of *M. galloprovincialis*, it appears that only a few individuals accumulated high Cu (Fig. 1C). This may have been caused by most mussels undergoing rapid depuration of Cu from the hemolymph to clean external water during the 1 h respirometry period. *Mytilus* mussels tend to close their valves in response to toxicant exposure, minimizing uptake (de Zwaan and Eertman, 1996), but internal Cu accumulation observed in the current study suggests that valve closure was not utilized for the entire exposure period. The high variability between Cu concentrations in *M. galloprovincialis* tissues may then reflect individual differences in the proportion of time spent with valves closed to protect the soft tissues from Cu exposure, and/or differences in Cu depuration rates. Of note, emersion did not affect Cu accumulation in the gills, foot, or hemolymph of *M. galloprovincialis* in this study which is contradictory to previous work on the sea cucumber *Cucumaria miniata* where it was found that 6 h of emersion influenced bioaccumulation of Cu, perhaps due to inhibited excretion during air exposure. Overall, tissue-specific Cu bioaccumulation patterns in this study confirm that the gills are the major site of Cu uptake and reflect the variable sensitivity of mussels' responses to toxicant exposure.

M. galloprovincialis exposed to Cu accumulated the metal in their shells which was paralleled by decreased shell Ca concentrations (Fig. 2). Bivalve shells consist of up to 99 % $CaCO_3$ (Murphy et al., 2018). $CaCO_3$ deposition requires (1) Ca transport from hemolymph across mantle epithelia using Ca channels, Ca-ATPases, and/or paracellular diffusion, (2) HCO_3 synthesis catalyzed by mantle cell CA, and (3) crystallization of $CaCO_3$ in the extrapallial space (EPS) between the mantle and shell (Zhao et al., 2017). Increased waterborne Cu uptake may interfere with any or all of these steps in the $CaCO_3$ deposition

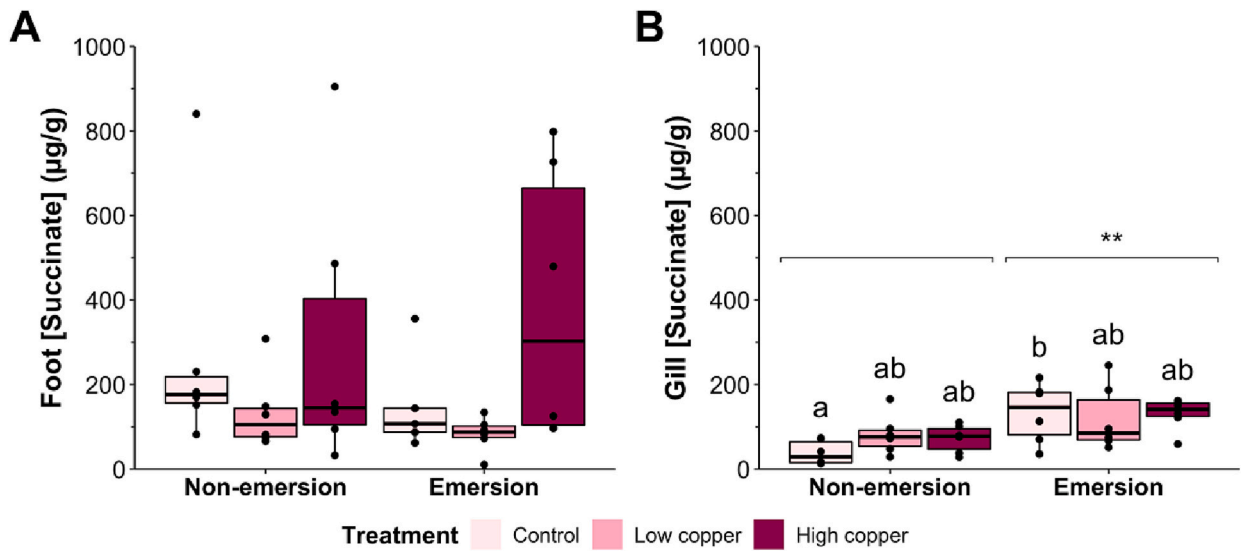

Fig. 5. Succinate levels in the (A) foot and (B) gill of non-emersed and emersed *Mytilus galloprovincialis* after exposure to control, low copper (Cu), and high Cu. Individual datapoints represent succinate accumulation by each individual mussel (n = 6). Horizontal lines within boxplots represent the median, upper and lower hinges represent the 25th and 75th percentiles (Q1 and Q3), respectively, and whiskers extend to the most extreme datapoints within Q1 − 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR. Outliers are denoted by individual datapoints outside the whiskers. Letters represent significant differences across all 6 group/treatment combinations, and stars denote significance between the non-emersed and emersed groups as determined with a two-way ANOVA (** denoting $p < 0.001$).

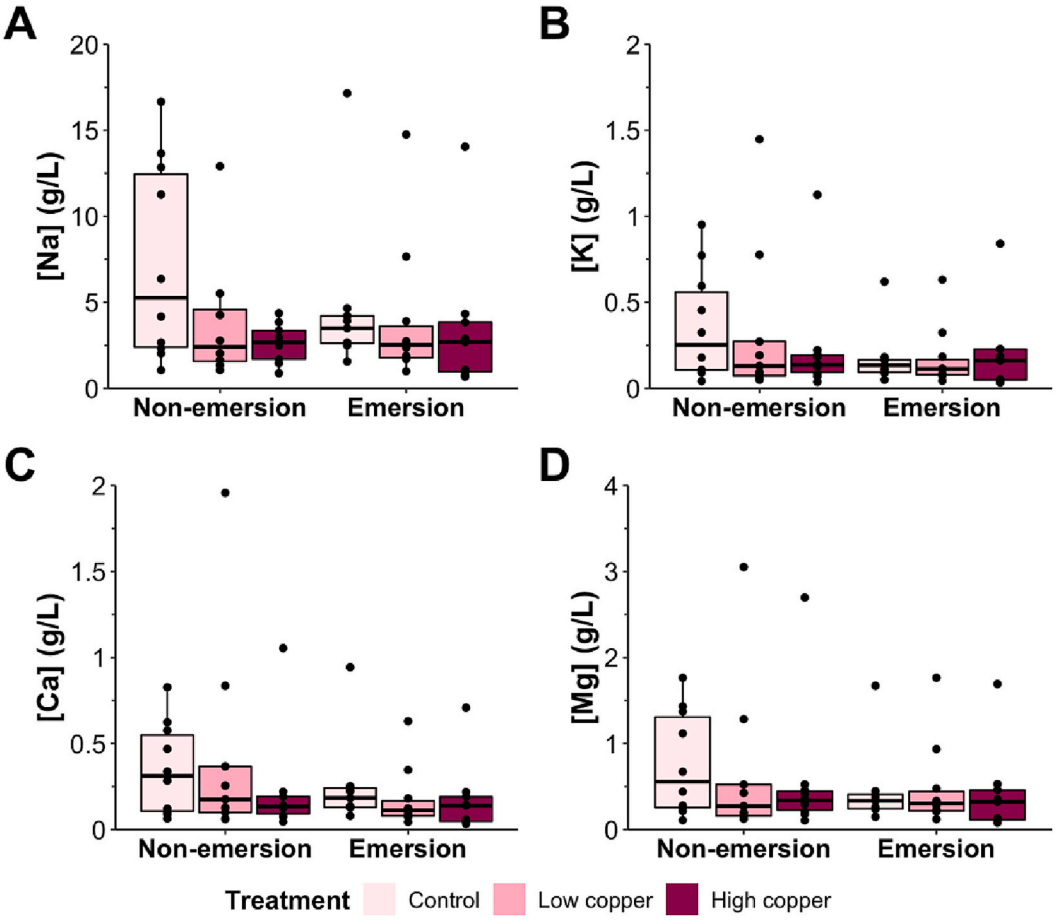

Fig. 6. Concentrations of (A) sodium, (B) potassium, (C) calcium, and (D) magnesium in *Mytilus galloprovincialis* hemolymph after 96 h of exposure to control, low copper (Cu) or high Cu conditions and with or without a 6 h emersion period. Individual datapoints represent ion levels of each individual mussel (n = 9 to 10). Horizontal lines within boxplots represent the median, upper and lower hinges represent the 25th and 75th percentiles, respectively, and upper and lower whiskers extend to the most extreme datapoints within quartile Q1 − 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR. Outliers are denoted by individual datapoints outside the whiskers.

pathway. First, Cu may diffuse paracellularly into the EPS, mimic Ca and be transported from the hemolymph to EPS using Ca uptake proteins, and/or inhibit Ca-ATPases, decreasing Ca transport (Viarengo et al., 1996; Zhao et al., 2017). This is supported by a previous study in which inhibition of Ca transport proteins decreased Cu deposition into shells of the mussel *Corbicula fluminea* (Zhao et al., 2017). Second, Cu may inhibit mantle CA, decreasing available HCO_3 for deposition (Lionetto et al., 2016; Wilbur and Jodrey, 1955). Thirdly, excess Cu in the EPS may outcompete Ca, precipitate with available HCO_3, and be deposited into the shell, though this has yet to be quantified in marine bivalves. These mechanisms of Cu uptake and Ca inhibition could have led to the observed high Cu and low Ca in *M. galloprovincialis* shells after Cu exposure. Interestingly, Cu deposition into bivalve shells (which are biologically inactive) may be a detoxification strategy to decrease the Cu load in other tissues (Zhao et al., 2017). On the other hand, long-term Cu exposure may impair CA-dependent $CaCO_3$ crystallization and inhibit shell growth (Wilbur and Jodrey, 1955). These results suggest *M. galloprovincialis* may be able to incorporate Cu into the shells which may decrease toxicity to internal soft tissues but impair shell growth over long-term exposures to high Cu concentrations. Future research may include evaluations of the speed and extent of Cu deposition into mussel shells during Cu exposure.

Emersion slightly lowered both Cu and Ca concentrations in *M. galloprovincialis* shells (Fig. 2). Induction of anaerobic metabolism caused by reduced oxygen uptake during valve closure is a common adaptation to tidal emersion in mussels (Shick et al., 1986), and anaerobiosis may produce acidic byproducts and cause respiratory acidosis (Fields et al., 2014; Allen et al., 2021). Acidosis may be buffered by resorbing $CaCO_3$ (and perhaps $CuCO_3$) from the shell (Deith, 1985). Decreased Cu and Ca in the shells of emersed mussels may have been triggered by partial induction of anaerobic pathways as a mechanism to buffer respiratory acidosis (see Section 4.5). Cu exposure may also induce acidosis by inhibiting CA (Bielmyer et al., 2005; Boitel and Truchot, 1989), which could explain the slightly lower Cu and Ca content in some of the mussel shells after the mixed Cu and emersion exposure in this study (Fig. 2). However, Cu concentrations in soft tissues and hemolymph after emersion did not increase, so remobilization of Cu from shell deposits caused by anaerobiosis was likely minimal. Overall, patterns of Cu and Ca incorporation into *M. galloprovincialis* shells suggest that both Cu and emersion may independently decrease shell growth by inhibiting calcification, and the combination of the two stressors may have larger effects.

4.3. Byssogenesis

Cu exposure decreased *M. galloprovincialis* byssogenesis (Fig. 3), similar to a previous study in which *Mytilus edulis* produced less byssal attachments upon exposure to 200 μg/L Cu (Sunila, 1981). Reduced byssus production may be attributed to two possible mechanisms: (1) valve closure upon exposure to a toxicant lowering the mussel's ability to secrete new byssal threads, and/or (2) metabolic stress incurred by Cu exposure reducing available energy stores for this process (Sunila, 1981; Ait Ayad et al., 2011; Babarro and Reiriz, 2010; Young, 1985). The byssal gland is located in the muscular foot, which extends from the shell and briefly seals to hard substrates (e.g., rocks or shells of other mussels) where it ejects proteinaceous threads and forms strong attachment plaques (Babarro and Reiriz, 2010; Lu et al., 2013). Byssogenesis may decrease during toxicant exposure because mussel valves need to be slightly open to allow the foot to extend and produce byssi, but a common behavioural response to waterborne toxicants like Cu is to close the valves to protect the soft tissue from uptake and toxicity (Sunila, 1981). On the other hand, even when toxic chemicals are present, mussels tend to produce some byssi especially if completely unattached from the substrate (Ait Ayad et al., 2011). Proceeding with byssogenesis when toxicants like Cu are present means the foot and internal tissues are not protected from exposure (Rajagopal et al., 2005). Both valve closure and Cu toxicity can cause metabolic stress (the first by reducing oxygen uptake, and the second by producing ROS, inhibiting respiratory enzymes like carbonic anhydrase, and damaging gill tissue) leading to increased reliance on anaerobic metabolism and decreased ATP production (Copper, 2011; Wang and Widdows, 1993; de Zwaan and Eertman, 1996; Brown and Newell, 1972; Giacomin et al., 2014). Byssogenesis is an energetically expensive process (Babarro and Reiriz, 2010), so a Cu-induced decrease in available energy stores may also lead to lower byssus production, which has been observed with other toxicants (e.g. Cypermethrin (Ait Ayad et al., 2011)). Low Cu levels used in this study were not enough to damage byssogenesis, perhaps because exposure to low Cu may allow assimilation of Cu into enzymes like cytochrome *c* oxidase which are vital for ATP production without overwhelming detoxification processes (Rainbow, 2007; Solomon and Lowery, 1993). Strong byssal attachments are important for survival of sessile *M. galloprovincialis* in harsh environments like the intertidal zone, and they are also necessary to form dense aggregations of mussels (called mussel beds) which provide extensive habitat for other small organisms like polychaetes and amphipods (Inoue et al., 2021; Borthagaray and Carranza, 2007). Interestingly, byssi also contribute to the biofouling ability and invasive capacity of mussels because the adhesive threads allow them to attach to ships and other human-made structures that may be less desirable habitats to other organisms (Inoue et al., 2021). The use of Cu in antifouling coatings - which damage many other species and thus are restricted in many waters - lowers the invasiveness of mussels by inhibiting byssal attachment (Crowe et al., 2000). Results of this study suggest that elevated Cu exposure in intertidal environments like salt marshes may have a range of impacts from smaller-scale (e.g., decreased fitness and survival of individual mussels) to larger-scale effects (e.g., loss of entire communities due to mussel bed damage, or prevention of expansion of invasive species).

4.4. Oxygen uptake and ammonia excretion

Control *M. galloprovincialis* $\dot{M}O_2$ measurements in this study were similar to rates found in previous studies of this species (Anestis et al., 2010; Fernández-Reiriz et al., 2012). Contrary to anticipated results, emersion had no effect on $\dot{M}O_2$ (Fig. 4A). We expected emersed mussels to have higher $\dot{M}O_2$ upon re-immersion than non-emersed mussels because this pattern has been observed in *Mytilus* spp. (Shick et al., 1986; Bayne et al., 1976) and other bivalves (Byrne et al., 1990; Yin et al., 2017). While air has higher oxygen content than water and is technically hyperoxic, emersion can collapse the delicate gills of aquatic animals which require submersion in water to maintain their structure and function, resulting in hypoxia (Crowe et al., 2000; Glover et al., 2013; Webb, 2021). Additionally, bivalves tend to close their shells during air exposure as a strategy to avoid desiccation of their soft tissues, which prevents oxygen uptake and creates an internal hypoxic environment (de Zwaan and Eertman, 1996; Yin et al., 2017). Hypoxia is stressful for mussels because they mainly depend on aerobic respiration to produce the energy necessary for physiological processes, so when oxygen is unavailable, they increase dependence on anaerobic metabolism which produces less ATP (Shick et al., 1986; Bayne et al., 1976). Upon re-immersion after air exposure, $\dot{M}O_2$ typically increases because tissues must be replenished with oxygen to make up for the hypoxic energy deficit (Bayne et al., 1976). Post-emersion reoxygenation may temporarily elevate internal ROS levels and cause oxidative stress (Hermes-Lima et al., 2015). However, during emersion some mussels periodically gape their valves to keep the gills in contact with the oxygen-rich atmosphere and maintain some oxygen uptake, though it is diminished compared to during immersion in water (Shick et al., 1986; Bayne et al., 1976). Valve gaping can cause desiccation of gills and other soft tissues, but this may be an effective strategy to overcome oxygen stress by maintaining some level of aerobic respiration during short-term air exposure (i.e., 6 h). Thus, effects of emersion on $\dot{M}O_2$ in this study may have been absent because *M. galloprovincialis* was capable of taking up

enough oxygen from the air to sustain normal physiological processes, rendering increased $\dot{M}O_2$ and reoxygenation of tissues upon re-immersion unnecessary. However, gaping could not be observed in this study due to the opacity of experimental containers. *M. californianus* was previously shown to be capable of oxygen uptake via gaping during air exposure (Bayne et al., 1976), but oxygen uptake and gaping behaviours during emersion would have to be measured to confirm this for *M. galloprovincialis*.

M. galloprovincialis exposed to Cu had higher $\dot{M}O_2$ than the controls (Fig. 4A), which is indicative of moderate (not extreme) stress (Sokolova et al., 2012). Previous studies have shown that Cu caused metabolic depression in *Mytilus* spp., and these effects have been attributed to reduced gas exchange at the gills due to several possible mechanisms: ROS damage, inhibition of carbonic anhydrase (CA), or increased mucus production (Brown and Newell, 1972; Scott and Major, 1972; Sunila, 1981; Jorge et al., 2016). The first mechanism occurs when excess Cu within tissues like the gills undergoes redox cycling, producing ROS which can cause oxidative stress and necrosis of gill tissue, such as tearing of interfilamentary junctions as observed in *Mytilus edulis* (Sunila, 1981; Spicer and Weber, 1991). The second mechanism of reduced gas exchange and impaired metabolism is caused by Cu binding to histidine residues and/or displacing the native zinc cofactor of CA, an enzyme that catalyzes the reversible conversion of carbon dioxide and water to bicarbonate and a proton (H^+). CA is integral for gas exchange, ammonia excretion, pH balance, ion transport, and even deposition of calcium carbonate ($CaCO_3$) in bivalves to form their shells; its inhibition can cause disturbances to all of these processes (Lionetto et al., 2016; Jorge et al., 2016; de Polo and Scrimshaw, 2012). In particular, Cu can decrease $\dot{M}O_2$ by inhibiting bivalve gill CA (Santini et al., 2011). Thirdly, high mucous production at the gills was observed after acute exposure to at least 300 μg/L Cu in mussels *M. edulis*, *Perna viridis*, and *Septifer virgatus*, and was predicted to be a detoxification method because high Cu levels were measured within the mucous; however, mucous increases the diffusive distance across the respiratory epithelia and thus may decrease gas exchange (Scott and Major, 1972; Sze and Lee, 1995). It is important to note that high Cu concentrations (300–500 μg/L) were used for the aforementioned studies in which metabolic suppression was observed, but Cu levels in the current study were lower and more environmentally relevant to CSMR (~35 and 160 μg/L). $\dot{M}O_2$ may increase during or after moderate stress exposure due to increased energetic costs of stress protection, such as upregulation of metallothioneins (MTs) which capture excess Cu and ROS to prevent further toxicity or heat shock proteins (HSPs) which can protect other proteins from metal damage (Sokolova et al., 2012; Magesky and Pelletier, 2018). Indeed, the mussels exposed to the highest-stress treatment in this study (high Cu followed by emersion) had the highest $\dot{M}O_2$, though this was only significantly higher than the non-emersed control (Fig. 4A). The Cu levels and/or the durations of exposure to Cu and emersion seem to have been enough to induce compensatory post-stress reoxygenation reflected by increased $\dot{M}O_2$ but were not enough to cause extreme stress to *M. galloprovincialis* which would likely have caused decreased $\dot{M}O_2$.

A further indicator that the Cu levels or durations of exposure and emersion used in this study were not enough to induce extreme stress in *M. galloprovincialis* is that AER was unaffected by Cu, emersion, or interactions of the two stressors (Fig. 4B). During emersion ammonia, a toxic nitrogenous waste product of protein catabolism, typically builds up within intertidal animals because submersion in water is required to create diffusive gradients for excretion (Weihrauch and Allen, 2018). Upon re-immersion, the accumulated ammonia may be rapidly excreted causing high AER, as observed in the mussel *M. edulis* and the crab *Carcinus maenas* (Durand and Regnault, 1998; de Vooys and de Zwaan, 1978). However, another study of emersed *M. edulis* showed low internal ammonia accumulation and low re-immersion AER, and authors proposed that some ammonia was returned to the amino acid pool to conserve energy and maintain normal amino acid levels after emersion, which may have also occurred in this study (Sadok et al., 1999). It appears that 6 h of emersion in the current study was not long enough for ammonia to accumulate within *M. galloprovincialis* to levels that required compensatory fast excretion upon re-immersion, similar to previous findings of Sadok et al. and reflecting this species' high tolerance to tidal emersion (Sadok et al., 1999).

Cu also did not affect AER, indicating that *M. galloprovincialis*'s nitrogen metabolism is not very sensitive to Cu exposure (Fig. 4B). In marine osmoconformers, damage to ammonia excretion has been proposed to be the main mechanism of Cu toxicity (Copper, 2011; Grosell et al., 2007). Cu may decrease ammonia excretion in bivalves by (1) inhibiting proteins such as CA or Rhesus (Rh)-like channels which are involved with ammonia excretion, (2) decreasing excretory organ ciliary activity, or (3) damaging excretory tissue which increases the distance for ammonia diffusion (Brown and Newell, 1972; Wilson-McNeal et al., 2020; Giacomin et al., 2014; Jorge et al., 2016; Santini et al., 2011; Goswami et al., 2014). As previously mentioned, CA is an established target of Cu and is involved not only in gas exchange but also ammonia excretion; thus, its inhibition may be tied to reduced AER (Grosell et al., 2007; de Polo and Scrimshaw, 2012). Cu may also block Rh-like passive ammonia channels present in the excretory epithelia of mussels (i.e., the gills and plicate organ - a small, thin, highly folded structure located at the base of the gills of mytilid mussels), possibly by interacting with histidine residues in the pores of these channels (Wilson-McNeal et al., 2020; Thomsen et al., 2016; Lim et al., 2015). Rh-assisted ammonia excretion is facilitated by movement of gill and plicate organ cilia, which stir the gill boundary layer to prevent ammonia from concentrating and altering the diffusive gradient across the epithelia (Thomsen et al., 2016). This ciliary movement is dependent on dynein ATPase (Wais-Steider and Satir, 1979). Cu targets ATPases (Copper, 2011), so it may inhibit excretory organ cilia and thus interfere with ammonia diffusion; indeed, Cu-induced inhibition of gill cilia was previously observed in *M. edulis* (Brown and Newell, 1972). Lastly, Cu toxicity may cause necrosis of excretory tissue and cause mucous aggregation at the gills, both processes which may effectively increase diffusive distance from hemolymph to water and decrease excretion rates (Spicer and Weber, 1991). However, none of these effects of Cu on ammonia excretion were present in the current study as AER was not affected by either stressor (Fig. 4B). Since Cu exposure followed by emersion was expected to result in inhibition of ammonia excretion due to excretory pathway damage leading to the inability to excrete internally accumulated ammonia, our results suggest that *M. galloprovincialis* have high tolerance not only to tidal emersion but also to Cu exposure. Ammonia concentrations within the mantle cavities and hemolymph of the mussels were not evaluated in this study, but these measures would have been useful to confirm if ammonia was being produced but not excreted under stressful conditions.

M. galloprovincialis AQ was also unaffected by Cu exposure and emersion (Fig. 4C), providing further evidence for their high tolerance to these stressors. This metric is useful for estimating the proportion of protein catabolism contributing to overall energy production; a higher AQ indicates more protein degradation which produces ammonia as a waste product, while a low AQ implies that metabolism is being fuelled more by carbohydrate and lipid breakdown (Kutty, 1972; Rahmah et al., 2020; Sinha et al., 2012). Although there were no significant AQ differences between groups/treatments in this study, there appears to be a trend toward slightly lower AQs in both high Cu-exposed groups, suggesting that some of these mussels had higher dependence on carbohydrate/lipid breakdown to fuel their metabolism. *M. edulis* seem to heavily rely on carbohydrates to fuel early anaerobiosis which could be a strategy to initially conserve protein reserves before turning to protein catabolism upon extended (i.e., multiple days) exposure to anaerobic conditions (de Zwaan and Wijsman, 1976; Sadok et al., 1999). Thus, lower AQs may imply some induction of anaerobic pathways after Cu exposure in *M. galloprovincialis*. However, AQs alone may not be reliable indicators of internal energy balance (Bayne et al., 1976), and analysis

of anaerobic end products provides more insight into the metabolic strategies utilized by organisms under stress.

4.5. Succinate

While lactate is commonly measured as a marker of anaerobic metabolism, lactate is only a minor end product of bivalve anaerobiosis and measures of other intermediate or end products (e.g., succinate, propionate, pyruvate, alanine) provide more accurate estimates of oxygen stress (de Zwaan and Wijsman, 1976; Livingstone, 1983). Emersion induced the production of succinate in previous studies of *M. galloprovincialis* and other bivalves, indicative of increased anaerobiosis due to valve closure and inhibited oxygen uptake (de Vooys, 1979; Shick et al., 1986; Bacchiocchi and Principato, 2000; Zurburg and RHM, 1980). In the current study, succinate levels significantly increased after emersion in *M. galloprovincialis* gills (Fig. 5B), but succinate production in the foot was unaffected by emersion (Fig. 5A). These results suggest that 6 h of emersion was sufficient to cause increased, but not total, dependence on anaerobic metabolism. Since bivalves are highly tolerant to emersion, whole-body anaerobiosis may not occur during short periods of air exposure; in particular, *M. galloprovincialis* gradually accumulates succinate, reaching high levels only after at least 24 h of emersion, reflecting only a moderate reliance on anaerobiosis during emersion (de Vooys, 1979). As previously mentioned, mussels can take up some oxygen via intermittent gaping during emersion (Shick et al., 1986; Bayne et al., 1976), which could explain the observation from this study that *M. galloprovincialis* appears to only partially switch to anaerobic metabolism after a short period of air exposure (Fig. 5).

Cu exposure also has the potential to induce anaerobic pathways as shown by increased levels of anaerobic end products in the clam *Mesodesma mactroides* (Giacomin et al., 2014) and the crab *Carcinus maenas* (Boitel and Truchot, 1989). However, *M. galloprovincialis* succinate production in the foot and gills was unaffected by Cu exposure, indicating that Cu concentrations used in this study were not high enough to induce significant anaerobiosis (Fig. 5). Despite insignificant differences between treatments, results follow a slight trend toward increased succinate in the foot of mussels exposed to high Cu in this study (Fig. 5A), suggesting that Cu may have partially inhibited aerobic metabolism but only in some of the mussels.

Researchers have postulated that mussels may be somewhat dependent on anaerobic pathways to produce energy even under normoxic/non-stressful conditions (de Zwaan and Wijsman, 1976). Indeed, a few mussels unexposed to either stressor in this study still produced high levels of succinate (Fig. 5A). In general, *M. galloprovincialis* displayed a broad range in anaerobic capacity, reflected by highly variable succinate levels between individuals regardless of emersion or Cu treatment. High variability in metabolic responses between individuals may contribute to this species' invasive success in the intertidal zone, because variability reflects intraspecies plasticity which contributes to a species' ability to withstand short-term environmental fluctuations as well as adapt to changes in environmental conditions over time (Tanner and Dowd, 2019).

4.6. Hemolymph ion concentrations

Dysregulation of *M. galloprovincialis* hemolymph cation concentrations was not observed in this study after Cu exposure, emersion, or both stressors (Fig. 6). Impaired osmoregulation by inhibited NKA is the major source of Cu toxicity in freshwater, but for marine osmoconformers Cu is less likely to target proteins involved with osmoregulatory processes (Copper, 2011). Still, osmoconforming invertebrates including bivalves may selectively regulate internal levels of ions like Ca and Mg, thus ionoregulatory proteins like Mg- and Ca-ATPases may also be targeted by Cu in marine contexts (Viarengo et al., 1996; Jorge et al., 2016). Indeed, Cu exposure impaired regulation of Ca and Mg by the osmoconforming and ionoregulating clam *Mesodesma mactroides* (Jorge et al., 2016). However, the absence of effects of Cu on ionoregulation in the current study supports other research on the sea cucumber *Cucumaria miniata* and the crab *Carcinus maenas*, which are also osmoconformers and showed no effects of Cu on iono- or osmoregulation (Lowes et al., 2023; Boitel and Truchot, 1989). It is possible that the Cu concentrations used were not high enough to cause ionoregulatory damage; alternatively, *M. galloprovincialis* may not depend on ionoregulation which could be an adaptation to the intertidal zone where salinity is highly variable. Intertidal invertebrates may experience ionic dysregulation during tidal emersion because air exposure may cause evaporative water loss (Allen et al., 2021). Valve closure by intertidal mussels during emersion allows the mussels to retain internal fluids, preventing dehydration and concentration of internal ions (Bayne et al., 1976). Thus, *M. galloprovincialis*'s hemolymph ion levels were likely unaffected by either Cu exposure or emersion because of their behavioural response to stress (valve closure) and/or because they are strong osmo- and iono-conformers with little need to regulate these ions.

5. Conclusion

Overall, results of this study indicate that *M. galloprovincialis* is very tolerant to both emersion and Cu exposure. Cu and Ca accumulation patterns in the mussel shells suggest that cycles of Cu and air exposure have the potential to alter bivalve shell growth, but assimilation of the metal into the shells may diminish its toxic effects. Indeed, the metabolic response of the mussels to Cu exposure was indicative of moderate (not severe) stress. 6 h of emersion appeared to slightly increase *M. galloprovincialis*'s dependence on anaerobic metabolism but had no other effects, and this high tolerance to tidal emersion was not affected by Cu exposure, contradicting our hypothesis that the metal would damage compensatory physiological processes used to withstand periods of air exposure. However, this study included acute Cu exposure and one submersion/emersion cycle which could have obscured potential long-term interactive effects of the two stressors. Further studies of tidal stressors on marine invertebrates using chronic toxicant exposure and environmentally realistic repeated tide cycles are recommended.

Carpinteria Salt Marsh is an extreme intertidal environment with broad fluctuations in temperature and oxygen availability to the organisms which inhabit its flood channels, including *M. galloprovincialis*. Based on results of the current study, these mussels are likely not damaged by the daily environmental challenges they face because they are able to withstand hypoxia during tidal emersion and Cu exposure. This may be a promising sign for survival of intertidal species and the overall health of such ecosystems in spite of anthropogenic activities, but tolerance of invasive species to stressors may also be worrying because invasive organisms may outcompete less resilient species over time and alter community structures (Nicastro et al., 2010). Additionally, conditions of the world's oceans are predicted to be altered in the near future by anthropogenic-driven climate change (Finke et al., 2007). The intertidal zone is particularly vulnerable to global climate change which is expected to cause factors like salinity, pH, temperature, and tidal heights to fluctuate more dramatically. Combinations of toxicant release and increased variation of environmental conditions may damage some populations but remain within the range of tolerance of other species. For example, *M. galloprovincialis* has been found to exhibit rapid adaptive responses to regional temperature changes, which allow this species to successfully invade new habitats and may mean they will easily be able to cope with extreme tidal fluctuations in the future (Han and Dong, 2020). The current study also shows that this species is highly tolerant to both Cu exposure and hypoxia. These results highlight the need for further studies of mixed stressors on intertidal organisms, as not all animals may be able to withstand the effects of anthropogenic influence and climate change on global coastlines.

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CRediT authorship contribution statement

HML – Lead, executed and designed experiment, analyzed data, wrote MS.

EE – Designed, executed and helped with all major experiments, data analysis and edited MS.

KS – Performed water chemistry analysis, data analysis, wrote sections and edited MS.

DSA – Performed water chemistry analysis, data analysis, wrote sections and edited MS.

TAB – Designed experiment with HML, supervised, helped write, and edited MS.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tamzin Blewett reports financial support was provided by Natural Sciences and Engineering Research Council of Canada.

Data availability

Data will be made available on request.

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