Impacts of Combined Natural and Anthropogenic Stressors on Pacific Intertidal Invertebrates

by

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Abstract

The intertidal zone provides extensive habitat around the world for countless species, but there are many challenges associated with life in this region. In particular, intertidal organisms may be subjected to hours of air exposure during low tide. Tidal emersion from water is potentially a major source of stress to aquatic animals because it may cause desiccation and inhibit several aspects of their physiology including oxygen uptake, ammonia excretion, and osmoregulation. Still, organisms live within their range of tolerance to environmental stressors, and intertidal animals have strategies to cope with emersion. For example, water-breathing invertebrates may switch to anaerobic metabolism, which is less energetically efficient, when they are unable to take up oxygen from air. Although intertidal organisms are adapted to withstand extreme environmental fluctuations, natural stressors like tidal emersion are not the only challenges that they face; they are also vulnerable to anthropogenic toxicant release. One major toxicant that poses a threat to the intertidal zone is copper (Cu), which is essential for life at low concentrations but toxic at high levels. Cu damages physiological processes including gas exchange, metabolism, ammonia excretion, and osmoregulation. Cu-induced damage to these pathways may be lethal to marine invertebrates. In the context of the intertidal zone, this is worrying because several natural and anthropogenic stressors including Cu and emersion may be simultaneously present. Mixed stressors may have additive or synergistic effects, especially when the stressors target similar processes in an organism. Since both Cu and emersion may affect the same physiological processes (e.g., metabolism and ammonia excretion), it is possible that Cu exposure prior to tidal emersion may alter intertidal organisms' ability to overcome the challenges presented by air exposure.

For my thesis, I studied the combination of Cu exposure and tidal emersion on two

intertidal marine invertebrates which use different strategies to cope with air exposure: (1) the orange sea cucumber *Cucumaria miniata* and (2) the Mediterranean mussel *Mytilus galloprovincialis*. I hypothesized that environmentally relevant concentrations of Cu would decrease the tolerance of both species to tidal emersion, but that effects would be smaller in *M. galloprovincialis* because it is able to protect its soft tissues from external stress by closing its valves, conferring high tolerance to a broad range of environmental conditions. To test my hypothesis, I conducted the same experiment on both species: 96 h acute exposure to Cu followed by a 6 h period of air exposure to mimic tidal emersion. I measured tissue-specific Cu bioaccumulation, oxygen uptake rates, ammonia excretion rates, ammonia quotients, levels of end products of anaerobic metabolism, and ion concentrations of internal fluids after exposure to the two stressors. I expected the combination of Cu exposure and emersion to result in decreased oxygen uptake and ammonia excretion rates, induction of anaerobiosis, and osmotic dysregulation in both organisms. Contrary to my hypothesis, analyses of these metrics revealed that while Cu toxicity and emersion each had individual negative effects on both of the study organisms, Cu did not seem to alter the ability of either animal to cope with tidal air exposure. My results speak to the high tolerance of both species to the environmental stressors they face and provide further understanding of how multiple stressors interact to affect the physiology of intertidal animals. This study may be used inform predictions of the distribution and survival of species in our changing climate.

Preface

This thesis is an original work by Hannah Morgan Lowes. Chapter 2 has been submitted to Science of the Total Environment as H.M. Lowes, A.M. Weinrauch, I.A. Bouyoucos, R.A. Griffin, D. Kononovs, D.S. Alessi, and T.A. Blewett, "Copper exposure does not alter the ability of intertidal sea cucumber *Cucumaria miniata* to tolerate emersion during low tide". I was responsible for experiment planning, data collection, analysis, and manuscript composition. A.M.W. and I.A.B. assisted with data collection, statistical analysis, and manuscript editing. R.A.G. assisted with data collection and manuscript editing. D.K., supervised by D.S.A., collected ICP-MS data. T.A.B. was the supervisory author, formed the experiment concept, and assisted with experiment planning, data collection, and manuscript editing. Chapter 3 has not been previously published but is being prepared for submission to Royal Society of Chemistry's Environmental Science: Processes and Impacts. I was responsible for experiment planning, data collection, statistical analyses, and manuscript composition. E.J.E. assisted with experiment planning, data collection, and manuscript editing. K.N.S., supervised by D.S.A., collected ICP-MS data. T.A.B. was the supervisory author, contributed to the experiment concept, and assisted with manuscript editing.

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List of Abbreviations

AER	Ammonia excretion rate
ANOVA	Analysis of variance
AQ	Ammonia quotient
ATP	Adenosine triphosphate
B.D.L.	Below detectable limit
Bi	Bismuth
BLM	Biotic ligand model
Ca	Calcium
CA	Carbonic anhydrase
$CaCO_3$	Calcium carbonate
CDFW	California Department of Fish and Wildlife
Cl	Chloride
CO_2	Carbon dioxide
CSMR	Carpinteria Salt Marsh Reserve
Cu	Copper
Cu^+	Monovalent copper ion
Cu^{2+}	Divalent copper ion
DMT	Divalent metal transporter
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon

DOLT-5	Dogfish liver reference material for trace metals and other constituents
EPHOC	Excess post-hypoxia oxygen consumption
EPS	Extrapallial space
Ge	Germanium
GSH	Glutathione
H^+	Proton
H_2	Hydrogen gas
HCl	Hydrochloric acid
HCO_3	Bicarbonate
He	Helium
HNO_3	Nitric acid
HSD	Honest significant difference
HSP	Heat shock protein
ICP-MS/MS	Inductively coupled plasma-mass spectrometry/mass spectrometry
In	Indium
IQR	Interquartile range
K	Potassium
KOH	Potassium hydroxide
LOD	Limit of detection
Lu	Lutetium
Mg	Magnesium
$\dot{M}O_2$	Oxygen uptake rate
MT	Metallothionein

n	Sample size
Na	Sodium
NaOH	Sodium hydroxide
NH_3	Ammonia
NH_4^+	Ammonium ion
NKA	Sodium/potassium ATPase
O_2	Oxygen gas
OH^-	Hydroxyl radical
Q1	Quartile 1/25th percentile
Q3	Quartile 3/75th percentile
Rh	Rhesus
RMR	Routine metabolic rate
ROS	Reactive oxygen species
S.E.M.	Standard error of the mean
Sc	Scandium
S.D.	Standard deviation
TDS	Total dissolved solids
UCSB	University of California, Santa Barbara
µg/L	Micrograms per litre

Chapter 1: Introduction

1. Stressors in the intertidal zone

The intertidal zone, or the area between high and low tide, provides habitat for countless species from a diverse selection of phyla around the world. Animals inhabiting this region are exposed to dramatic fluctuations in environmental conditions throughout the tide cycle, including temperature, salinity, pH, and oxygen availability (Finke et al., 2007; Helmuth et al., 2006; Truchot and Duhamel-Jouve, 1980). For example, tide pools may rapidly reach temperatures much higher than the ocean when low tide coincides with the hottest time of day, and evaporation or rainfall may respectively raise or lower pool salinities, potentially causing physiological stress for organisms inhabiting these pools (Truchot and Duhamel-Jouve, 1980). If stagnant tide pools contain a high density of animals, they can also reach extremely low dissolved oxygen (DO) concentrations as the organisms consume the available oxygen, which may be another source of stress (Truchot and Duhamel-Jouve, 1980). Additionally, intertidal organisms that live outside of tide pools experience repeated cycles of aquatic and terrestrial conditions because they are submersed in water during high tide but experience emersion (air exposure) during low tide (Finke et al., 2007).

1.1. Tidal emersion

Air has a low heat capacity and thus emersed temperatures vary much more than in the water, so thermal stress is one potential consequence of tidal emersion (Blanchette et al., 2016). Periods of emersion are also physiologically stressful because aquatic animals must contend with the higher oxygen content but lower density of air relative to water (Glover et al., 2013; Webb, 2021). Many aquatic animals rely on submersion in water to physically support their delicate

respiratory organs (i.e., gills) and maintain oxygen uptake, because in air these organs may collapse which decreases the available area for gas exchange (Glover et al., 2013; Weinrauch and Blewett, 2019). Tidal emersion may result in gill or even whole-body desiccation, especially for soft-bodied invertebrates. However, animals tend to inhabit regions where environmental conditions are within their ranges of tolerance, so it is unusual for stressors associated with the tide cycle to inflict great damage on intertidal organisms (Crowe et al., 2000). Indeed, the major problems presented by emersion are typically sublethal for intertidal organisms because they have mechanisms to tolerate these stresses (Finke et al., 2007).

The main physiological stresses caused by emersion (other than thermal stress) are related to the reduced physical support provided by air and/or the absence of diffusive gradients across an organism's epithelia from the internal fluid to the water, which are required for many physiological processes (Glover et al., 2013; Wilkie, 2002). Specifically, the major problems of interest to this study are (1) desiccation, (2) inhibited aerobic metabolism, (3) impaired ammonia excretion, and (4) osmoregulatory damage (Allen et al., 2021; Crowe et al., 2000).

1.1.1. Desiccation

First, desiccation may occur when tissues or organs like the gills of water-breathing animals collapse without the support of water, or evaporative water loss may dry out these tissues which require moisture to function (Allen et al., 2021; Glover et al., 2013). Gills are responsible for the majority of most marine organisms' gas exchange, pH balance, excretion of ammonia and other waste products, and osmoregulation, so if the gills (or equivalent organs) are desiccated, all of these processes may be irreversibly disrupted (Allen et al., 2021). Bivalves such as the mussel *Mytilus edulis* close their valves to protect their soft tissues from desiccation, and a side effect of valve closure is temporary inhibition of oxygen uptake leading to hypoxia

(Fields et al., 2014; Nicastro et al., 2010; Shick et al., 1986). Thus, whether an animal has behavioural strategies to minimize desiccation like closing its valves or if its respiratory epithelia are physically damaged by air exposure, emersion almost always leads to the second problem: inhibition of aerobic metabolism.

1.1.2. Aerobic metabolism

Aerobic pathways are the preferred methods of energy generation because they are more efficient (i.e., yield much more ATP per unit of substrate consumed) than anaerobic pathways, and anaerobiosis produces toxic end products such as lactate (de Zwaan and Wijsman, 1976; Sokolova et al., 2012). ATP is necessary for survival because it is used for basal processes such as regulation of proteins and ion balance, and it is also important for growth and reproduction (Sokolova et al., 2012). If tidal emersion results in gill damage and inhibition of oxygen uptake by an organism, it must compensate for this either by using other routes to acquire oxygen or by facultatively switching to anaerobic metabolism to produce energy. For example, the sea cucumber *Holothuria forskali* may take up some oxygen across the integument when its respiratory tissue is unable to do so (Astall and Jones, 1991). Similarly, high intertidal mussels (e..g, *Mytilus californianus*) periodically gape their valves during air exposure and are able to maintain some oxygen uptake at the gills and possibly the mantle (Bayne et al., 1976). Using alternative routes of oxygen uptake usually provides enough oxygen to maintain some aerobic processes and prevent an entire switch to anaerobiosis (McMahon, 1988). However, organisms which do not utilize alternative gas exchange routes make a facultative shift to anaerobic metabolism during emersion. For instance, low intertidal mussels including *Mytilus galloprovincialis* rarely gape their valves which creates a hypoxic environment within their shell; thus they heavily rely on anaerobic pathways in air, evidenced by low oxygen consumption rates

and accumulation of anaerobic end products (Anestis et al., 2010; Babarro et al., 2007; McMahon, 1988). Since emersion alters the metabolism of most animals, they must compensate for low energy production when returned to normal conditions (i.e., re-immersion in water during high tide). Regardless of the strategy to cope with inhibited oxygen uptake, aquatic animals that undergo periods of emersion/hypoxia show a pattern of increased oxygen uptake upon re-immersion in water to reoxygenate internal tissues and compensate for energy deficits (Bayne et al., 1976; Curley et al., 2021). Re-immersion also allows these organisms to utilize the gradient from internal fluids to water for other physiological processes like ammonia excretion.

1.1.3. Ammonia excretion

Most nitrogenous waste is passively excreted across the gills/excretory epithelia so submersion in water is necessary to create a gradient across the epithelia conducive to passive transport (Weihrauch and Allen, 2018; Wilkie, 2002). Thus, emersion inhibits excretion and causes internal buildup of ammonia, which is a toxic waste product of protein catabolism (Sadok et al., 1999; Wright, 1995). Marine animals may avoid potential stress caused by ammonia accumulation by increasing reliance on carbohydrates and lipids as catabolic substrates rather than proteins, which reduces ammonia production (Randall and Tsui, 2002; Sadok et al., 1999). Alternatively, animals may tolerate accumulated ammonia during emersion and rapidly excrete it upon high tide re-immersion, resulting in high ammonia excretion rates as observed in the mussel *M. edulis* (de Vooys and de Zwaan, 1978) and the crabs *Necora puber* and *Carcinus maenas* (Durand and Regnault, 1998). These strategies allow intertidal organisms to withstand inhibition of ammonia excretion during emersion.

1.1.4. Osmoregulation

Lastly, osmoregulation may be inhibited by tidal air exposure because transfer of ions also depends on submersion to allow transport between internal fluids and the surrounding water. In addition, evaporative water loss may occur when an organism is exposed to air for extended periods, which increases hemolymph ion concentrations (Allen et al., 2021). This may lead to further dehydration and desiccation of tissues such as the gills, which may then affect other physiological processes like oxygen uptake (Bayne et al., 1976). Many marine invertebrates are osmoconformers but some (e.g., the clam *Mesodesma mactroides* and sea cucumber *Apostichopus japonicus*) may regulate circulatory fluid ion levels, so air exposure may still inflict osmotic damage and lead to further desiccation of intertidal invertebrates (Bayne et al., 1976; Geng et al., 2016; Jorge et al., 2016). Osmotic dysregulation is not the major effect of emersion for osmoconformers, but it may still contribute to overall physiological stress experienced by intertidal animals during air exposure.

1.2. Anthropogenic toxicants

Extreme environmental fluctuations in the intertidal zone present many potential sources of stress to organisms that inhabit these areas, and variations in stressful conditions from low- to high-intertidal regions are major drivers of species distribution (Crowe et al., 2000). In particular, tidal emersion introduces many natural challenges to the physiology of aquatic animals, but they are adapted to survive these adverse conditions. However, coastal areas are also altered by human activities. A multitude of anthropogenic toxicants are frequently released into marine ecosystems, where organisms are less likely to be tolerant to their effects. Waterborne toxicants are of great concern to aquatic animals because they are in constant contact with the external water (and thus the toxicant) during submersion (Wood, 2011). Natural and

anthropogenic stressors both have high potential to impact distribution and survival of intertidal species, so both must be studied to understand the full picture of the daily challenges they face.

2. Copper as a waterborne anthropogenic toxicant

Copper (Cu) is an essential trace metal, meaning it is required for life at low concentrations but becomes toxic at high concentrations (Grosell, 2011). Cu is a common toxicant in aquatic environments because it runs off into surface waters from countless anthropogenic sources, including but not limited to mining, agricultural, industrial, and urban activities (Crowe et al., 2000). For example, Cu is used in pesticides, antifouling paint on boats, and automobile brake pads. Water bodies located adjacent to or downstream of Cu inputs, such as intertidal areas, receive the highest concentrations of this metal. Offshore dissolved Cu concentrations are typically quite low, ranging from < 1 to 25 µg/L (B.C. Ministry of Environment and Climate Change Strategy, 2019), but intertidal areas and estuaries may reach 100 µg/L or more due to their proximity to anthropogenic inputs (US EPA, 2007). However, total dissolved Cu concentrations may cause overestimations in environmental risk assessments because Cu speciation affects its toxicity (de Polo and Scrimshaw, 2012). Free ionic Cu (Cu^{2+}) is considered most bioavailable to aquatic organisms, but less than half of total dissolved Cu is present as free Cu^{2+} because it tends to complex with organics or anions (e.g., carbonate). Complexation lowers its bioavailability and toxicity to water-breathing organisms, and may result in partitioning of Cu into sediments (Grosell, 2011). Other aspects of water chemistry including salinity and pH may alter Cu's speciation, bioavailability, and toxicity (de Polo and Scrimshaw, 2012). High salinity in marine environments may lower the toxicity of Cu (compared to freshwater) because inorganic complexation increases in seawater, and cations like sodium (Na) and calcium (Ca) can outcompete Cu at binding sites and decrease its uptake

(Paquin et al., 2002). High pH encourages formation of Cu carbonates and organic complexes and thus also decreases Cu toxicity by lowering the proportion of free Cu (Paquin et al., 2000). In the fathead minnow *Pimephales promelas*, increases in Ca, pH, and dissolved organic carbon (DOC) all decreased Cu toxicity, although authors suggested that free Cu may not be the only species able to exert toxic effects on aquatic organisms (Erickson et al., 1996). For example, Cu hydroxides may also be toxic (Erickson et al., 1996; Grosell et al., 2007).

Cu toxicity has been a major topic of study in the past few decades, possibly because it is one of the most common metals reported to impair water quality (Reiley, 2007). As such, Cu uptake by aquatic organisms and mechanisms of toxicity are relatively well understood especially in freshwater environments. A biotic ligand model (BLM) for Cu has been developed, which incorporates the water chemistry parameters described above to predict the speciation and bioavailability of the metal and its toxicity to a range of freshwater organisms (US EPA, 2007). BLM predictions are used to develop water quality guidelines and to conduct metal risk assessments (Paquin et al., 2002). Cu BLMs suggest that gills of aquatic animals are the major uptake site for Cu, and that accumulation in the gills correlates with the concentration of free ionic Cu (Paquin et al., 2000). However, the BLM has yet to be firmly established for marine contexts due to the higher salinity and pH compared to freshwater which alter Cu speciation and toxicity (Grosell, 2011).

2.1. Copper uptake, transport, and accumulation

While Cu toxicity varies between freshwater and saltwater environments, routes of uptake, internal transport, metabolism, and excretion are generally the same. Most waterborne Cu uptake occurs across the respiratory epithelia, where apical divalent metal transporters (DMTs) may transport divalent Cu into epithelial cells (Bury et al., 2003). Additionally, it has

been proposed that reductase proteins at fish gills may reduce divalent Cu to the monovalent form (Cu^{+}), which can also enter the gills via copper transporters like Ctr1 (Grosell, 2011). Of particular importance in freshwater, Cu acts as an ion mimic and may take advantage of ion channels (e.g., Na, Ca; Grosell, 2011; Wood, 2011). Dissolved Cu may also be taken up across digestive epithelia using similar transporters, especially for marine animals because they live in hyperosmotic conditions and must continuously drink water to avoid dehydration (Grosell, 2011). In soft-bodied marine invertebrates, the integument may also be a site of metal uptake (Blewett and Leonard, 2017; Deb and Fukushima, 1999), but this has not yet been confirmed for Cu. Once Cu enters epithelial cells, it may be transported across basolateral membranes by a Cu-ATPase, as observed in the sea bream *Sparus aurata* (Minghetti et al., 2010). Alternatively, it may diffuse directly from the external water to the circulatory fluid using paracellular pathways between epithelial cells (Wood, 2011). The metal then binds plasma proteins (e.g., hemoglobin, ceruloplasmin or equivalents) and is transported around the body for delivery to various tissues (Deb and Fukushima, 1999; Harris and Gitlin, 1996). Cu is delivered to the liver, kidney, and muscle, and later to the gut and gills for excretion (Wood, 2011). Cu is essential for many physiological processes; for example, it is a cofactor in cytochrome *c* oxidase which is vital for mitochondrial oxidative phosphorylation (Grosell, 2011). Thus, some Cu is incorporated into essential enzymes or stored in these tissues, but excess Cu must be detoxified and excreted. Detoxification occurs when metallothionein (MT, a metal-scavenging protein) binds free Cu, and excess Cu may also be sequestered into lysosomes or insoluble granules for excretion (Viarengo and Nott, 1993). Metal excretion is less well understood than accumulation and detoxification, but likely routes of Cu excretion are biliary (from the liver or hepatopancreas), urinary (from the kidney), and branchial (from the gills; Grosell, 2011). Some marine invertebrates may be unable

to excrete Cu and instead heavily rely on detoxification of excess levels (Rainbow, 2007). Due to these uptake, transport, and excretion patterns, Cu tends to accumulate to the highest concentrations in the liver, respiratory tissue, and circulatory fluid, followed by the muscle. However, accumulation does not necessarily result in toxicity because organisms have mechanisms to tightly regulate Cu homeostasis (e.g., MTs). Toxicity occurs when uptake rates overwhelm an organism's capacity to detoxify and excrete the metal (Rainbow, 2007).

2.2. Copper toxicity

Cu exerts toxicity both generally and at specific targets. Cu has high oxidation-reduction (redox) potential which makes it useful for oxidative phosphorylation but also contributes to its toxicity (Grosell, 2011). Excess cellular Cu undergoes redox cycling, producing reactive oxygen species (ROS) such as hydroxyl radicals (OH^-) which can cause oxidative damage to DNA, lipids, and proteins (Grosell, 2011; Magesky and Pelletier, 2018). Antioxidant enzymes like glutathione (GSH) protect cells by scavenging ROS, but another aspect of Cu that contributes to its toxicity is its tendency to bind histidine, cysteine, and methionine residues of proteins, potentially rendering them non-functional (Grosell, 2011; Harris and Gitlin, 1996). Thus, Cu may contribute to oxidative stress both by producing ROS and by binding and inhibiting antioxidant enzymes like GSH (Grosell, 2011). Excess Cu may also bind and inhibit other important proteins including carbonic anhydrase (CA), which catalyzes the hydration of carbon dioxide to bicarbonate and a proton and ties together gas exchange, acid-base balance, ammonia excretion, and osmoregulation (Bielmyer et al., 2005; Grosell et al., 2007; Santini et al., 2011). Na/K-ATPase (NKA), which maintains electrochemical gradients across cell membranes and is important for osmoregulation, is another potential target of Cu (de Boeck et al., 2001). Inhibition of these proteins and/or oxidative stress damages several physiological

processes of aquatic organisms. Specifically, Cu mainly affects aerobic metabolism, ammonia excretion, and osmoregulation. Damage to these processes causes stress and can inhibit growth and survival of organisms (Grosell, 2011).

2.2.1. Aerobic metabolism

Cu damages aerobic metabolism of marine animals by inhibiting gas exchange or damaging respiratory proteins like hemoglobin. Inhibited gas exchange may be due to oxidative stress causing physical damage to respiratory tissue. For example, Cu exposure caused tearing, thickening, and necrosis of gill tissue in the spiny lobster *Panulirus homarus* (Maharajan et al., 2012) and mucous accumulation on the gills of mussels *Mytilus edulis* and *Perna viridis* (Goswami et al., 2014; Sunila, 1981). Both of these outcomes effectively increase the diffusive distance across the branchial epithelia, decreasing the animals' capacity for oxygen uptake (Spicer and Weber, 1991). Cu may also affect oxygen uptake by binding and inhibiting CA which is important for gas exchange (de Polo and Scrimshaw, 2012; Grosell et al., 2007; Lionetto et al., 2016). Cu may indirectly inhibit gas exchange in bivalves because they may close their valves in response to toxicant exposure which cuts off oxygen uptake (de Zwaan and Eertman, 1996). Lastly, Cu is known to bind invertebrate respiratory pigments like hemoglobin, and may inhibit oxygen delivery to tissues (Deb and Fukushima, 1999). Any or all of these effects of Cu on oxygen uptake may result in a partial or total switch to anaerobic metabolism, which is less energetically efficient than aerobic pathways (Giacomin et al., 2014). Thus, Cu exposure typically lowers the aerobic capacity of marine organisms, resulting in respiratory depression (e.g., Scott and Major, 1972).

2.2.2. Ammonia excretion

Perhaps the most important toxic effect of Cu on marine osmoconformers is on ammonia excretion (Grosell, 2011; Grosell et al., 2007). Since ammonia is a toxic waste product that damages neuroregulatory and metabolic pathways, it must be excreted to avoid internal accumulation (Wright, 1995). A commonly observed effect of Cu exposure in marine animals is increased ammonia in the plasma (e.g., Grosell et al., 2003), indicative of inhibited ammonia excretion. Effects of Cu on gas exchange are tied to effects on ammonia excretion for organisms which use the same organs for both (i.e., gills or equivalent organs). Increased diffusive distance across respiratory/excretory epithelia and inhibition of CA (which is also integral for ammonia excretion) damage this process. Additionally, Cu may bind and inhibit other proteins involved with ammonia excretion, such as Rhesus (Rh) channels which transport ammonia across apical membranes of excretory tissue (Thomsen et al., 2016). Finally, ammonium (NH_4^+) transport across fish gills may be tied to ionic Na transport (e.g., NH_4^+ may mimic H^+ and be excreted via apical Na/H^+ exchange proteins), and a similar mechanism for excretion may exist in invertebrates (Wilkie, 2002). Since Cu mimics Na and impacts Na transport, it may prevent exchange of Na for NH_4^+ and thus inhibit its excretion. Whatever the target(s) of Cu in ammonia excretion pathways, exposure causes internal ammonia accumulation which may itself have severe toxic effects, and this is the major toxic route of Cu in marine organisms (Grosell, 2011; Grosell et al., 2007).

2.2.3. Osmoregulation

In freshwater, the main mechanism of Cu toxicity is osmoregulatory disturbance caused by inhibition of CA and NKA (Grosell et al., 2007). Cu inhibits Na uptake and alters the vital electrochemical gradients across biological membranes. However, saltwater organisms have

different osmoregulatory strategies; in particular, many marine invertebrates are osmoconformers, and thus Na gradients are not as important for survival. Some marine invertebrate osmoconformers may regulate ion concentrations in their circulatory fluid, and ionoregulatory disturbances due to Cu exposure have been observed (e.g., in the clam *Mesodesma mactroides*; Jorge et al., 2016), but this is not Cu's major mechanism of toxicity in marine environments. One might expect marine osmoconformers to be the least sensitive to Cu exposure due to competitive inhibition of Cu uptake by saltwater ions and the relative unimportance of Na gradients, but osmoconformers are just as sensitive to Cu as osmoregulators (Grosell et al., 2007) due to metabolic disturbance and inhibition of nitrogenous waste excretion. Overall, Cu exposure causes damage by inducing oxidative stress and inhibiting metabolic, excretory, and osmoregulatory pathways, and long-term exposure may impact sensory, hormonal, and immune function, reproductive output, growth, and survival (Grosell, 2011).

3. Mixed natural and anthropogenic stressors in the intertidal zone

A multitude of previous studies have established that mixed stressors may have additive or even synergistic effects on marine organisms (for meta-analysis of studies see Crain et al., 2008). Intertidal animals that are exposed to tidal stressors like air exposure and changes in salinity, pH, and temperature are also vulnerable to toxicant exposure. Many combinations of natural stressors related to the tide cycle and anthropogenic toxicants like Cu have been studied. For example, pH fluctuations increase DNA damage and oxidative stress in *M. edulis* exposed to Cu (Wilson-McNeal et al., 2020), and salinity changes may increase or decrease Cu toxicity depending on the osmoregulatory strategy utilized by an organism (Grosell et al., 2007; Holan et al., 2019). Additionally, small water temperature increases (2–4 °C) heightened the toxicity of Cu to multiple subantarctic marine invertebrate species (Holan et al., 2019). The combination of

Cu exposure and tidal emersion warrants study because they both affect similar physiological processes (i.e., metabolism, ammonia excretion, osmoregulation), but interactions of these two stressors on intertidal animals have not yet been evaluated. Studying effects of mixed tidal and anthropogenic stressors is vital for predictions of future distribution and survival of intertidal species because fluctuations in tidal conditions are expected to become more extreme due to global climate change (Finke et al., 2007).

4. Intertidal marine invertebrates as model organisms in multi-stressor research

Marine invertebrates are often overlooked in research, but they are ecologically important members of marine food webs and possess roles such as nutrient cyclers, water filters, ecosystem engineers, and bioindicators (Borthagaray and Carranza, 2007; Eriksson et al., 2012; Inoue et al., 2021). They are of particular interest in the current study of Cu toxicity and emersion because invertebrates that inhabit the intertidal zone have diverse physiological mechanisms to tolerate tidal air exposure, even among related species. For example, mid-intertidal mussels experience shorter periods of emersion and are less tolerant to aerial desiccation than high intertidal mussels of the same genus, and tend to close their valves for a much higher proportion of emersion periods (McMahon, 1988). Anatomical and physiological adaptations to the ecosystems marine invertebrates inhabit are understudied in many species, so researching stress responses of a variety of organisms spanning multiple phyla improves general knowledge of invertebrate physiology. Lastly, because intertidal invertebrates are at the forefront of the marine-terrestrial interface, they may be especially vulnerable to mixed stressors as well as increased variability or intensity of these stressors due to anthropogenic activities and climate change (Finke et al., 2007).

The two species selected for the current analysis of exposure to Cu and emersion were the orange sea cucumber *Cucumaria miniata* and the Mediterranean mussel *Mytilus galloprovincialis*. Both of these species inhabit the mid-intertidal region and thus may experience tidal air exposure for 6 hours or more. Both may also be exposed to Cu due to anthropogenic inputs, and Cu likely targets the same physiological processes for both species. However, their anatomy differs quite drastically and thus their responses to tidal stressors and toxicants may vary. For instance, *C. miniata* uses the respiratory pigment hemoglobin to efficiently transport oxygen to its tissues which may be an adaptation to tolerate oxygen fluctuations (Fontaine and Lambert, 1973; Weinrauch and Blewett, 2019), but *M. galloprovincialis* has no respiratory pigment. On the other hand, the mussel can close its valves to protect its soft tissues, but the sea cucumber cannot avoid exposing its soft body to the external environmental conditions (including presence of a toxicant). If intertidal conditions become more extreme as global and regional climates change, and distribution and survival of species are affected, decreased heterogeneity of species within intertidal communities may become an issue if some organisms (like mussels) are more tolerant to environmental fluctuations (Deutsch et al., 2015).

5. Thesis aims

Repeated cycles of waterborne toxicant exposure during high tide and emersion during low tide (in addition to other stressors like temperature fluctuations) have the potential to create a consistently stressful environment for intertidal invertebrates. Multi-stressor studies using stressors that originate from different sources (e.g., climate-driven environmental variability and anthropogenic pollution) but have the same targets (e.g., metabolic damage) are understudied in the current literature (Simmons et al., 2021). Lethal or even sublethal combined

effects of stressors on individual organisms may end up altering populations and community structures, which could reduce species richness and damage overall ecosystem health (Deutsch et al., 2015; Simmons et al., 2021).

The main goal of my thesis was to determine if exposing *C. miniata* and *M. galloprovincialis* to environmentally relevant concentrations of Cu results in physiological damage that decreases their ability to tolerate tidal emersion. This goal was accompanied by several smaller aims, namely: (1) to improve current knowledge of general *C. miniata* physiology including routine oxygen uptake rates, (2) to evaluate *C. miniata*'s response to Cu toxicity which has not yet been studied, and (3) to generally compare responses of the two study species to the same multi-stressor exposure methods to add to current knowledge of how physiology contributes to stress tolerance. I hypothesized that Cu would damage aerobic metabolism, ammonia excretion, and ionoregulation of both species and thus damage their capacity to compensate for tidal air exposure using strategies supported by these physiological processes. I also hypothesized that *M. galloprovincialis* might have a reduced (or different) response to the combination of the two stressors than *C. miniata* due to its ability to protect its soft tissues from Cu exposure by closing its valves and its established high tolerance to a broad range of environmental conditions (Han and Dong, 2020). Broadly, I anticipated that results would increase knowledge about the tolerance or sensitivity of the two species to mixed stressors in the intertidal zone, which could be extrapolated to population, community, or ecosystem-level effects. Results may be applied to future contexts because temperature, pH, salinity, and ocean levels are expected to fluctuate more dramatically in the intertidal zone due to climate change (Finke et al., 2007). Results of my thesis will increase knowledge of marine invertebrate

physiology and plasticity and allow predictions of their distribution and survival in a changing world.

Chapter 2: Copper exposure does not alter the ability of intertidal sea cucumber *Cucumaria miniata* to tolerate emersion during low tide[1]

1. Introduction

The intertidal zone along the coastlines of the world's oceans provides habitat for an abundance of species from several different phyla. These organisms are subject to extreme daily variation of oxygen availability, temperature, salinity, and pH (Crowe et al., 2000; Finke et al., 2007; Truchot and Duhamel-Jouve, 1980). In particular, oxygen availability is heavily influenced by tidal cycles of immersion/emersion which may expose organisms to air for hours during low tide. This is a source of potential stress for aquatic animals that are dependent on submersion in water for oxygen uptake. Furthermore, depletion of dissolved oxygen (DO) is also observed, especially within tidal pools that remain stagnant until refreshed by the incoming tide (Herreid, 1980). Physiological tolerance to such variable conditions in the intertidal zone is a major driver of species distribution (Deutsch et al., 2015), and since organisms typically live within their range of tolerance to potential sources of stress, intertidal animals may have evolved mechanisms to cope with air exposure and DO fluctuation (Crowe et al., 2000). For example, during emersion many intertidal invertebrates use anaerobic pathways when limited oxygen levels mean aerobic metabolism is minimized or impossible (Byrne et al., 1990; Crowe et al., 2000; Fields et al., 2014; De Zwaan and Eertman, 1996). Anaerobiosis is less energetically efficient than aerobic metabolism and can mostly only be used for metabolic maintenance or survival until conditions improve (i.e., submersion later in the tide cycle; Livingstone, 1991; Sokolova et al., 2012).

[1] In submission to Science of the Total Environment as "Copper exposure does not alter the ability of intertidal sea cucumber *Cucumaria miniata* to tolerate emersion during low tide" by Hannah M. Lowes, Alyssa M. Weinrauch, Ian A. Bouyoucos, Robert A. Griffin, Daniels Kononovs, Daniel S. Alessi, Tamzin A. Blewett

Previous studies have also shown that marine invertebrates are unable to excrete ammonia when exposed to air, potentially causing a toxic buildup of this nitrogenous waste product which must be tolerated until it can be rapidly excreted upon re-immersion in water (Durand and Regnault, 1998). While air exposure during the tide cycle introduces obstacles to physiological processes, natural tidal stressors are not the only challenges faced by intertidal organisms, as coastal regions are also influenced by anthropogenic activity.

Countless sources of stress have been introduced to aquatic animals by human activities, a significant one being exposure to toxicants such as metals. Copper (Cu) is common in coastal areas due to its prevalence in agricultural, industrial, and urban runoff (Crowe et al., 2000). Cu is found in pesticides, effluent from mines, antifouling coatings on boats, and wear of automobile brake pads, all of which can end up in marine waters. Cu concentrations in seawater typically range from 1 - 25 μg/L but may be higher in intertidal regions due to their proximity to anthropogenic inputs (B.C. Ministry of Environment and Climate Change Strategy, 2019). While low concentrations of Cu are essential for a number of physiological processes, most notably for oxidative phosphorylation because it is the metal cofactor in mitochondrial cytochrome *c* oxidase, high concentrations are toxic to aquatic life (Crowe et al., 2000; Grosell, 2011). In the aquatic environment the bioavailability, and thus toxicity of Cu has the potential to change due to complexation with organic and inorganic species. These complexes reduce the concentration of the most bioavailable form of Cu, the divalent cation Cu^{2+} (Grosell, 2011). In general, some divalent Cu^{2+} is reduced to monovalent Cu^{+} at biological membranes such as the gills, and both ionic forms of Cu can cross these surfaces by mimicking major cations including sodium (Na^{+}) and calcium (Ca^{2+}) and using their respective ion channels (Grosell, 2011). Some Cu bioaccumulates in respiratory tissue and exerts toxic effects there, such as binding proteins like

Na/K ATPase or Ca ATPase, decreasing their activity and damaging osmoregulation (Boyle et al., 2013; Viarengo et al., 1996). However, Cu is also transported around the body via the blood and unloaded at a variety of tissues including the liver, muscle, and reproductive tissue (Deb and Fukushima, 1999). Accumulation of essential trace metals like Cu at these sites does not necessarily result in toxicity; rather, toxicity occurs when uptake rates overcome an animal's capacity to detoxify and excrete the metal, resulting in excess Cu (Rainbow, 2007). Excess Cu undergoes oxidation-reduction (redox) cycling, produces reactive oxygen species (ROS), and causes oxidative damage to DNA and tissues (Grosell, 2011). It can also bind and inhibit proteins like carbonic anhydrase (CA), which is essential for ion transport, gas exchange, pH balance, and ammonia excretion (De Polo and Scrimshaw, 2011; Grosell, 2011). Cu-induced oxidative stress and protein interference result in damage to a broad range of processes including respiration, metabolism, ammonia excretion, acid-base balance, and osmoregulation (Bielmyer et al., 2005; Cheung and Cheung, 1995; Giacomin et al., 2014; Li et al., 2016; Magesky and Pelletier, 2018; Zimmer et al., 2012). Specifically, Cu has been found to depress metabolic rates, induce anaerobic metabolism, and inhibit ammonia excretion of marine invertebrates, and these effects may be lethal at environmentally relevant Cu concentrations (Cheung and Cheung, 1995; Giacomin et al., 2014; Grosell et al., 2007; Holan et al., 2016; Li et al., 2016). Thus, Cu is a potent environmental toxicant with the potential to damage individual organisms, populations, and ecosystems.

Multiple natural and anthropogenic stressors are present in variable environments like the intertidal zone, and effects of combined stressors are typically additive or synergistic (Crain et al., 2008). Variations in environmental conditions such as salinity, pH, and temperature can alter Cu toxicity, and interactions of these stressors and Cu exposure on marine invertebrates have

been studied, but the combined effects of air exposure and Cu toxicity have not yet been examined (Grosell et al., 2007; Lee et al., 2010; Wilson-McNeal et al., 2020). Both tidal air exposure and Cu toxicity can alter physiological processes like aerobic metabolism and ammonia excretion, so it is vital to study how the two stressors might interact to affect intertidal organisms. For these animals, which alter metabolic strategies during tidal air exposure but are also vulnerable to exposure to anthropogenic toxicants, Cu exposure has the potential to inhibit their ability to cope with emersion.

One such organism that might be impacted by these combined effects is the orange sea cucumber, *Cucumaria miniata. C. miniata* is an intertidal holothurian which wedges into crevices between rocks and suspension feeds by extending oral tentacles into the water column (Kozloff, 1993). Sea cucumbers are valuable members of marine food webs and are important nutrient cyclers in the intertidal zone (Eriksson et al., 2012; Weinrauch and Blewett, 2019). They use a pair of elaborately branched internal structures called respiratory trees coupled with the respiratory pigment hemoglobin to efficiently take up oxygen from water and transport it around the body, but during prolonged air exposure respiratory organs may collapse and become essentially non-functional for aerobic metabolism in spite of air's high oxygen content (Astall and Jones, 1991; Cook et al., 2015; Fontaine and Lambert, 1973; Huo et al., 2018; Lambert, 1997; Weinrauch and Blewett, 2019). In these situations, sea cucumbers may take up minimal oxygen across the integument, but their thick skin is less conducive to gas exchange (Astall and Jones, 1991). Thus, it has been hypothesized that the animals may switch to less energetically efficient anaerobic pathways to maintain metabolic requirements and survive under stress (Livingstone, 1991; Weinrauch and Blewett, 2019). Anaerobic metabolism results in production of lactate in tissues such as muscles and respiratory organs and may trigger breakdown of

glycogen to glucose to fuel metabolism (Carlsson and Gäde, 1981; Ellington and Hammon, 1977; Hernández-Palomares et al., 2018; Livingstone, 1991; Smith and Lawrence, 1987; Weinrauch and Blewett, 2019). Since tidal air exposure can trigger anaerobiosis, it was hypothesized by Weinrauch and Blewett (2019) that sea cucumbers like *C. miniata* would accumulate lactate and glucose after emersion. However, after 6 h of emersion, *C. miniata* produced minimal lactate and maintained regular glucose levels, indicating aerobic capacity was not heavily impacted during emersion (Weinrauch and Blewett, 2019). The authors suggested that *C. miniata*'s ability to maintain aerobic pathways during emersion reflect the animal's high tolerance to its extremely variable intertidal habitat (Weinrauch and Blewett, 2019). While *C. miniata* is tolerant to emersion, the combined stress of adding an anthropogenic toxicant like Cu could change its relationship to air exposure, particularly because both emersion and Cu toxicity affect physiological processes like metabolism and ammonia excretion in other animals (Cheung and Cheung, 1995; Grosell et al., 2007; Li et al., 2016). Effects of Cu exposure on *C. miniata* have not been evaluated, the metabolic rate of *C. miniata* before and after air exposure has not yet been examined, and it remains to be studied whether the addition of anthropogenic toxicants like Cu will alter *C. miniata*'s response to tidal air exposure.

The main objective of the current study was to examine physiological responses of *C. miniata* to a combination of tidal emersion and Cu exposure. Individual sea cucumbers were subjected to Cu exposure for 96 hours, followed by 6 hours of air exposure, after which whole-animal oxygen uptake rates ($\dot{M}O_2$) and ammonia excretion rates (AER) were assessed. Tissue samples were collected to evaluate the tissue-specific distribution of Cu. Lactate measurements in the introvert retractor muscle (which pulls the feeding tentacles and pharynx into the body cavity) and respiratory tree were taken as a proxy for anaerobic metabolism (Berg et al., 2002).

These measures provide insight into which energetic pathways the sea cucumbers may be using under conditions of submersion/emersion and with/without Cu exposure, and if any damage to regular physiological processes has occurred. While holothurians are typically osmoconformers, under some conditions marine invertebrates have been observed to regulate ions in the hemolymph, so we also measured concentrations of major ions in their coelomic fluid to determine if any osmoregulatory processes were affected by Cu toxicity (Geng et al., 2016; Jorge et al., 2016). Overall, we hypothesized that Cu accumulation would negatively affect the ability of *C. miniata* to tolerate tidal emersion, and that this would be evident in measures of $\dot{M}O_2$, AER, and lactate. We predicted that the combination of the two stressors to have additive effects and result in decreased $\dot{M}O_2$ and AER compared to either of the individual stressors. We also anticipated that Cu toxicity and air exposure would result in lactate production in the muscle and respiratory tree of *C. miniata*, reflecting a switch to anaerobic metabolism under these conditions. Lastly, we predicted that Cu exposure might alter osmoregulation of the coelomic fluid, resulting in changes of concentrations of major ions sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg). Results of this study will advance knowledge of *C. miniata* physiology and provide insight into how multiple stressors can impact marine invertebrates.

2. Methods

2.1. Animal care

All procedures were approved by the Bamfield Animal Research Ethics Board (Animal Use Protocol RS-21-02) and followed the Guidelines of the Canadian Council on Animal Care. 60 orange sea cucumbers (*C. miniata)* with mass 107 ± 5.8 g (all values presented are means ± standard error of the mean, S.E.M., unless otherwise noted) were obtained by SCUBA from the intertidal zone of Ross Islets, British Columbia (B.C.), Canada, in early June 2021 under

Department of Fisheries and Oceans Canada capture permit XR 176 2021. Animals were transported in aerated water tanks to Bamfield Marine Sciences Centre (Bamfield, B.C.) and then held in 200 L tanks maintained with flow-through sea water under constant aeration and a natural light cycle (~14 h light: 10 h dark), with a mean temperature of 12.0 °C and salinity of 35 ppt. Water chemistry (in mM) was previously measured as Na, 475; K, 11; Ca, 10; Mg, 47; Cl, 515 (Blewett and Goss, 2017). Animals were acclimated to these conditions and were not fed for 7 d before experimentation.

2.2. Experimental Cu and emersion exposures

Cu stock solution (1 g/L) was made by adding copper (II) sulfate pentahydrate (Sigma Aldrich, St. Louis, MO, USA) to 1 L of deionised water (ELGA) and acidifying with 0.1% trace metal grade nitric acid (HNO_3, Sigma Aldrich) to prevent the loss of Cu to the stock container and to encourage Cu^{2+} species to be dominant (John and Leventhal, 2004). Cu stock was then diluted with seawater and the following concentrations were made up in 50 L aerated containers: 45 L of pure seawater with no Cu ("control"), 45 L of 20 µg/L Cu ("low Cu"), and 45 L of 200 µg/L Cu ("high Cu"), with measured values in Table 1. All exposure containers were placed in a 200 L recirculating wet table with flow through Bamfield inlet water to keep water temperature maintained at 12.1 ± 0.11 °C. Water samples were collected from each container before and after the experiments for inorganic analysis.

Individual *C. miniata* were randomly placed in one of three Cu treatments for 96 h: control, low Cu, or high Cu water with 20 replicates per treatment (10 used for non-emersion, 10 for emersion). All exposures were conducted in 1 L aerated containers held in a recirculating water bath where the temperature was maintained at 12.1 ± 0.07 °C, and a complete water change for all replicates was performed at 48 h. After 96 h, 10 replicates of each Cu treatment

(control, low Cu, or high Cu) were air exposed for a 6 h period by placing the sea cucumbers on seawater dampened paper towel in an empty container held in the same recirculating water bath to maintain temperature. Water samples for inorganic analysis were collected from each experimental chamber upon addition of the sea cucumbers and again at the end of the 96-h exposure period, with results found in Table 1.

2.3. Water chemistry analysis

Water samples from treatment stock containers and sea cucumber exposure chambers 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. Concentrations of Cu and other inorganics were then measured using inductively coupled plasma-mass spectrometry (ICP-MS/MS). Samples with concentrations below the Cu detection limit of 5.11 µg/L are reported as B.D.L. (below detectable limits).

2.4. Cu accumulation and inorganic analysis

Tissues (body wall, introvert retractor muscle, respiratory tree) and coelomic fluid samples were prepared for inorganic analysis by digesting in 2 N HNO_3 (Sigma Aldrich) and incubating for 48 hours at 65 °C in 20 mL plastic scintillation vials. All samples were vortexed 24 hours into the incubation. The digested samples were then diluted to ~2300 ppm total dissolved solids (TDS) using a solution of 2% HNO3 and 0.5% HCl, and concentrations of Cu and other inorganics (Na, K, Ca, Mg) were measured using ICP-MS/MS.

2.5. Oxygen uptake rates

Following treatment (i.e., exposure to Cu and/or emersion), sea cucumbers were gently transferred to 1.2 L hermetically sealed containers containing fresh seawater. Routine oxygen

uptake rates ($\dot{M}O_2$) were measured as proxies for routine metabolic rate (RMR) using a closed respirometry protocol over a single one-hour period with constant vigorous mixing. One hour of closed respirometry was sufficient for individuals to reduce air saturation within respirometers by > 10% (Svendsen et al., 2016). Oxygen concentration was recorded using robust fibre-optic oxygen probes on a Witrox 4-channel oxygen meter and Autoresp software (Loligo Systems; Tjele, Denmark). Rates of oxygen decline with coefficients of determination (R^2) greater than 0.9 were extracted for the last 50 minutes of measurement using the 'calc_rate()' function in the R package, 'respR' (Harianto et al., 2019; R Core Team, 2020). Background respiration rate was negligible over 2-h periods (Weinrauch and Blewett, 2019) and containers were rinsed with HNO_3, bleach, and clean seawater between sea cucumbers, so background was not accounted for in this study. Oxygen uptake rate was calculated as:

$$\dot{M}O_2 = \frac{\Delta SO_2 \times V}{m \times \Delta t}$$

where, ΔSO_2 is the change in % air saturation, V is the total volume of the respirometer (minus the organism mass) in L, Δt is the 50-minute time period over which uptake was measured (h), m is the mass of the organism (kg). Values were mass corrected using the metabolic scaling exponent of 0.81 from Hughes et al. (2011).

2.6. Ammonia excretion rates

Water ammonia was determined using the salicylate hypochlorite methods of Verdouw et al. (1978) in duplicate using clear, flat bottom 96-well plates. Briefly, 160 µL of sample was combined with 40 µL each of sodium salicylate (0.25 M), sodium nitroprusside (0.76 mM) and sodium citrate (1.4 M prepared in 1 N NaOH combined at a 1:2 ratio with 6% bleach immediately before use). Samples were incubated at room temperature in the dark for 60 min

prior to reading at A595 nm and compared to a standard curve of prepared ammonium chloride. Ammonia excretion rate (AER; μmol/kg/h) was determined using the following equation:

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

where, Amm_f and Amm_i are the final and initial ammonia concentrations (μM), respectively, V represents container volume (L), m represents animal mass (kg) and t represents flux time period (h). Of note, Amm_i values were below the detectable limits of the assay and as such, were set as zero for these calculations. The ammonia quotient (amount of ammonia excreted to oxygen consumed) was also calculated by dividing each AER by the unscaled $\dot{M}O_2$.

2.7. Tissue lactate analysis

L(+)-lactate concentrations in *C. miniata* introvert retractor muscle and respiratory tree samples were determined using a lactate assay kit according to manufacturer instructions (Sigma Aldrich). Samples were ground to a fine powder on liquid nitrogen using a mortar and pestle, homogenized in 1 M perchloric acid, and centrifuged at 13000 x *g* for 2 minutes. The supernatant was then adjusted to pH 7-8 using 2 M KOH and centrifuged at 13000 x *g* for 10 minutes. Sample absorbances were measured in triplicate at 570 nm in a 96-well clear bottom plate using SpectraMax M2 and SoftMax Pro (v. 7.1). Lactate concentrations were calculated from an L(+)-lactic acid standard curve using R (v. 4.2.0).

2.8. Data presentation and analyses

Statistical analyses were performed using R (v. 4.2.0), and packages 'rstatix' and 'multcompView' were used to check parametric assumptions and conduct all tests unless otherwise stated (Graves et al., 2019; Kassambara, 2021). For all analyses an alpha value of 0.05 was considered significant. For analysis of Cu accumulation in the coelomic fluid and body

tissues, none of the datasets met normality assumptions for parametric analyses, so data were square root transformed to undergo parametric analysis. Cu concentrations were then modelled as a function of the interaction of group (non-emersed or emersed) and treatment (control, low Cu, or high Cu) using two-way analysis of variance (ANOVA). Pairwise *post hoc* comparisons for each dataset were performed using Tukey's honest significant difference (HSD) tests.

Owing to missing samples, oxygen uptake rates and ammonia quotients were not available for the non-emersed high Cu treatment group. Therefore, oxygen uptake rates and ammonia quotients were analysed in two ways: 1) testing for an effect of low or no Cu exposure between control and emersion groups; 2) testing for an effect of Cu (none, low, or high) only within the emersion groups. For analysis of oxygen uptake rates and ammonia quotients, response variables were modelled as a function of the interaction of treatment (i.e., non-emersion or emersion) and Cu exposure (i.e., none or low) using least squares regression. A constant variance structure was applied to the interaction of treatment and exposure – using the 'varIdent()' weight in the 'nlme' R package (Pinheiro et al., 2018) – because of heterogeneous variances between experimental groups. For analysis of oxygen uptake rates, mass corrected RMR of animals in the emersion treatment only was modelled as a function of Cu exposure (i.e., none, low, or high) using least squares regression with a constant variance structure applied to the Cu exposure variable. Ammonia quotients did not meet the assumptions of parametric analyses for analysis 2 and, as such, ammonia quotient was modelled as a function of Cu exposure (i.e., none, low, or high) for animals within the emersion treatment only using a Kruskal-Wallis test. Ammonia excretion rates met the assumptions for Gaussian linear models; therefore, ammonia excretion rate was modelled as a function of the interaction of treatment (i.e., control or emersion) and Cu exposure (i.e., none, low, or high), with mass (in kg) as a covariate

using ANOVA. For these tests, pairwise *post hoc* comparisons were made with the 'lsmeans' R package (Lenth, 2016).

Lactate levels in *C. miniata* muscle and respiratory tree were square root transformed upon failing the normality assumption for parametric analysis. Lactate concentrations were then modelled for each tissue as functions of the interaction of group and treatment using two-way ANOVA. Tukey's HSD *post hoc* tests were performed to determine pairwise differences.

For analysis of ion concentrations (Na, K, Ca, and Mg) in coelomic fluid, all datasets passed normality and homoscedasticity assumptions except K, which was log-transformed to meet these assumptions for parametric analysis. Concentrations of these inorganics were then modelled as a function of group and treatment with two-way ANOVA.

3. Results

3.1. Water chemistry

Measured Cu concentrations were found to be roughly equivalent to the nominal values in the samples taken at the beginning of the exposure period (Table 1). All experimental and stock controls had Cu levels below the detection limit of 5.11 µg/L. The non-emersion and emersion low Cu were initially measured at 24.00 ± 12.22 and 25.35 ± 12.93 µg/L, respectively, and the non-emersion and emersion high Cu were initially measured at 222.1 ± 6.22 and 227.0 ± 8.97 µg/L. Following the 96-h exposure period, Cu levels were about half the initial measurements in both non-emersion and emersion high Cu treatments and were much lower to the point of being undetected in both groups of low Cu treatments (Table 1).

3.2. Cu accumulation

The bioaccumulation of Cu in *C. miniata* coelomic fluid was significantly affected by group ($F_{(1,54)} = 76.025$, $p < 0.001$), treatment ($F_{(2,54)} = 316.497$, $p < 0.001$), and interaction of group and treatment ($F_{(2,54)} = 80.599$, $p < 0.001$). Coelomic fluid Cu bioaccumulation showed a significant increase from non-emersion and emersion control values of 0.30 ± 0.06 and 0.31 ± 0.05 mg/L, respectively, to 1.32 ± 0.01 and 1.52 ± 0.04 mg/L in the non-emersed low and high Cu exposures, but in the emersion group Cu only significantly increased to 1.53 ± 0.04 mg/L in the high Cu treatment (Figure 2.1A).

In the introvert retractor muscle, Cu accumulation was dependent on group ($F_{(1,50)} = 19.274$, $p < 0.001$), treatment ($F_{(2,50)} = 28.606$, $p < 0.001$), and interaction of the two factors ($F_{(2,50)} = 9.364$, $p < 0.001$). Muscle [Cu] was about 4-fold higher (though with higher variability) at 13.25 ± 1.399 mg/kg in the non-emersion high Cu treatment than all other treatments which did not differ from each other (Figure 2.1B).

Accumulation of Cu in the body wall was also significantly affected by group ($F_{(1,48)} = 30.924$, $p < 0.001$), treatment ($F_{(2,48)} = 26.440$, $p < 0.001$), and interactions ($F_{(2,48)} = 16.937$, $p < 0.001$), and followed a trend similar to that of the coelomic fluid, with highest Cu levels in both high Cu treatments (non-emersion, 6.37 ± 1.00 mg/kg; emersion, 5.93 ± 0.54 mg/kg; Figure 2.1C). The emersion low Cu body wall had the lowest Cu accumulation of all treatments at 0.74 ± 0.11 mg/kg, but it did not significantly differ from the emersion control.

Finally, the respiratory tree of the non-emersion high Cu treatment showed nearly 3-fold higher Cu accumulation (21.0 ± 3.13 mg/kg) than in the control and low Cu treatments (7.89 ± 1.52 and 8.34 ± 2.18 mg/kg, respectively) with very high variation (from ~5 to 40 mg/kg Cu) between individuals (Figure 2.1D). Group ($F_{(1,51)} = 3.694$, $p = 0.049$) and treatment ($F_{(2,51)} =$

15.497, $p < 0.001$) had significant effects on Cu accumulation in the respiratory tree, but the interaction of group and treatment did not ($F_{(2,51)} = 0.751$, $p = 0.477$).

3.3. Oxygen uptake and ammonia excretion

Oxygen uptake rate ($\dot{M}O_2$) was significantly higher and more variable in emersed sea cucumbers than in non-emersed ($F_{(1,22)} = 6.290$, $p = 0.020$), but neither Cu treatment ($F_{(1,22)} = 6.290$, $p = 0.113$) nor interaction of factors ($F_{(1,22)} = 0.074$, $p = 0.788$) affected $\dot{M}O_2$ (Figure 2.2A). Within the emersion group, there were no significant $\dot{M}O_2$ differences between control, low Cu, and high Cu treatments ($F_{(2,18)} = 0.456$, $p = 0.641$), but there was a slight trend toward decreased $\dot{M}O_2$ from control 866.8 ± 202.5 to 764.7 ± 137.6 μmol/kg$^{0.81}$/h with decreased variation in the highest Cu treatment (Figure 2.2A).

Overall ammonia excretion rate (AER) was significantly higher and more variable in emersed than in non-emersed sea cucumbers regardless of treatment ($F_{(1,40)} = 6.705$, $p = 0.013$; Figure 2.2B). Cu treatment also had a significant effect on AER ($F_{(2,40)} = 9.659$, $p < 0.001$), but the interaction of group and treatment was insignificant ($F_{(2,40)} = 1.655$, $p = 0.204$). Both non-emersion and emersion high Cu treatments had significantly lower AER (301.3 ± 57.28 and 293.3 ± 74.26 μmol/kg/h, respectively) than control or low Cu treatments (non-emersion, 497 ± 96.05 and 434.2 ± 73.50 μmol/kg/h; emersion, 740.1 ± 150.3 and 833.9 ± 72.62 μmol/kg/h), and the control and lower Cu treatments did not differ from each other.

The ammonia quotient (AQ) was not different between the non-emersed and emersed sea cucumbers ($F_{(1,21)} = 2.266$, $p = 0.147$), and neither treatment ($F_{(2,21)} = 0.418$, $p = 0.525$) nor interactions of group and treatment ($F_{(2,21)} = 0.164$, $p = 0.690$) affected the non-emersion control or low Cu AQs of 0.92 ± 0.26 and 0.68 ± 0.17, respectively (Figure 2.2). Within the emersion group, there were no significant differences in AQ between treatments ($\chi^2 = 4.788$, df = 2, $p =$

0.091). However, there is a trend towards a lower ammonia quotient in the high Cu treatment, which at 0.44 ± 0.09 was less than half the quotient of the control (1.21 ± 0.43) or low Cu (1.23 ± 0.36) treatments.

3.4. Lactate

While lactate concentration in the introvert retractor muscles of emersed sea cucumbers exposed to high Cu appeared to be 6-fold higher at 57.3 ± 40.8 μg/g than all other treatments (which ranged from emersion low Cu 3.76 ± 1.09 μg/g to non-emersion high Cu 9.96 ± 5.36 μg/g), there was no effect of group ($F_{(1,30)} = 1.140$, $p = 0.294$), treatment ($F_{(2,30)} = 2.447$, $p = 0.104$), or interactions ($F_{(2,30)} = 1.375$, $p = 0.268$) on muscle lactate (Figure 2.3A).

Emersion had no effect on respiratory tree lactate concentrations ($F_{(1,30)} = 1.135$, $p = 0.295$), but treatment ($F_{(2,30)} = 26.916$, $p < 0.001$) and interactions ($F_{(2,30)} = 3.669$, $p = 0.038$) had significant effects. High Cu significantly increased lactate concentrations in the respiratory tree to 12.5 ± 6.82 μg/g (non-emersion) and 27.2 ± 7.20 μg/g (emersion), but the non-emersion group was not significantly different from the controls or low Cu treatments which all contained < 1 μg/g lactate (Figure 2.3B).

3.5. Coelomic fluid cation levels

In *C. miniata* coelomic fluid, the concentration of Na was not affected by group ($F_{(1,54)} = 0.400$, $p = 0.530$), treatment ($F_{(2,54)} = 2.253$, $p = 0.115$), or interactions ($F_{(2,54)} = 0.529$, $p = 0.592$; Figure 2.4A). K in the coelomic fluid was independent of group ($F_{(1,54)} = 0.002$, $p = 0.969$), treatment ($F_{(2,54)} = 1.717$, $p = 0.189$), and interactions ($F_{(2,54)} = 0.421$, $p = 0.659$; Figure 2.4B). Ca was also independent of all factors (group: $F_{(1,54)} = 0.398$, $p = 0.531$; treatment: $F_{(2,54)} = 0.121$, $p = 0.887$, interactions: $F_{(2,54)} = 0.874$, $p = 0.423$; Figure 2.4C). Mg followed the same

trend as the other ions, and no effects of group ($F_{(1,54)} = 2.882$, $p = 0.095$), treatment ($F_{(2,54)} = 1.001$, $p = 0.374$), or interactions ($F_{(2,54)} = 1.367$, $p = 0.264$) were found (Figure 2.4D).

4. Discussion

While *C. miniata* bioaccumulated Cu in the coelomic fluid, introvert retractor muscle, body wall, and respiratory tree (Figure 2.1), this did not result in major effects on $\dot{M}O_2$ and AER, and neither additive nor synergistic effects of Cu toxicity and air exposure were observed. Results did not support our predictions as oxygen uptake and AER did not decrease to the greatest extent following exposure to the combined stressors (Figure 2.2), and lactate analysis suggested that high Cu exposure triggered some anaerobiosis, but air exposure had no significant effect on lactate production (Figure 2.3). Lastly, osmoregulatory disturbances by individual or multiple stressors in this study were not observed in measures of coelomic fluid ion concentrations (Figure 2.4). Overall, results of this study show that while Cu can cause damage to physiological processes, exposure to environmentally relevant concentrations of Cu likely does not alter *C. miniata*'s strategies to cope with air exposure.

4.1. Water chemistry

Both the control and high Cu stocks displayed steady concentrations over the course of 96 hours (Table 1). Of note, the low Cu stock initially had B.D.L. Cu levels, but this is likely due to an issue in water mixing since the samples taken from the low Cu treatment containers had expected Cu levels and these were filled directly from the low Cu stock. The final 96 h measurement of Cu in both non-emersion and emersion exposures was lower than the initial time zero exposure concentration, likely due to *C. miniata* accumulating Cu from the water. Indeed, bioaccumulation was measured and showed a significant increase in Cu accumulation in Cu conditions (Figure 2.1).

4.2. Cu accumulation

Results of this study show that Cu accumulated to the highest concentrations in the respiratory tree compared to the other tissue and fluid samples (Figure 2.1). This was expected because dissolved Cu tends to accumulate most at the site(s) of uptake, and the main route of soluble Cu uptake in aquatic animals is across the respiratory surface which is used for gas, ion, and waste exchange (Grosell, 2011; Wood, 2011). In *C. miniata*, this organ is the respiratory tree, which is analogous to the gills in fish and other invertebrate species with some differences; for example, fish gills are externally exposed to the environment but the respiratory tree is situated inside the body cavity of holothurians (Astall and Jones, 1991; Wood, 2011). While the respiratory tree in *C. miniata* is internal, the exchange of gas and ions between seawater and the coelomic fluid of the internal body cavity is constant because water is constantly pumped in and out of the organ by the cloaca, thus this organ is directly exposed to waterborne Cu (Dolmatov et al., 2011). Fish gills contain numerous exchangers and channels for ions including Na and Ca, and Cu mimics these ions so it can be transported across the epithelia and accumulate to high concentrations within these tissues (Grosell, 2011). Since respiratory trees have many similarities to gills (Geng et al., 2016), Cu may also enter holothurian respiratory tissue by taking advantage of ion channels with ion mimicry. Fish gills also contain apical metal transporters including the divalent metal transporter DMT1 which brings environmental Cu into the gill epithelial cells, so it is possible that similar transporters exist in invertebrate respiratory tissue (Bury et al., 2003; Grosell, 2011). It has also been hypothesized that dissolved Cu can diffuse across gill epithelium via paracellular pathways, but this has yet to be studied in marine invertebrates like *C. miniata* (Wood, 2011). Thus, there are several potential pathways of Cu uptake into the respiratory tree,

all of which could lead to high bioaccumulation of Cu in this organ, as seen in this study (Figure 2.1D).

The body wall of *C. miniata* also accumulated Cu, though to lower concentrations than the respiratory tree (Figure 2.1C). This tissue consists of an outer integumentary layer which is constantly exposed to the environmental milieu, and the body wall is otherwise extremely thick and tough as it is composed of mutable collagenous tissue and muscle used for locomotion (Mo et al., 2016; Wang et al., 2020). The thickness of the body wall makes it a considerable barrier to external contaminants like Cu, but the invertebrate integument has been proposed as a secondary site of ion, amino acid, and organic compound exchange (Gomme, 2001). These roles are similar to those of fish gills, which are known to accumulate metals via ionic mimicry, so it is possible that *C. miniata*'s integument allowed accumulation of Cu in the body wall (Blewett and Leonard, 2017). Cu was found to passively adsorb to the body surface of the shrimp *Palaemon elegans* without uptake across the epithelia, which also could have occurred in this study (White and Rainbow, 1982). However, since Cu accumulated less in the body wall than in the respiratory tree, transport across the integument or adsorption to the body wall are likely minor sources of whole-body Cu accumulation for *C. miniata* (Figure 2.1C).

The sea cucumbers' coelomic fluid also accumulated Cu, which was expected because the circulatory fluid is often one of the highest sites of metal accumulation after the respiratory tissue (Figure 2.1A; Deb and Fukushima, 1999). Once Cu crosses epithelial barriers, it is transported (possibly via an ortholog to the basolateral Cu-ATPase found in sea bream *Sparus aurata*) to the circulatory fluid (Grosell, 2011; Minghetti et al., 2010). Here, Cu binds hemocytes which are found in invertebrates including *C. miniata* and is transported around the body (Deb and Fukushima, 1999; Fontaine and Lambert, 1973). Circulating hemocytes end up delivering

some Cu to internal tissues such as liver or muscle; indeed, in this study, Cu accumulation was also observed in the introvert retractor muscle (Figure 2.1B; Deb and Fukushima, 1999). In general, Cu accumulated in all tissues and coelomic fluid in this study, and observed differences in accumulation patterns between groups are attributable to the 6 h emersion period.

Two potential effects of emersion on Cu accumulation are suggested from this study: (1) 6 hours of emersion may be long enough for some tissue-specific detoxification, transport, and excretion of the metal, but (2) both Cu exposure and emersion may alter animals' metabolic strategies and thus decrease available energy stores for these processes (which requires further validation in lab research; Rainbow et al., 1990; Sokolova et al., 2012). Detoxification is a process where Cu is sequestered by proteins including metallothioneins, which prevent the metal from causing damage (Amiard et al., 2006; Telahigue et al., 2018). Cu may then be stored in lysosomes or insoluble granules and excreted from respiratory or gut tissue (Viarengo and Nott, 1992); for example, formation and excretion of metal-containing insoluble granules has been observed in gastropod gills (e.g., *Littorina littorea*; Mason and Nott, 1981) and bivalve kidneys (e.g., *Mytilus edulis*; George and Pirie, 1980). Cu concentrations may temporarily increase in a tissue-dependent manner after uptake during the time between detoxification/storage and excretion, and then levels decrease after excretion (Rainbow et al., 1990). This pattern was found in the low Cu treatment, where Cu accumulation in both coelomic fluid and body wall was higher in non-emersed sea cucumbers than in those emersed (Figure 2.1), suggesting that the emersion period was long enough to allow for some removal of Cu from fluid and tissues to occur. This was also observed in the muscle of *C. miniata* exposed to high Cu. On the other hand, among the sea cucumbers exposed to high Cu, there was no difference in Cu accumulation in coelomic fluid, body wall, or respiratory tree between non-emersed and emersed groups

(Figure 2.1). This could be a result of diminished excretion caused by Cu-and emersion-induced metabolic alterations leading to low available energy for excretory processes (Sokolova et al., 2012). Excretion of Cu can be energetically expensive, particularly when uptake rates are high due to high exposure levels (i.e., ~200 µg/L Cu in this study; Rainbow et al., 1990; Sokolova et al., 2012). Although *C. miniata* have been found to be metabolically tolerant to emersion (Weinrauch and Blewett, 2019), results of this study suggest that the combination of energetic stresses caused by both emersion and Cu exposure may allow metal accumulation in this species by decreasing their capacity to excrete the metal.

Overall, while it appears that *C. miniata* exposed to low Cu only accumulated Cu in some tissues and were able to maintain metal excretory function after the emersion period, cucumbers exposed to high Cu accumulated the metal in all tissues and after emersion they may have been unable to regulate internal Cu levels using excretion. However, this hypothesis would need to be confirmed by measuring actual Cu excretion rates. In this study sea cucumbers were only exposed to Cu for 96 h, but in the environment, animals may be chronically exposed and experience repeated periods of tidal emersion. Accumulation patterns may vary in more realistic scenarios which should be studied for further understanding. As previously mentioned, Cu toxicity only occurs when uptake rates overwhelm detoxification and excretion, so observed accumulation patterns may provide valuable information about where toxicity may be occurring (Rainbow, 2007). However, physiological parameters must also be measured to evaluate whether observed Cu levels are within the organism's range of tolerance or are in excess and causing damage.

4.3. Oxygen uptake and ammonia excretion

Control non-emersion $\dot{M}O_2$ in this study was comparable to *C. miniata* basal $\dot{M}O_2$ previously measured by Weinrauch and Blewett (2019). Low Cu (~20 µg/L) had no effect on $\dot{M}O_2$, and within the emersed group high Cu also had no effect (Figure 2.2A). Decreased respiration is the most common effect of sublethal Cu exposure on marine invertebrates, including the sea cucumber *Apostichopus japonicus* (Li et al., 2016). This may be caused by respiratory tissue damage (e.g., Maharajan et al., 2012), increased mucus production as a stress response which increases diffusive distance for gas exchange (e.g., Scott and Major, 1972), inhibition of cellular respiration (e.g., Jorge et al., 2016), or behavioural response to toxicant exposure (e.g., closure of valves by mussels, see Sunila, 1981). However, increased $\dot{M}O_2$ after metal exposure has also been observed (e.g., Cheung and Cheung, 1995) and may be caused by increased energetic demand to upregulate heat shock proteins (HSPs) which are induced under stressful conditions to repair denatured proteins and maintain their activity (Magesky and Pelletier, 2018). Since the main organ used for oxygen uptake in holothurians is the respiratory tree, and this was also a major site of Cu uptake and accumulation, it was anticipated that *C. miniata*'s oxygen uptake (and aerobic metabolism, which requires a high oxygen load) would be affected by Cu-induced damage. Absence of effects, either positive or negative, of Cu on *C. miniata* $\dot{M}O_2$ indicate that either Cu levels or duration of exposure used in this study were insufficient to alter oxygen homeostasis. Exposure to Cu levels of 20 and 80 µg/L for 96 h respectively lowered oxygen consumption and survival of sea cucumber *Apostochopus japonicus*, so *C. miniata* appears to be more tolerant to Cu exposure than this species (Li et al., 2016).

However, emersion increased $\dot{M}O_2$ of both control and low Cu-exposed *C. miniata* in this study (Figure 2.2A), which may reflect a replenishment of tissue oxygen levels due to an "oxygen debt" acquired during air exposure upon re-immersion in water for $\dot{M}O_2$ measurements (Bayne et al., 1976). Increased $\dot{M}O_2$ upon a return to normoxia from hypoxia (termed excess post-hypoxia oxygen consumption or EPHOC) occurs in fish such as *Cyprinus carpio,* and this has also been observed in marine invertebrates (Bayne et al., 1976; Genz et al., 2013; Shick et al., 1986). Although air has much higher oxygen content than water resulting in a hyperoxic environment (Webb, 2021), emersion is often described as hypoxic or anoxic for intertidal invertebrates because most rely on submersion in water to maintain the physical integrity and function of their respiratory organs (in this case the respiratory tree; Weihrauch and Allen, 2018; Wilkie, 2002). Hypoxia causes stress for organisms because oxygen is necessary for ATP production via oxidative phosphorylation which provides the energy essential for most cellular processes (Sokolova et al., 2012). Since sea cucumbers may take up some oxygen across the integument when the respiratory tree is unable to function (Astall and Jones, 1991), emersion is likely hypoxic or even normoxic, depending on the level of oxygen uptake at the skin compared to the respiratory tree (which is efficient at gas exchange but prone to collapse during emersion; Weinrauch and Blewett, 2019). Additionally, *C. miniata* is unique among holothurians because its coelomic fluid contains the respiratory pigment hemoglobin (Fontaine and Lambert, 1973), which increases oxygen carrying capacity and has been hypothesized to be an adaptation to maintain aerobic function during environmental oxygen fluctuations (Weinrauch and Blewett, 2017). Since *C. miniata* showed a compensatory increase in $\dot{M}O_2$ upon re-immersion, emersion may be hypoxic for this species in spite of potential oxygen uptake across the integument and the presence of a respiratory pigment.

Of note, both control and low Cu-exposed emersed *C. miniata* displayed high variability in $\dot{M}O_2$ measurements, ranging from 174.5 to 1999 μmol $O_2/kg^{0.81}/h$ (Figure 2.2A). This could mean the level of oxygen uptake via the integument and thus ability to maintain regular tissue oxygen levels during emersion broadly varies between individuals, resulting in some *C. miniata* more able to withstand emersion than others. Combined effects of high Cu and emersion could not be evaluated due to missing non-emersion/high Cu replicates (some individuals plugged outflow tubes and interfered with oxygen readings), so it was not possible to determine if these stressors interact to affect *C. miniata*'s oxygen uptake (Figure 2.2A).

Cu exposure decreased *C. miniata* AER in this study (Figure 2.2B), consistent with previous findings in fish and invertebrates (e.g., Blanchard and Grosell, 2006; Wilson-McNeal et al., 2020). In fact, in marine osmoconformers like sea cucumbers, impaired ammonia excretion has been proposed as the major route of Cu toxicity (Grosell et al., 2007). Cu exposure can affect ammonia excretion in three possible ways: the first by inhibiting the enzyme carbonic anhydrase (CA, which catalyzes the reversible hydration of carbon dioxide to bicarbonate and a proton (H^+) and is critical for multiple processes including acid-base balance, ammonia excretion, and respiratory gas exchange) by displacing its zinc cofactor or binding other sites (de Polo and Scrimshaw, 2012; Grosell et al., 2007; Lim et al., 2015; Lionetto et al., 2016). The second way Cu affects ammonia excretion is through Na-mimicking behaviour. Na and ammonium (NH_4^+) transport are tied in many species, so disruption of Na transport could also contribute to potential toxicity by internal accumulation of ammonia, though this is less likely in marine osmoconformers with no need to maintain Na gradients across epithelia (Hunter and Kirschner, 1986; Wilkie, 2002). Thirdly, Cu may bind and inhibit rhesus (Rh) proteins which facilitate ammonia excretion (Grosell, 2011; Lim et al., 2015; Weihrauch and Allen, 2018). CA is the most

likely target, because it is the common denominator of ammonia excretion, ion regulation, pH balance, and gas exchange, and all of these processes have been reported to be affected by Cu (Grosell et al., 2007). Any of these potential targets of Cu could have resulted in the decreased AER observed in the high Cu treatments, and low Cu in this study was not sufficient to reduce AER in *C. miniata* (Figure 2.2B).

AER increased in *C. miniata* after emersion (Figure 2.2B), which has also been observed in crabs *Carcinus maenas* and *Necora puber* (Durand and Regnault, 1998). As previously mentioned, *C. miniata* requires submersion in water to maintain physical integrity and function of the respiratory tree (which also functions in excretion), and to keep in contact with the required gradients for diffusion of ammonia and other waste products from the body into the water (Weihrauch and Allen, 2018; Wilkie, 2002). Thus, ammonia excretion is mostly halted during emersion when excretory organs may be collapsed and ammonia gradients are not present. In these situations, intertidal animals may retain ammonia until re-immersed in water, when excretory function returns and they are able to quickly excrete built up ammonia, resulting in high AER (Durand and Regnault, 1998). No interactive effects of Cu treatment and emersion on *C. miniata* AER were found, so the combination of the two stressors was neither additive nor synergistic.

While it is beneficial to examine both $\dot{M}O_2$ and AER individually, the ammonia quotient (AQ; the proportion of ammonia excreted to oxygen consumed) is also a useful factor to consider as it can provide insight into which substrates are being used to fuel the animal's aerobic metabolism (Kutty, 1972). In general, a higher AQ indicates a higher fraction of metabolism is being fuelled by protein degradation and/or anaerobic pathways, while a lower AQ can mean higher turnover of carbohydrates and lipids and/or ammonia retention (De Boeck et al., 2001;

Kombat et al., 2021; Kutty, 1972; Magesky and Pelletier, 2018; Rahmah et al., 2020; Sinha et al., 2012). Organisms under stress (like toxicant exposure or hypoxia) typically exhibit higher AQs (e.g., Li et al., 2020). In this study, neither Cu exposure nor emersion had significant effects on AQ, and the two stressors did not have any interactive effects (Figure 2.2C). However, emersion appears to have caused high variation in AQs in control and low Cu treatments (0.32 to 3.32, and 0.46 to 3.94, respectively), meaning some individuals may have been more reliant on protein catabolism and/or anaerobic metabolism than others within the same group (Figure 2.2C). High Cu-exposed *C. miniata* showed lower AQ variation from 0.25 to 0.67 (Figure 2.2C). This suggests individual *C. miniata* have highly variable metabolic responses to air exposure, but this variation may be diminished by exposure to high Cu levels. High variation of these fitness-related physiological parameters within a species is reflective of the plasticity of that species, which is essential for a population to adapt to long-term shifts in environmental conditions (i.e., climate change; Tanner and Dowd, 2019). Thus, Cu exposure may have the potential to negatively affect the population-wide ability of intertidal invertebrates like *C. miniata* to adapt to changing environmental conditions.

Overall, high $\dot{M}O_2$, increased ammonia excretion, and unchanged ammonia quotients after emersion reflect *C. miniata*'s mechanisms to tolerate air exposure. Decreased AER after exposure to high Cu reflects damage to ammonia excretion pathways, and unchanged AQs suggest that the substrates used to fuel metabolism were unaffected by Cu exposure and emersion.

4.4. Lactate

Lactate is a major product of anaerobic metabolism, so it can be measured and interpreted as a marker of the switch from aerobic to anaerobic pathways under conditions of insufficient

oxygen uptake and/or delivery around the body (Berg et al., 2002; Weinrauch and Blewett, 2019). It was previously found that *C. miniata* produced low lactate levels in the body wall across normoxic, anoxic, and hypoxic treatments, and therefore did not seem to switch to anaerobic metabolism during 6 h of emersion (Weinrauch and Blewett, 2019). This is consistent with lactate concentrations in the introvert retractor muscle and respiratory tree of both the non-emersed and emersed controls in this study (Figure 2.3). The low Cu treatment also did not induce lactate production in either group or tissue, indicating that the ~20 μg/L exposure over 96 h was insufficient to induce anaerobic metabolism. Interestingly, although lactate production in the muscle did not vary between groups or treatments, the spread of individual responses to high Cu suggests that aerobic pathways of some *C. miniata* were unaffected (low lactate production), but others may have been inhibited (high lactate production; Figure 2.3A).

However, high Cu treatment followed by emersion seemed to induce anaerobic metabolism in *C. miniata*, reflected by lactate levels of 27.2 ± 7.20 μg/g measured in the respiratory tree – significantly higher than both control and low Cu, which all had less than 5 μg lactate/g of tissue (Figure 2.3B). Both hypoxia and Cu exposure have been found to trigger lactate production due to anaerobic metabolism in marine invertebrates (e.g., Ellington and Hammen, 1977; Giacomin et al., 2014). Increased reliance on anaerobic pathways after each of these stressors is typically attributed to damage to respiratory organs which disrupts oxygen uptake (Brown and Newell, 1972; Giacomin et al., 2014), so it is possible that Cu toxicity and air exposure caused some physical damage to the respiratory tree, restricting aerobic gas exchange and forcing the sea cucumbers to use these alternative pathways (Astall and Jones, 1991; Cook et al., 2015; Huo et al., 2018). However, Cu exposure was likely the main (or only) cause of respiratory damage since lactate production did not differ between the non-emersion and

emersion groups (Figure 2.3). Another way Cu can affect aerobic metabolism is by binding and disrupting respiratory proteins such as hemocyanin or hemoglobin, which are important for oxygen storage and transport via circulatory fluid to various tissues around the body (Deb and Fukushima, 1999). If *C. miniata*'s hemoglobin was damaged by Cu exposure, oxygen transport and delivery may have been inhibited which could also lead to a facultative switch to anaerobic metabolism and increased lactate production (Deb and Fukushima, 1999; Huo et al., 2018; Ellington and Hammen, 1977). In future studies, it would be useful to examine *C. miniata*'s hemoglobin for Cu-induced damage, and further research could include conducting similar experiments on other marine invertebrates like crustaceans which utilize the Cu-based respiratory pigment hemocyanin and may be better adapted to overcoming Cu exposure.

4.5. Coelomic fluid cation levels

Cation concentrations in *C. miniata* coelomic fluid were roughly equivalent to those found in seawater and coelomic fluid of other sea cucumber species (Figure 2.4; Blewett and Goss, 2017; Castellano et al., 2018). Neither Cu exposure nor emersion influenced the concentrations of the major ions Na, K, Ca, and Mg in the coelomic fluid (Figure 2.4), reflective of a previous study on the European shore crab *Carcinus maenas* (Boitel and Truchot, 1989). In freshwater animals, the main mechanism of toxicity by Cu is by impairing osmoregulation, which can be lethal (Grosell et al., 2007). Cu impairs osmoregulation by targeting ion channels and ATPases, including Na/K, Ca/Mg, Ca, and Mg ATPases, but its effects can be complex; at low concentrations Cu may increase expression of these proteins, but at higher concentrations it can block their activity (Boyle et al., 2013; Viarengo et al., 1996). Osmoregulation is not expected to be the main target of Cu in seawater because most marine invertebrates are osmoconformers, but some have been found to regulate levels of specific ions in their lymph, so

it is possible that Cu could affect ionoregulation in these animals (Geng et al., 2016; Jorge et al., 2016). For example, Cu was found to affect internal regulation of divalent cations Ca^{2+} and Mg^{2+} in the clam *Mesodesma mactroides* in spite of it being an osmoconformer (Jorge et al., 2016). Emersion may also affect internal ion concentrations due to evaporation and water loss (e.g., Allen et al., 2021), but this was not observed in this study, likely because of the short time period of emersion. *C. miniata* were previously observed to retain internal fluids during emersion while other holothurians released fluids, possibly reflecting another mechanism of tolerance to tidal emersion that could result in maintenance of regular coelomic fluid ion levels in this species (Weinrauch and Blewett, 2019). The absence of effects of emersion or Cu exposure on coelomic fluid ion concentrations suggest that these stressors were not present for long enough (or for Cu, concentrations were not high enough) to induce damage to any osmoregulatory processes that may exist in *C. miniata*.

5. Conclusions

Overall, results of this study suggest that, while acute Cu exposure and subsequent accumulation has the potential to damage many physiological processes in *C. miniata*, it does not affect *C. miniata*'s high tolerance for periods of tidal air exposure. Cu was shown to accumulate non-specifically throughout several tissues and affect ammonia excretion. While emersed sea cucumbers utilized increased metabolic and ammonia excretion rates upon re-immersion in water, Cu exposure did not interact with hypoxia to affect these compensatory processes. Lactate analysis indicated that individuals exposed to high Cu may have increased dependence on anaerobic metabolism, but emersion had no effect on lactate production regardless of Cu exposure. These findings do not support our hypothesis that Cu exposure and accumulation would have a negative effect on *C. miniata*'s adaptive response to air exposure. However, it is

possible that further studies of chronic, longer-term exposure to Cu and repeated cycles of emersion and immersion may reveal interactive effects of Cu toxicity and tidal air exposure that could have been obscured by the short duration of this study. Also, similar studies using non-essential metals like cadmium (Cd) are recommended, since these metals are more difficult for aquatic animals including sea cucumbers to regulate and are also known to cause toxicity by impacting processes like ammonia excretion (Charan-Dixon et al., 2017). *C. miniata* or other intertidal organisms may be less tolerant to non-essential metal exposure than to Cu exposure (Depledge and Rainbow, 1990), and there may be higher potential for interactions between non-essential metals and other stressors.

Marine invertebrates are extremely diverse and abundant, and there are large gaps in knowledge about the physiology of many species including *C. miniata* and other sea cucumbers. Studying the physiological responses of such animals to both toxicants and natural environmental stressors will provide baseline knowledge that may be extrapolated to future climate scenarios. Potential additive or synergistic effects of multiple stressors are worrying, especially in the intertidal zone as this area is particularly vulnerable to both toxicant exposure and swings in natural stressors like air exposure, temperature, salinity, and pH, all of which are predicted to become more extreme due to climate change (Finke et al., 2007; Grosell et al., 2007; Holan et al., 2019; Lee et al., 2010). Since these factors dictate intertidal species distribution, investigating their combined effects will contribute to knowledge of the degree of harm caused by anthropogenic toxicant release and may inform predictions of distribution and survival of these organisms in the future. This information may also be used to impose strict limits on release of toxicants and increase remediation of contaminated sites to protect sensitive marine species, both today and in the future.

Tables and figures

Table 2.1. Measured copper (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 10 replicates per group. Concentrations below the detection limit of 5.11 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	B.D.L.	B.D.L.
	Low Cu	20	24.00 ± 12.22	B.D.L.
	High Cu	200	222.1 ± 6.22	111.1 ± 14.21
Emersion	**Control**	0	B.D.L.	B.D.L.
	Low Cu	20	25.35 ± 12.93	B.D.L.
	High Cu	200	227.0 ± 8.97	106.6 ± 13.36
Stock	**Control**	0	B.D.L.	B.D.L.
	Low Cu	20	B.D.L.	26.18 ± 26.18
	High Cu	200	236.8 ± 3.78	235.3 ± 1.61

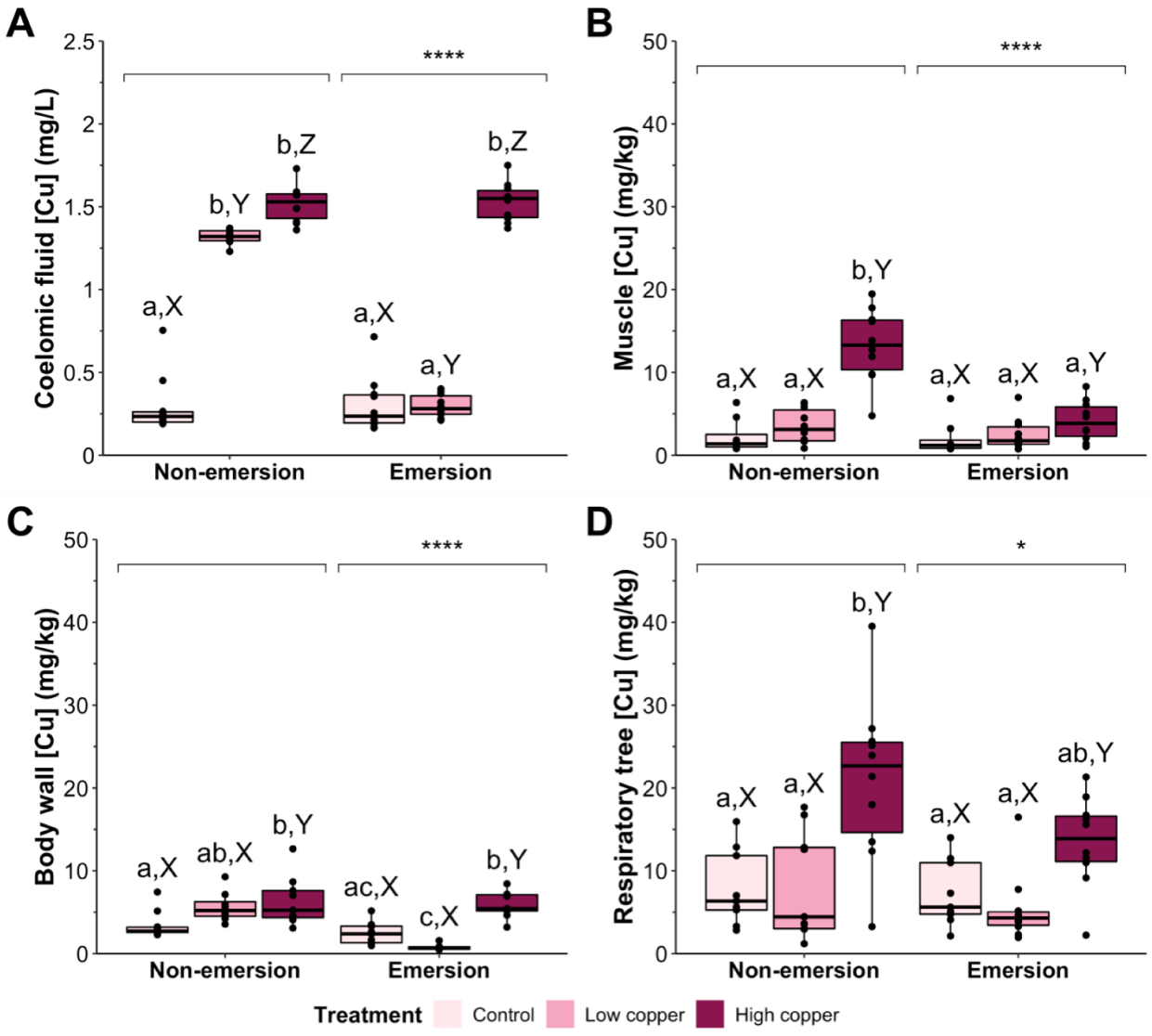

Figure 2.1. Copper (Cu) accumulation in *Cucumaria miniata* (A) coelomic fluid, (B) introvert retractor muscle, (C) body wall, and (D) respiratory tree following 96 h of control, low Cu, or high Cu exposure, and with or without 6-h of emersion after the exposure period. Individual datapoints represent Cu levels of each sea cucumber (n = 8 to 10). Horizontal lines within boxplots represent the median of 10 replicates per treatment, 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, uppercase letters denote differences between the 3 Cu treatments, and stars denote differences between the 2 emersion groups as determined with a two-way ANOVA (* denotes $p < 0.05$, and **** denotes $p < 0.0001$).

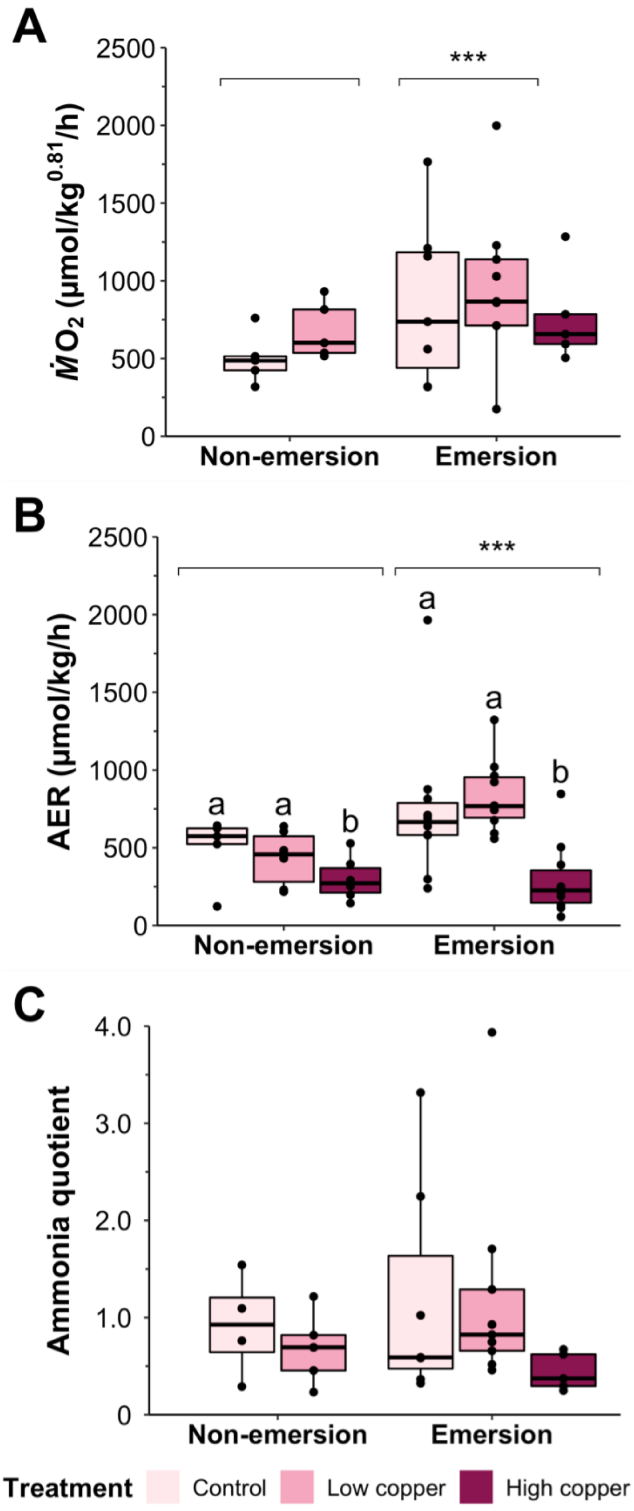

Figure 2.2. (A) Mass-scaled oxygen consumption rate ($\dot{M}O_2$), (B) ammonia excretion rate (AER), and (C) ammonia quotient (AQ) of *Cucumaria miniata* exposed to 96 h of control, low

copper (Cu), or high Cu water with or without 6 h of emersion following the exposure. Individual datapoints represent measures of each sea cucumber (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 upper and lower whiskers extend to the most extreme datapoints within Q1 – 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR. Individual datapoints outside the whiskers represent outliers. Lowercase letters denote significant differences ($p < 0.05$) between Cu treatments and stars denote differences between the 2 emersion groups (for $\dot{M}O_2$, only including control and low Cu treatments, but AER including all treatments; *** means $p < 0.001$).

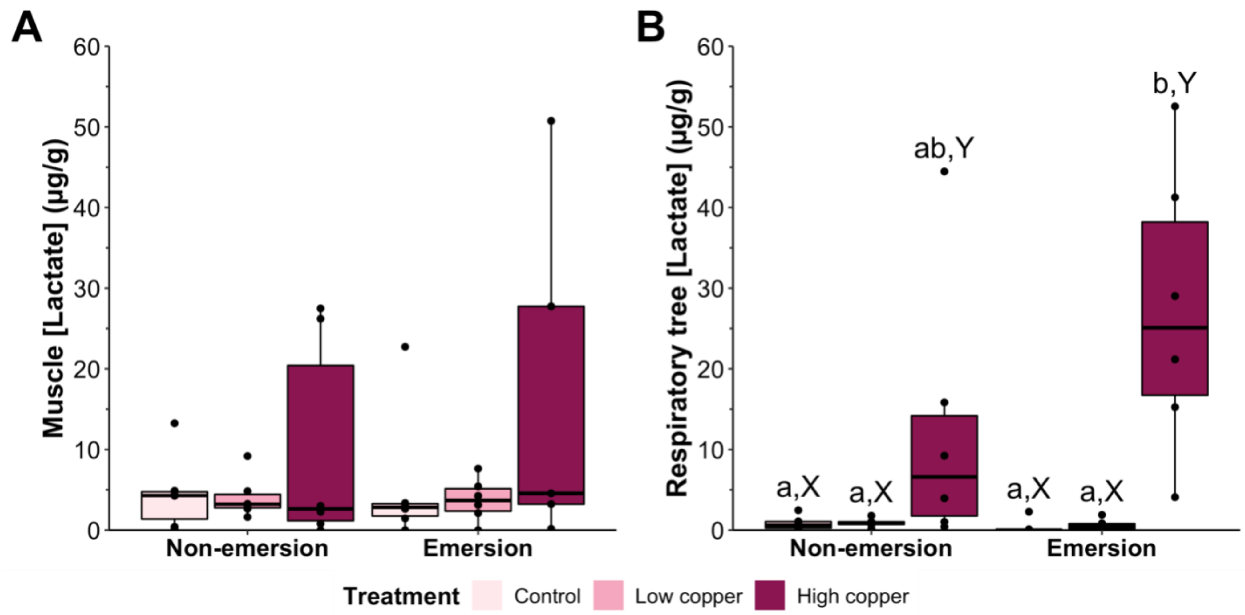

Figure 2.3. Lactate content in the (A) introvert retractor muscle and (B) respiratory tree of *Cucumaria miniata* exposed to 96 h of control, low copper (Cu), or high Cu conditions with or without 6 h of emersion from water following the exposures. Individual datapoints represent lactate levels of each sea cucumber (n = 6). Horizontal lines within boxplots represent the median, upper and lower hinges represent the 25th and 75th percentiles (Q1 and Q3), respectively, and upper and lower whiskers extend to the most extreme datapoints within Q1 – 1.5 * interquartile range (IQR) and Q3 + 1.5 * IQR. Individual datapoints outside the whiskers are outliers. Lowercase letters represent significant differences between all 6 group/treatments, and uppercase letters represent differences between the 3 Cu treatments determined with a two-way ANOVA ($p < 0.05$).

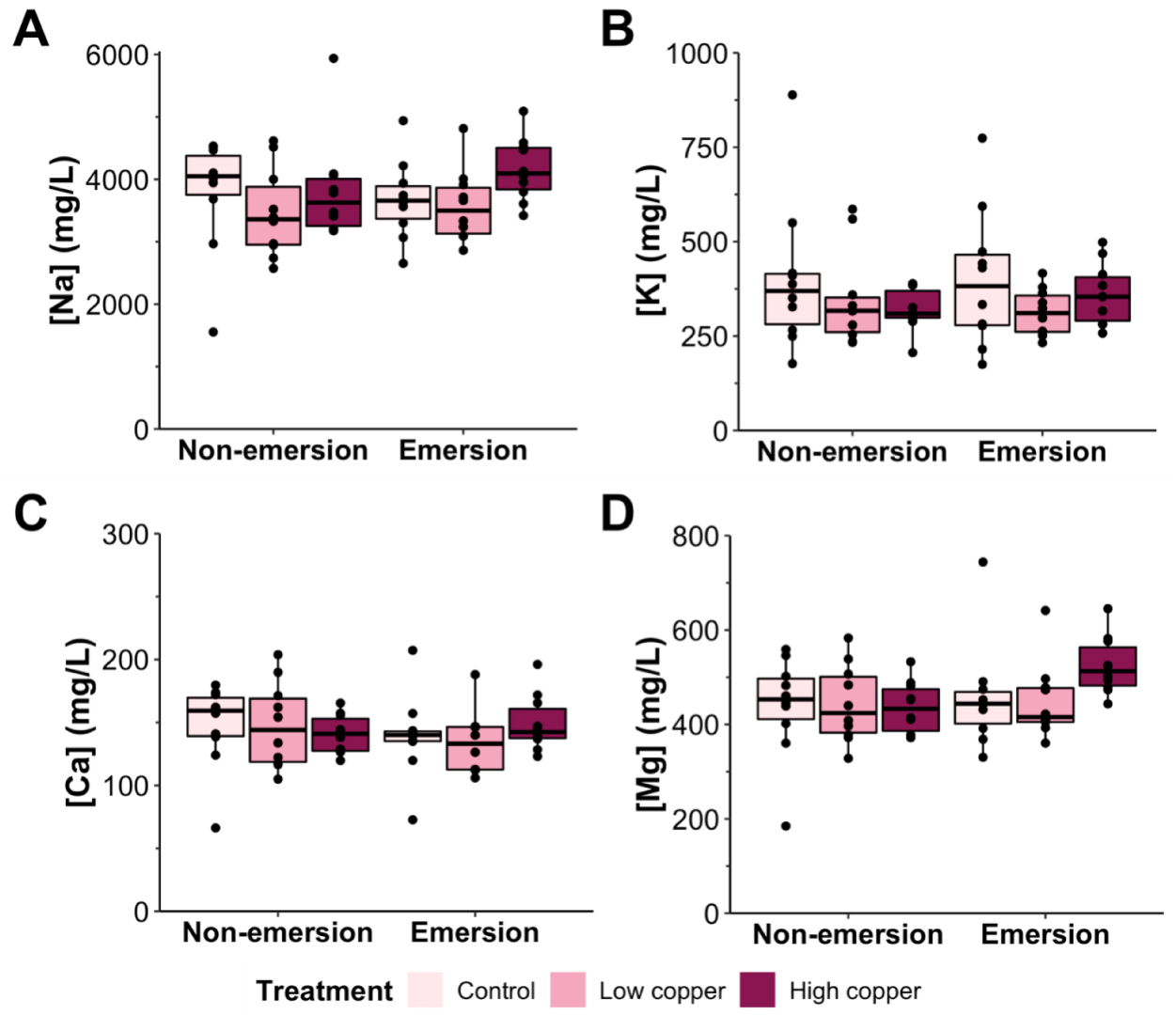

Figure 2.4. Concentrations of (A) sodium, (B) potassium, (C) calcium, and (D) magnesium in *Cucumaria miniata* coelomic fluid. Individual datapoints represent ion concentrations for each sea cucumber (n = 8 to 10). Horizontal lines within boxplots represent the median, upper and lower hinges represent 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.

Chapter 3: Copper toxicity does not affect low tide emersion tolerance of *Mytilus galloprovincialis*[2]

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 them are also vulnerable to anthropogenic toxicant exposure (Blake and Olin, 2022; Cao et al., 2006; Kirwan et al., 2016; Nelson and Zavaleta, 2012).

In California, it is estimated that at least 91% of their 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 wetland sites, 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, a portion of the channel is 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;

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Kraskura and Heard, UCSB, personal communication). Such conditions present several challenges for the physiological performance and survival of 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 abiotic conditions, animals in Carpinteria Salt Marsh must contend with anthropogenic input of pollutants. The marsh receives watershed 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 outside of these tightly regulated concentrations, higher environmental doses will enhance toxicity (Grosell, 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). Concentrations in the water column typically range from 1-25 μg/L but may be much higher near densely populated areas (B.C. Ministry of Environment and Climate Change Strategy, 2019). Cu tends to partition into sediment to higher concentrations than in the water column; in Carpinteria Salt Marsh, sediment samples have been found to contain up to 67.5 μg/g of Cu, and levels reach upwards of 200 μg/g in sediments of other California salt marshes (Cao et al., 2006; Hwang et al., 2006; 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 (Grosell, 2011; Rader et al., 2019). In seawater, Cu may also complex with anions to form Cu carbonates, which are likely nontoxic, or Cu hydroxides, which may exert toxicity along with free Cu (Blanchard and Grosell, 2006; Erickson et al., 1995). 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 (Grosell, 2011). The metal can also be ingested via the water or sediment, or bound to DOC (Grosell, 2011; Wood, 2011). Cu crosses epithelial membranes (e.g., gills) by mimicking transport routes for essential ions like sodium (Na) and calcium (Ca), and fish gills have divalent metal transporters (DMT1) and Cu transporters (CTR1) that may also be used for Cu uptake (Grosell, 2011). After crossing epithelial surfaces, excess Cu undergoes oxidation-reduction (redox) cycling, producing reactive oxygen species (ROS) which cause DNA damage and lipid peroxidation (Grosell, 2011). 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 (Grosell, 2011; Harris and Gitlin, 1996). Cu toxicity results in damage to several physiological processes including respiration, metabolism, ammonia excretion, osmoregulation, and acid-base balance (Grosell, 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 (Grosell, 2011). 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 tide 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 could be problematic for intertidal salt marsh animals because these environments typically present several simultaneous stressors, such as air exposure. 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 grasp the 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 invade new habitats in part due to its high tolerance to a range of thermal conditions (Han and Dong, 2020). In spite of their invasiveness, these mussels are now 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 mostly sessile and thus cannot avoid 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 (Chapter 2), but mussels have drastically different anatomy and thus their physiological responses to mixed stressors may differ. 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 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 (Andrade et al., 2019; Durand and Regnault, 1998; Grosell et al., 2007), 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 will advance knowledge of how multiple stressors that alter similar physiological processes in intertidal animals may interact to decrease their fitness and survival. Additionally, mixed stressor studies may provide insight into the success of invasive species like *M. galloprovincialis* which are highly tolerant of a broad range of environmental conditions (Han and Dong, 2020). 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 sensitive intertidal communities is vital to inform regulations regarding toxicant release and may provide a basis for further predictions of future survival and distribution of species such as *M. galloprovincialis*. In particular, results will 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-213430003-21346-001). 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 Na, 11 K, 11 Ca, 52 Mg.

2.2. Experimental Cu and emersion exposures

Experimental design and procedures were based on previous experiments (Chapter 2), with some adjustments. Cu stock solution (1 g/L) 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). 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 in table 1.

Individual *M. galloprovincialis* were placed in 400 mL plastic containers and randomly assigned to control, low Cu, or high Cu seawater for 96 h. As in Chapter 2, 20 replicates per Cu treatment (one mussel per container), were used for the experiment. All containers were held in a flow-through water table where temperature was maintained at 14.2 ± 0.10 °C and were constantly aerated during the treatment. A complete water change was conducted 48 h into the treatment. After 96 h, 10 mussels from each Cu treatment were removed from water for 6 h in their empty original containers floating in the same water table to maintain temperature. The 10 submersed replicates from each Cu treatment were immediately removed for 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.

2.3. Water chemistry

Water samples (10 mL) were collected from each stock (control, low Cu, and high Cu) several times throughout the Cu exposure experiments for inorganic analysis. Water samples were also collected from each experimental mussel container at the beginning of the experiments and at the end of the 96 h exposure period. All 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 were measured using inductively coupled plasma-mass spectrometry (ICP-MS/MS). For ICP-MS/MS analyses, standards were prepared in a matrix of 2% HNO_3 and 0.5% HCl and covered a range of 0.0005-500 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 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. Recovery of the cations ranged from 72.0 to 84.9%.

2.5. Oxygen uptake rates

Immediately after exposure to Cu and/or emersion, mussels were transported to one of four 160 mL sealed glass chambers placed within two flow-through tanks containing fresh seawater for a closed respirometry protocol with a constant water recirculation rate in the closed systems of about 1 L/min, 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 probes with a 4-channel FireSting optical oxygen meter (Pyroscience, Germany). 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 oxygen uptake slopes with coefficients of determination (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. (2016) and the following equation:

$$\dot{M}O_2 = (K_1 V_1 - K_2 V_2) \cdot M^{-1}$$

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. Values were mass-scaled using the allometric metabolic mass exponent of 0.715 described 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). Half 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 from each respirometry chamber before and after the 1 h measurement period were collected and immediately frozen at -20°C for analysis. Ammonia concentration was measured following the sodium salicylate-hypochlorite method of Verdouw et al. (1978) with some adjustments. 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 hour 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:

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

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 (Bacchiocchi and Principato, 2000; de Vooys, 1979; de Zwaan and Wijsman, 1976; Fields et al., 2014; Zurburg and Ebberink, 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 x *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 were 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

Cu concentrations measured 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, but the 96-h control water samples were below the Cu detection limit. After the 96-h exposure period, low Cu experimental water from both non-emersion and emersion groups had about half the initial Cu, and high Cu experimental water from both groups decreased to about a fifth of the 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 (Figure 3.1). Bioaccumulation of Cu in the gills was significantly affected by treatment (i.e., control, low Cu or high Cu; $F_{(2,52)} = 94.511$, $p < 0.0001$), but independent of group (i.e., non-emersion or emersion; $F_{(1,52)} = 0.227$, $p = 0.636$) and interactions of group and treatment ($F_{(2,52)} = 0.582$, $p = 0.563$). 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 (Figure 3.1A).

In the mussel foot, Cu accumulation was also dependent on treatment ($F_{(2,49)} = 13.467$, $p < 0.0001$) but independent of 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] 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 (Figure 3.1B).

Accumulation of Cu in the hemolymph was also significantly affected by treatment ($F_{(2,52)} = 6.114$, $p = 0.004$) but not by 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 (Figure 3.1C).

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; Figure 3.2A). Group ($F_{(1,54)} = 14.785$, $p < 0.001$), 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 group ($F_{(1,54)} = 4.790$, $p = 0.033$) and 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] in emersed mussels was 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; Figure 3.2B).

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; Figure 3.3). 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; Figure 3.4A). 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 group ($F_{(1,43)} = 0.640$, $p = 0.428$), 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 (Figure 3.4B).

AQ analysis also did not reveal any effects of group ($F_{(1,43)} = 0.072$, $p = 0.790$), 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; Figure 3.4C). The emersed high Cu mussels had the lowest AQ at 0.275 ± 0.048, though again this was insignificant (Figure 3.4C).

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 (Figure 3.5A). A few outliers produced high succinate in the controls (Figure 3.5A).

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 (Figure 3.5B). 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 (Figure 3.5B).

3.6. Hemolymph ion concentrations

M. galloprovincialis hemolymph ion concentrations were roughly equivalent to those measured in UCSB facility seawater (Figure 3.6; section 2.1). The mussels showed high

individual variation in Na, K, Ca, and Mg concentrations. Na concentrations were unaffected by group ($F_{(1,52)} = 0.500$, $p = 0.483$), 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.

4. Discussion

Overall, while Cu accumulation was observed in *M. galloprovincialis*'s shell and internal tissues, 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 towards the high tolerance of the mussel to multiple stressor exposures.

4.1. Water chemistry

All stocks had steady Cu concentrations over the 96-h exposure period, though the high Cu stock had slightly lower Cu than expected (Table 1). This may have been due to some adsorption of Cu to surfaces of containers or air stones under the salinity and pH conditions of seawater (John and Leventhal, 2004). Both non-emersion and emersion controls showed low but measurable Cu levels representing background Cu levels (Table 1). Cu was not detected in the 96 h controls, and both low and high Cu experimental exposures had lower Cu levels 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 (Figure 3.1).

4.2. Cu bioaccumulation and shell Ca concentrations

M. galloprovincialis gills accumulated the highest concentrations of Cu, followed by hemolymph and foot (Figure 3.1), 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 (Deb and Fukushima, 1999; Grosell, 2011). 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 (Bury et al., 2003, Grosell, 2011). 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, 1992). 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 (Figure 3.1). 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 (Deb and Fukushima, 1999; Torres-Duarte et al., 2019), and while there was some significant accumulation of Cu in hemolymph of *M. galloprovincialis*, it appears that only a few individuals accumulated high Cu (Figure 3.1C). This may have been caused by most mussels undergoing rapid depuration of Cu

from the hemolymph to external water under clean water conditions 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 the mussels did not close their valves 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 (Figure 3.2). Cu may adsorb to external surfaces of aquatic animals, as observed in a study of the shrimp *Palaemon elegans* (White and Rainbow, 1982), which may have contributed Cu to the measured concentrations in the shell samples, but Cu may also be assimilated into mussel shells via ion mimicry (Zhao et al., 2017). 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

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. However, this could not be confirmed in the current study because Cu loosely bound to the external shell surfaces was not differentiated from Cu incorporated into the shells. Future research may include comparisons of these two possible sources of Cu in shell samples, and 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 (Figure 3.2). 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 (Allen et al., 2021; Fields et al.,

2014). Acidosis may be buffered by resorbing $CaCO_3$ (and perhaps Cu CO_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 (Figure 3.2). 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 (Figure 3.3), similar to a previous study in which *Mytilus edulis* produced fewer 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 (Ait Ayad et al., 2011; Babarro and Reiriz, 2010; Sunila, 1981; 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 (Brown and Newell, 1972; de Zwaan and Eertman, 1996; Giacomin et al., 2014; Grosell, 2011; Wang and Widdows, 1993). 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 (Borthagaray and Carranza, 2007; Inoue et al., 2021). 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 these 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* $\dot{M}$O2 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 $\dot{M}O_2$ (Figure 3.4A). 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. (Bayne et al., 1976; Shick et al., 1986) 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 and predation 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 (Bayne et al., 1976; Shick et al., 1986). 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 (Bayne et al., 1976; Shick et al., 1986). 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 (Figure 3.4A), 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; Jorge et al., 2016; Scott and Major, 1972; Sunila, 1981). 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* (Spicer and Weber, 1991; Sunila, 1981). 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 (de Polo and Scrimshaw, 2012; Jorge et al., 2016; Lionetto et al., 2016). 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 (~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 (Magesky and Pelletier, 2018; Sokolova et al., 2012). 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 (Figure 3.4A). 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 $\dot{M}O_2$. However, it must be noted that 6 mussels exposed to high Cu spawned during respirometry (and were excluded from $\dot{M}O_2$ analysis), and spawning may be an indicator of stress (Petes et al., 2007). Thus, it seems that high Cu may have caused low to moderate stress in some mussels (reflected by increased $\dot{M}O_2$), but higher stress in others (reflected by spawning behaviour).

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 (Figure 3.4B). 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* (de Vooys and de Zwaan, 1978; Durand and Regnault, 1998). 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. (1999) and reflecting this species' high tolerance to tidal emersion.

Cu also did not affect AER, indicating that *M. galloprovincialis*'s nitrogen metabolism is not very sensitive to Cu exposure (Figure 3.4B). In marine osmoconformers, damage to ammonia excretion has been proposed to be the main mechanism of Cu toxicity (Grosell, 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; Giacomin et al., 2014; Goswami et al., 2014; Jorge et al., 2016; Santini et al., 2011; Wilson-McNeal et al., 2020). 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 (de Polo and Scrimshaw, 2012; Grosell et al., 2007). 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 (Lim et al., 2015; Thomsen et al., 2016; Wilson-McNeal et al., 2020). 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 (Grosell, 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 (Figure 3.4B). 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 (Figure 3.4C), 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 towards 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 production of succinate in previous studies of *M. galloprovincialis* and other bivalves, indicative of increased anaerobiosis due to valve closure and inhibited oxygen uptake (Bacchiocchi and Principato, 2000; de Vooys, 1979; Shick et al., 1986; Zurburg and Ebberink, 1980). In the current study, succinate levels significantly increased after emersion in *M. galloprovincialis* gills (Figure 3.5B), but succinate production in the foot was unaffected by emersion (Figure 3.5A). 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 (Bayne et al., 1976; Shick et al., 1986), 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 (Figure 3.5).

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 (Figure 3.5). 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 (Figure 3.5A), 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 (Figure 3.5A). 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 (Figure 3.6). 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 (Grosell et al., 2007). 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 (Jorge et al., 2016; Viarengo et al., 1996). 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 (Boitel and Truchot, 1989; Chapter 2). 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. Conclusions

Overall, results of this study suggest that *M. galloprovincialis* is very tolerant to emersion, but somewhat sensitive to Cu exposure. Cu accumulated in the gills, but assimilation of the metal into the shells may have diminished its adverse effects. Indeed, the metabolic response of the mussels to Cu exposure was indicative of moderate stress. 6 h of emersion appeared to slightly increase dependence on anaerobic metabolism but had no other effects. Cu decreased byssal thread production, possibly due to a decrease in available energy for this process. *M. galloprovincialis*'s high tolerance to tidal emersion was not affected by Cu exposure, contradicting our hypothesis that the metal would damage the physiological processes that

compensate for periods of air exposure. High tolerance to multiple stressors contributes to the invasiveness this species. However, this study only included acute Cu exposure and one submersion/emersion cycle which could have obscured potential 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 other 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 invasive 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.

Tables and figures

Table 3.1. 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

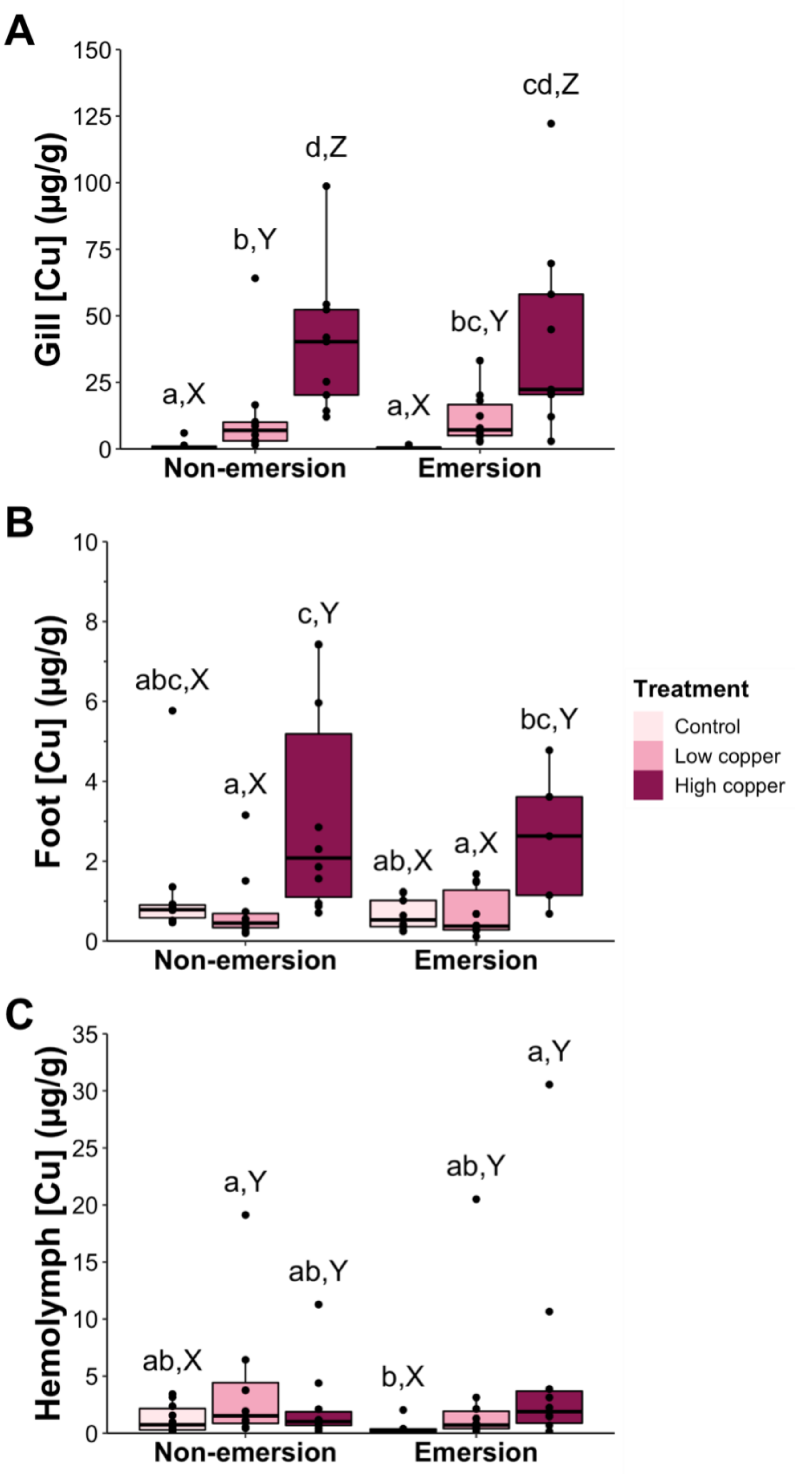

Figure 3.1. Copper (Cu) accumulation in the (A) gill, (B) foot, and (C) hemolymph of *Mytilus galloprovincialis* after 96 h of control, low Cu, or high Cu exposure, with or without 6 h of emersion following the exposures. Individual datapoints represent Cu bioaccumulated by each

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.

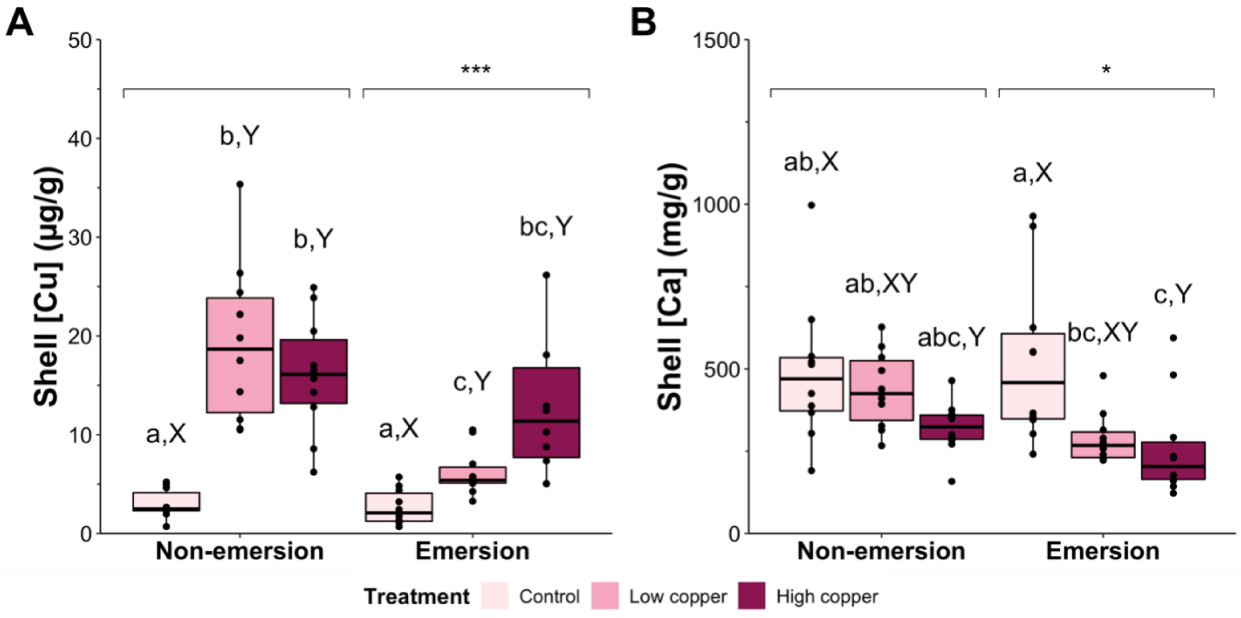

Figure 3.2. Concentrations of (A) copper (Cu) and (B) calcium (Ca) in the shells of *Mytilus galloprovincialis* exposed to 96 h of control, low Cu, or high Cu conditions and with or without 6 h of emersion. Individual datapoints represent ion content of each individual mussel's shell (n = 10). Horizontal 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 $p < 0.001$).

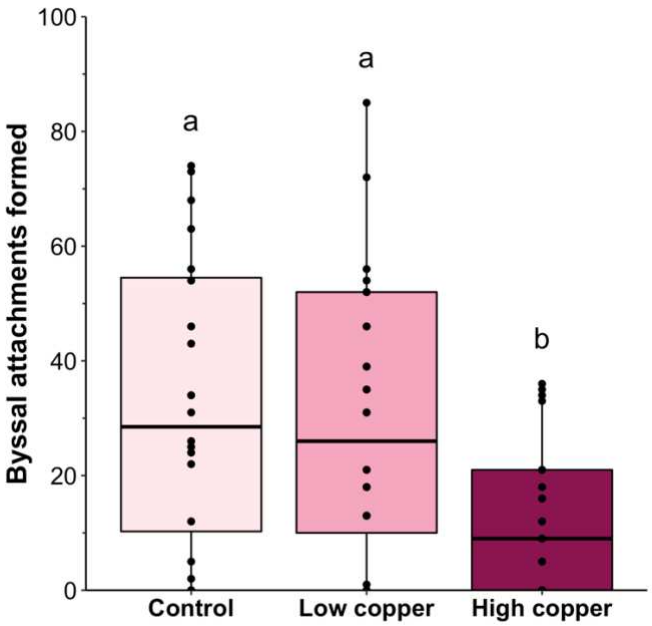

Figure 3.3. The number of new byssal threads produced and adhered to experimental containers by *Mytilus galloprovincialis* during 96 h of control, low copper (Cu), or high Cu exposure. Individual datapoints represent byssi produced by each individual mussel (n = 20). Horizontal lines within boxplots 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.

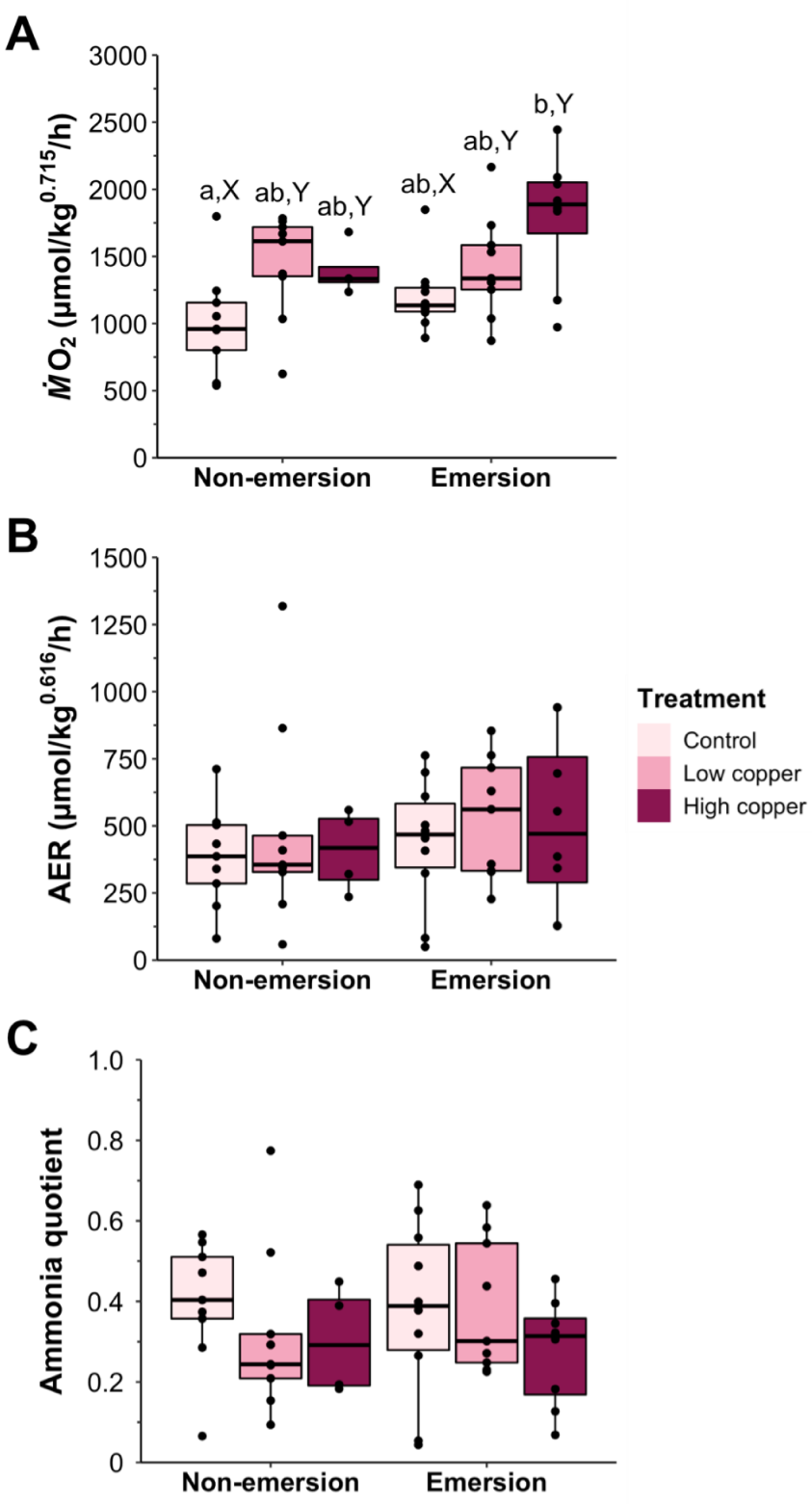

Figure 3.4. (A) Mass-scaled oxygen uptake rate ($\dot{M}O_2$), (B) mass-scaled ammonia excretion rate (AER), and (C) ammonia quotient of *Mytilus galloprovincialis* after 96 h of exposure to control,

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 ($p < 0.05$).

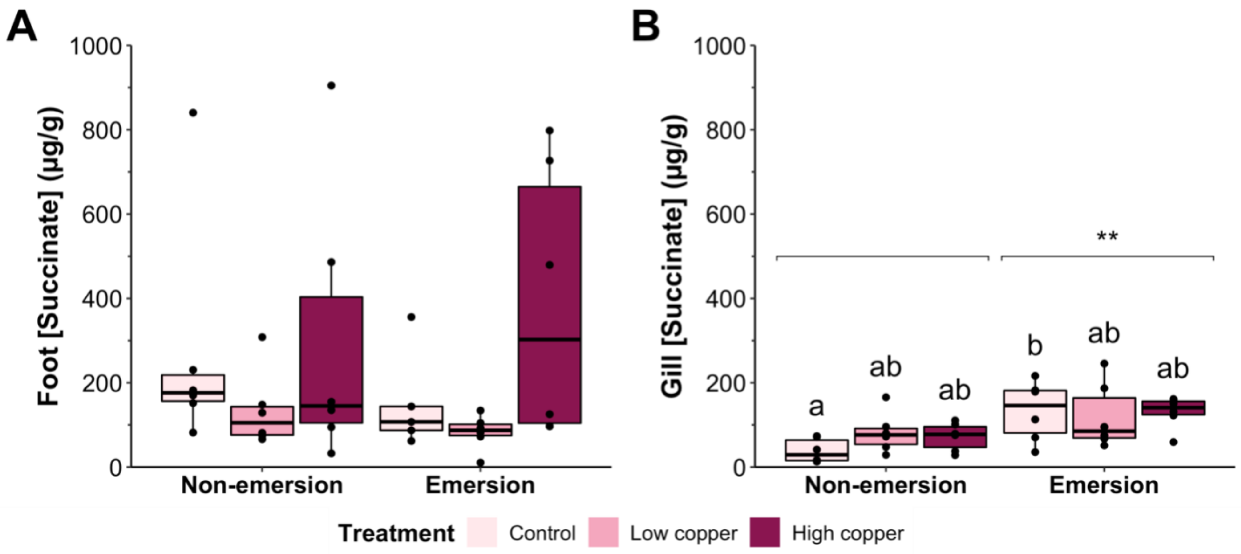

Figure 3.5. Succinate levels in the (A) foot and (B) gill of non-emersed and emersed *Mytilus galloprovincialis* after exposure to control, low copper (Cu), and high Cu. Individual datapoints represent succinate accumulation by each individual mussel (n = 6). Horizontal lines within boxplots represent 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 $p < 0.001$).

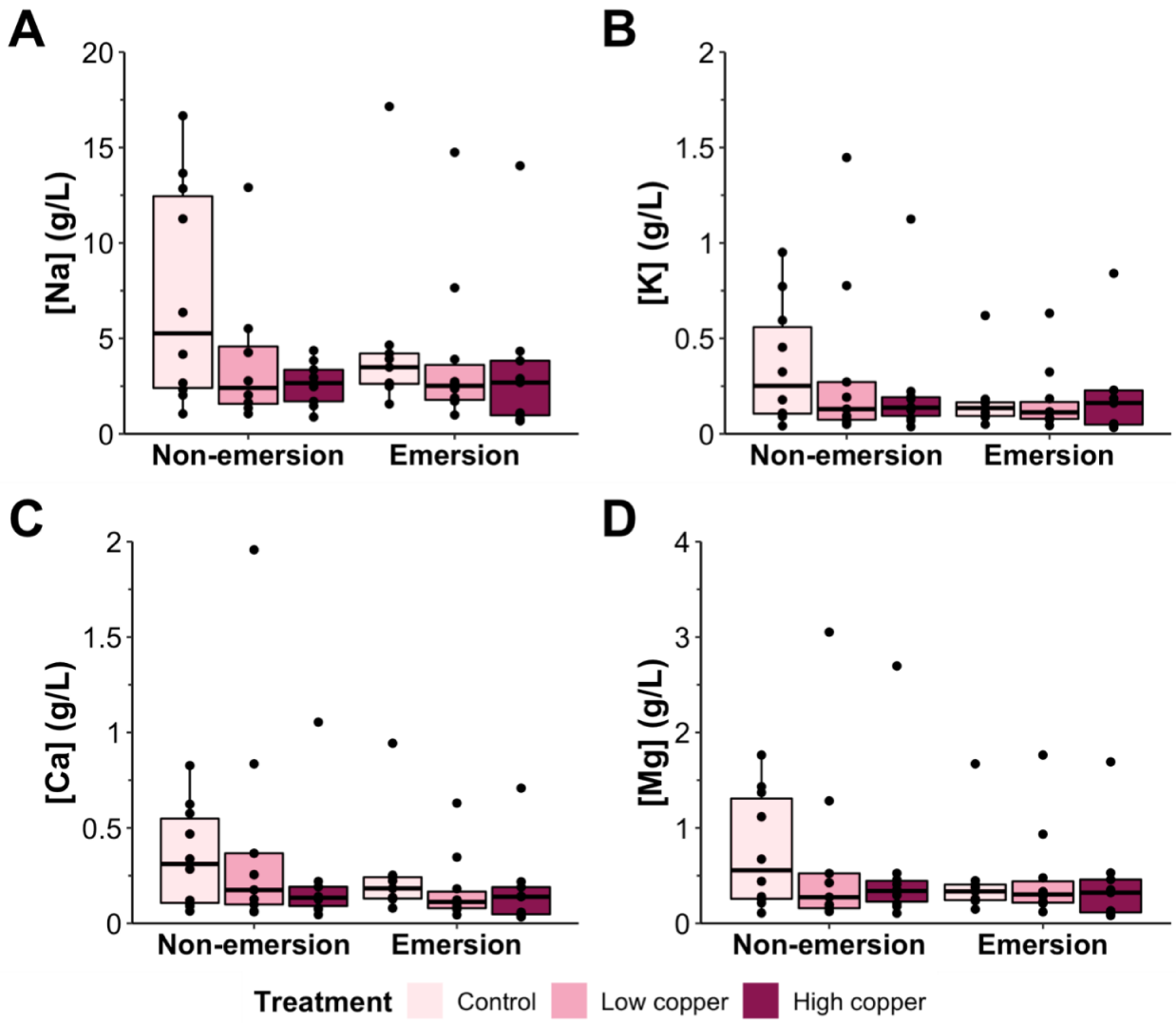

Figure 3.6. Concentrations of (A) sodium, (B) potassium, (C) calcium, and (D) magnesium in *Mytlus galloprovincialis* hemolymph after 96 h of exposure to control, low copper (Cu) or high Cu conditions and with or without a 6 h emersion period. Individual datapoints represent ion levels of each individual 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.

Chapter 4: Discussion and conclusions

1. Tolerance of intertidal species to mixed stressors

Contrary to the hypotheses of this thesis, results of experiments on *Cucumaria miniata* and *Mytilus galloprovincialis* suggested that both species have a high tolerance to tidal emersion and environmentally realistic concentrations of anthropogenic Cu exposure. *C. miniata* was sensitive to short-term emersion and acute high Cu exposure as separate stressors, but the two stressors were not found to interact to produce additive or synergistic physiological effects. Similarly, these stressors did not have any interactive effects on *M. galloprovincialis.* The mussels displayed some sensitivity to Cu exposure (in particular, byssus production was inhibited by Cu), but physiological processes were mostly unaffected by 6 hours of emersion, and Cu did not alter their response to emersion. *M. galloprovincialis* appeared to be more tolerant to both stressors, but it is important to note that high Cu treatment concentrations used for *M. galloprovincialis* were lower than those used for *C. miniata*, so the two studies may not be directly comparable. However, these findings still have major implications for future distribution and survival of these species.

In the near future, it is possible that factors like tide height/emersion time, salinity and pH of coastal waters, and both aquatic and terrestrial temperatures will fluctuate more dramatically due to climate change (Finke et al., 2007). If soft-bodied intertidal invertebrates like

sea cucumbers are tolerant to natural stressors like emersion and are able to survive exposure to realistic toxicant concentrations as shown in the current experiments, these animals may be well equipped to overcome current and future environmental fluctuations. On the other hand, species that are invasive and/or better adapted to the challenges presented by tidal variation in environmental conditions (like *M. galloprovincialis*) may be even less vulnerable to the effects of climate change and anthropogenic input of toxicants into aquatic environments. Thus, since some intertidal animals are likely more able to withstand both regional effects of climate change and exposure to a variety of toxicants, it is possible that these species may outcompete more sensitive species. This may lead to altered community structures in the future as species abundances in coastal regions change in response to varying conditions.

2. Thesis pitfalls

There were several factors of the experiments in both chapters 2 and 3 that created obstacles for drawing conclusions from the results. In chapter 2's experiments on *C. miniata*, the sea cucumbers were unexpectedly active in their respirometry chambers – some even plugged outflow tubes, interfering with oxygen readings and causing the non-emersed high Cu group to have too few replicates for metabolic analysis. This meant a full evaluation of Cu exposure's effects on *C. miniata*'s response to emersion could not be completed. I recommend using higher replicate numbers and using closed respirometry with stirred water instead of water pumps to avoid this potential problem. For chapter 3's work with *M. galloprovincialis*, the respirometry setup also presented some problems because there was potential Cu contamination between each batch of 4 mussels due to the flow-through system, and Cu bioaccumulation was measured after the respirometry period. Additionally, succinate samples from the mussel tissues were not standardized to protein content, which would have controlled for potential interference of water

or proteins in the absorbance readings. Lastly, actual Cu concentrations can be difficult to predict based on nominal values due to the metal's tendency to adsorb to surfaces. This was observed in chapter 3, where high Cu concentrations were lower than expected. These results meant the two studies were less comparable, as the same concentrations of Cu were not used for both species.

3. Recommendations for future research

Based on results of this study, future directions in the field of multi-stressor research on intertidal marine invertebrates may include similar studies using different toxicants (e.g., non-essential metals like cadmium, or persistent organic pollutants like microplastics). Toxicants with different mechanisms of action on aquatic organisms may have more potential to cause interactive (i.e., additive or synergistic) effects with the stresses caused by tidal emersion (Simmons et al., 2021). Additional endpoint measurements, such as carbonic anhydrase or antioxidant enzyme activity, ATP levels, and production of other intermediate and end products of anaerobic metabolism are recommended. While no interactive effects of Cu exposure and tidal emersion were observed in this study, acute toxicant exposure may not be reflective of longer-term, chronic exposure. Realistically, intertidal organisms undergo repeated cycles of exposure to various stressors (e.g., 6 h of toxicant exposure during submersion followed by 6 h of thermal and metabolic stress during emersion, and back to submersion, etc.). A complete understanding of future effects of tidal and anthropogenic stressors on intertidal organisms requires studies of chronic, cyclic variations in tidal conditions and intermittent toxicant exposure. For example, effects of longer-term Cu exposure and emersion cycles on mussel shell calcification should be evaluated since this study illuminated potential mechanisms of shell growth inhibition. Effects of more than two stressors on intertidal invertebrates should be evaluated, ideally incorporating as many stressors as are environmentally relevant, although this is much more logistically difficult.

Additionally, similar studies should be conducted on a variety of intertidal organisms with diverse physiology (e.g., the invasive green crab *Carcinus maenas*) to improve understanding of how mixed stressor effects on different populations dictate the health of intertidal ecosystems, both currently and in the future. Multi-stressor research is vital to increase knowledge of marine invertebrate physiology, and results of such studies are useful to predict the vulnerability of intertidal species in the context of a changing world.

4. Conclusion

Overall, results of this thesis provide further insight into the mechanisms intertidal organisms use to overcome the cyclic challenges they face due to the influences of tides and human activities. This study showed that although *C. miniata* and *M. galloprovincialis* survived acute Cu exposure, environmentally relevant concentrations still had sublethal effects which could be more damaging for organisms under chronic exposure to these conditions. Regardless, the high tolerance of both *C. miniata* and *M. galloprovincialis* to Cu exposure and tidal emersion has implications for future fitness and survival of these species. These two species may be capable of withstanding exposure to multiple stressors well into the future. Tolerance to adverse conditions is promising for their survival, but the higher capacity of some species to overcome challenges is worrying for future heterogeneity and health of coastal ecosystems.

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