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Copper alters hypoxia sensitivity and the behavioural emersion response in the amphibious fish *Kryptolebias marmoratus*

Tamzin A. Blewett[a,*], Robyn A. Simon[b], Andy J. Turko[b], Patricia A. Wright[b]

[a] Department of Chemistry and Biochemistry, Wilfrid Laurier University, Waterloo, Ontario, N2L 3C5, Canada
[b] Department of Integrative Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, N1G 2W1, Canada

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ABSTRACT

Elevated levels of metals have been reported in mangrove ecosystems worldwide. Mangrove fishes also routinely experience severe environmental stressors, such as hypoxia. In the amphibious fish *Kryptolebias marmoratus* (mangrove rivulus), a key behavioural response to avoid aquatic stress is to leave water (emersion). We hypothesized that copper (Cu) exposure would increase the sensitivity of this behavioural hypoxia avoidance response due to histopathological effects of Cu on gill structure and function. *K. marmoratus* were exposed to either control (no added Cu) or Cu (300 μg/L) for 96 h. Following this period, fish were exposed to an acute hypoxic challenge (decline in dissolved oxygen to ~0% over 15 min), and the emersion response was recorded. Gills were examined for histological changes. Fish exposed to Cu emersed at a higher dissolved oxygen level (7.5 ± 0.6%), relative to the control treatment group (5.8 ± 0.4%). Histological analysis showed that the gill surface area increased and the interlamellar cell mass (ILCM) was reduced following Cu exposure, contrary to our prediction. Overall, these data indicate that Cu induces hypoxia-like changes to gill morphology and increases the sensitivity of the hypoxia emersion response.

1. Introduction

Metals enter the aquatic environment through a myriad of industrial practices, such as mining, aquaculture and urban development (Bayen, 2012; Defew et al., 2005; Wood, 2012). In tropical countries, the impacts of metal contamination in near-coastal marine ecosystems is buffered somewhat by the presence of mangrove forests. These perform an important role as a sink for land-based contamination and therefore may protect marine settings from the highest concentrations of toxicants, at the risk of becoming extremely contaminated areas themselves (Bayen, 2012). Mangrove systems also filter macronutrients from agricultural run-off. These nutrients, such as nitrogen and phosphorus, support near-coastal aquatic plant/bacterial growth, and subsequently respiration. When respiration is coupled with tidal stagnation, mangrove settings can display reduced dissolved oxygen (DO) concentrations, resulting in hypoxia or anoxia (Bayen, 2012). This, in turn, impacts metal speciation, as hypoxia facilitates the formation of metal sulfides and other reduced chemical species that effectively trap trace metals in sediments and interstitial water, preventing them from exiting the mangrove system (Bayen, 2012; MacFarlane and Burchett, 2002). For example, the trace metal copper (Cu) can be found in mangrove sediments at concentrations as high as 4050 μg/g dw, and within waters sampled from mangrove habitats at concentrations up to 110 μg/L (Bayen, 2012). The highest concentrations of metals are found in mangrove regions associated with urban areas, where they may present a moderate to serious threat to the habitat (Defew et al., 2005).

Metal toxicants can exacerbate the effects of hypoxia in fishes by compromising the efficiency of oxygen uptake and/or oxygen sensing (Bayen, 2012; Handy, 2003). For example, in rainbow trout Cu causes thickening of the gill epithelium due to hypertrophy of pavement and chloride cells. This increases gas diffusive distance and thus hinders the ability of the adult fish to take up sufficient oxygen (van Heerden et al., 2004). In addition, Cu may interfere with blood oxygen transport through negative impacts on erythrocyte function in fishes (e.g. Baker, 1969; James and Sampath, 1995). Despite the presence of elevated Cu in mangrove habitats (Bayen, 2012), the effect of Cu on hypoxic responses in mangrove fishes has not yet been examined.

Behavioural avoidance is the first line of defense that animals use when confronted with unfavorable environmental conditions. For example, aquatic hypoxia drives the amphibious fish *Kryptolebias marmoratus* to leave the water or emerse (Regan et al., 2011). Aerial exposure induces a number of morphological changes in the gills of *K. marmoratus*, including an increased height of the interlamellar cell mass

* Correspondence to: Department of Biological Sciences, University of Alberta, 11455 Saskatchewan Drive, Edmonton, Alberta, T6G 2E9, Canada.
E-mail address: tamzin@ualberta.ca (T.A. Blewett).

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(ILCM) and an overall decrease in lamellar surface area (Ong et al., 2007; LeBlanc et al., 2010; Turko et al., 2012). These changes are concomitant with the utilization of skin and buccal/opercular surfaces to take up oxygen from the relatively oxygen-rich air (Cooper et al., 2012; Turko et al., 2014). The emersion response is an ideal bioassay for assessing the effects of Cu on the ability of *K. marmoratus* to respond to declining oxygen in its aquatic environment. Indeed, behavioural toxicology has emerged as a promising alternative to lethal endpoint studies. Animal behaviour integrates internal physiology with that of the external environment, bridging the field to laboratory divide (Pyle and Ford, 2017). Furthermore, any toxicological behavioural impairments may point to underlying physiological deficits and can be used to evaluate ecological risk, especially if this affects survival, growth or reproduction (Pyle and Ford, 2017).

Our objective was to determine if Cu exposure impaired the behavioural emersion response to hypoxia in *K. marmoratus*. *K. marmoratus* are self-fertilizing, amphibious, euryhaline fish that typically inhabit crab burrows or temporary pools in the mangrove forest that frequently experience low DO (Ellison et al., 2012; Taylor, 2012; Wright, 2012). We hypothesized that acute exposure to Cu in an aquatic environment will alter the behavioural hypoxia avoidance strategy of *K. marmoratus* due to histopathological effects of Cu on gill structure and function. If so, then Cu-exposed fish will have a reduced gill surface area and will emerse at a higher DO relative to control fish. Fish were exposed to Cu (300 μg/L) for 96 h, and the emersion response during a 15 min acute hypoxia challenge was video recorded. Gills were processed for histological examination.

2. Methods

2.1. Experimental organism

Kryptolebias marmoratus were procured from a colony in the Hagen Aqualab, University of Guelph, Ontario, Canada. Individuals were maintained in 60 mL of 15 ppt brackish water (Crystal Sea Marinemix; Marine Enterprises International, Inc., Baltimore, MD, U.S.A., mixed with reverse osmosis water) at 25 °C within separate 120 mL plastic cups (FisherBrand Collection Containers; Fisher Scientific) on a light:dark cycle of 12:12 h, as previously described (Frick and Wright, 2002). Water was replaced completely once per week. Feeding (*Artemia* nauplii) occurred 2 times a week, but fish were fasted 48 h ahead of exposures to reduce the effect of digestive processes on experimental outcomes. Animal care and experiments were conducted in accordance with approvals obtained from the University of Guelph Animal Care Committee (#2239).

2.2. Experimental protocol

Animals were randomly divided into 2 experimental groups: control (no Cu added; n = 30) and a Cu-exposed group (300 μg/L; n = 29). Each individual fish was placed in 60 mL of 15 ppt brackish water in 100 mL glass beakers (PYREX™ Griffin), at a 12:12 h light:dark cycle at 25 °C. Cu (nominally 300 μg/L) was added to Cu exposure groups from a stock solution of $CuSO_4{\cdot}6H_2O$ (Sigma Aldrich, Toronto, ON). All glassware was pre-washed with 10% HNO_3 (Nitric Acid, Sigma Aldrich) for 24 h to reduce the effect of contamination. Water was dosed 24 h prior to experimentation, to ensure Cu speciation equilibration. Fish were exposed to these solutions for 96 h, and throughout this period fish were prevented from emersing by the presence of a perforated parafilm covering. After 48 h, an 80% water change occurred. After 72 h, fish were transferred from the original 100 mL beakers into new 100 mL beakers of identical water composition containing oxygen sensing spots. During the first 72 h the chambers were not aerated but DO remained above 70%, however aeration was initiated from 72 to 96 h to prepare them for the hypoxia challenge during the last 24 h of Cu exposure or control period to allow fish to become accustomed to the water agitation before behavioural experiments began. Water samples were taken at 0 and 96 h of exposure for water chemistry measurements (i.e. salinity, Cu concentration, dissolved organic carbon (DOC); Table 1).

Table 1
Water chemistry of exposure conditions at 0 and 96 h, N = 4 per treatment, values are means (± S.E.M.). Asterisk denotes significant differences between treatments.

Condition	Control (0 h)	Control (96 h)	Cu Exposed (0 h)	Cu Exposed (96 h)
Salinity (ppt)	15	15	15	15
Cu (μg/L)	14.5 ± 4.1	14.0 ± 5.2	307.3 ± 44	300 ± 40
Dissolved Organic Carbon (mg/L)	0.9 ± 0.5	–	2.9 ± 0.1*	–

2.3. Emersion experiment

At the end of 96 h exposures, all fish from both control and Cu-exposure groups were challenged with acute hypoxia. Each exposure chamber was bubbled with a fine flowing stream of nitrogen (N_2; Vital Air, Guelph ON), decreasing DO within the water to 0% over a 15-min period (Regan et al., 2011). A flowmeter was used to ensure a constant flow rate of N_2. During this period, DO was measured using oxygen sensing spots via Loligo Systems OXY-REG respirometer and AutoResp Witrox software program, and emersion responses were recorded (Logitech Quickcam Pro, 381 Fremont, CA, USA), with the DO at the point of emersion monitored. All exposure chambers were covered in parafilm to ensure low DO values could be achieved. Emersion was defined as an attempt by the fish to leave the water (Regan et al., 2011). EC_{50} values were calculated by adding a line of best fit following logistic regression, using calculations defined in Regan et al. (2011).

2.4. Water chemistry

Unfiltered and filtered (0.45 μm syringe filter; Acrodisc: Pall Life Sciences, Houston, TX, USA) water samples (2 mL) were taken at 0 and 96 h for determination of total and dissolved water Cu. Water was immediately acidified with 2% HNO_3, and Cu determined via Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) against certified multi-element standards (SCP Science: PlasmaCAL Multielement Q.C. 4, Sigma–Aldrich Chemical Company, Oakville, ON, Canada). Because there was less than a 5% difference between filtered and unfiltered samples, only filtered water Cu concentrations are reported. A water sample (50 mL) was also collected at 96 h and filtered for DOC analysis. DOC was measured using a Shimadzu TOC-Vcph/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan).

2.5. Gill histology

Following behavioural assays, fish were euthanized in a 300 mg/L solution of tricaine methanesulfonate (MS 222), and fixed in 10% neutral buffered formalin (4 °C) for 24 h. Histological analysis was performed as previously described (Turko et al., 2011). Briefly, gills were decalcified for 1 h (20 °C) (Cal-Ex, Fisher Scientific), and dehydrated in a graded ethanol series. Gills were then routinely embedded in paraffin, sectioned in 4 μm increments, and stained with haematoxylin and eosin. For each fish, ten lamellae from each gill arch were used for morphometric analysis. These were randomly selected and observed on a Nikon Eclipse 90i epifluorescence microscope and measurements were taken with NIS Elements software (Nikon, Melville, NY, U.S.A). The height of the interlamellar cell mass (ILCM) was measured in addition to the total lamellar length (Ong et al., 2007). Percent lamellar

coverage was also determined. This was defined as the ILCM divided by the lamellar length, with this value then multiplied by 100 to achieve percent coverage.

2.6. Statistical analysis

Data were expressed as means ± S.E.M. Statistical analysis was performed with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA) and SigmaStat 3.5 (Systat Software Inc., San Jose, CA USA). All data were assessed using a Students paired *t*-test between control and Cu treatments. Significance for all statistical tests was accepted at $\alpha = 0.05$.

3. Results

3.1. Water chemistry

Cu concentrations in unexposed controls were 14.5 ± 4.1 and 14.0 ± 5.2 µg/L, while dissolved Cu concentrations in Cu-exposure groups were close to nominal at 298.5 ± 40 and 307.3 ± 44 µg/L (Table 1). Dissolved organic carbon was low in exposures without Cu (< 1 mg/L), but reached significantly higher concentrations of 2.9 mg/L in the Cu exposure group (Table 1).

3.2. Emersion response

Acute hypoxia challenge, where DO was rapidly decreased, led to emersion behaviour in all mangrove rivulus tested. However, animals exposed to Cu displayed a significantly higher emersion threshold. Fish exposed to Cu jumped out of water when the DO level was 7.5% air saturated, while control fish emersed at 5.8% (Fig. 1A). The relationship between the proportion of fish remaining immersed at each DO level also indicated a significant difference between control and Cu exposure groups (Fig. 1B). The effect concentration ($EC_{50;}$ DO at which 50% of rivulus emersed) was 5.01% air saturation for control fish and 6.72% air saturation for Cu-exposed fish (Fig. 1B).

3.3. Gill histology

In control fish, part of the space between lamellae was filled with cells, a characteristic termed the interlamellar cell mass (ILCM) (Fig. 2A). In the Cu exposure group, the ILCM was decreased relative to control fish (Fig. 2A and B), which significantly reduced lamellar coverage ($p < 0.05$) compared to control fish (Fig. 2B).

4. Discussion

Pre-exposure to Cu altered the sensitivity of the behavioural emersion strategy that *K. marmoratus* invoke in response to severe hypoxia. Cu-exposed fish emersed at a significantly higher DO relative to control fish, as predicted. Given these findings, we expected that Cu-induced histopathological changes enlarged ILCM coverage and impaired oxygen uptake, thus resulting in the increased hypoxia sensitivity. In contrast to this expectation, gill coverage was reduced in Cu-exposed fish relative to control fish. These findings are also in contrast to studies in other fish species where Cu exposure increased the ILCM (Griffitt et al., 2007), caused hypertrophy of the gill epithelium (van Heerden et al., 2004), branchial lesions (Daoust et al., 1984), and/or fusion of the lamellae (Pandey et al., 2008). However, these studies all document effects in freshwater fish, and in contrast there has been little investigation of histological effects of Cu on the gills of seawater teleosts (Grosell, 2012). Thus, relative to studies to date, the gills of *K. marmoratus* showed a unique response to elevated waterborne Cu, a response which may enhance oxygen uptake in contaminated waters.

4.1. Cu impacts the behavioural emersion response

We initially reasoned that Cu exposure would alter the behavioural hypoxia response due to histopathological effects on gill structure and function. Based on other studies (e.g. Sollid et al., 2003; van Heerden et al., 2004; Griffitt et al., 2007; Mitrovic et al., 2009; Tzaneva et al., 2011; Turko et al., 2012), we anticipated that an increase in the ILCM would reduce the oxygen diffusive capacity of the gill, and thus cause earlier emersion in response to acute hypoxia. However, in the current study Cu exposure led to reduced lamellar coverage and ILCM (Fig. 2A and B). In fact, Cu exposure showed similar gill remodeling responses to those seen following prolonged hypoxia exposure in mangrove rivulus (Turko et al., 2012). The decreased ILCM observed in Cu-exposed fish may be explained by the interaction of this element with hypoxia-inducible factor (HIF). HIF is ultimately responsible for many pathways (e.g. erythropoiesis, iron metabolism, angiogenesis, control of blood flow, glucose uptake, pH regulation and cell-cycle control) that assist in promoting oxygen delivery during hypoxic conditions (Semenza, 1999). HIF also performs an important role in survival and cell proliferation (Lee et al., 2004). Importantly, HIF 1-α may also play a role in gill remodelling in fish, with its expression associated with a reduction in ILCM in crucian carp (Sollid et al., 2006). Previous research has shown that metals, including Cu, stabilize HIF 1-α, mimicking the effects of hypoxia (Duyndam et al., 2001; Salnikow et al., 2003; Gao et al., 2002; Costa et al., 2003; Martin et al., 2005; Griffitt et al., 2007; Fitzgerald et al., 2016). It is therefore possible that the reduction in ILCM in the Cu group was a result of Cu's stabilization of HIF 1-α, leading to the typical hypoxia response of a decreased ILCM. Further research is required to confirm if HIF expression is involved in this gill response.

Cu-exposed fish emersed at a slightly but significantly higher water oxygen level relative to control fish, opposite to what we expected. Several species of fish have been shown to improve respiratory function in mangrove rivulus (Turko et al., 2012), and we thus expected that given the reduced ILCMs in Cu-exposed fish, these fish would be less sensitive to water oxygen levels. The discrepancy could be explained by an effect of Cu on physiological parameters that control oxygen delivery. Previous evidence in several species of fish has shown that Cu can cause haemolysis (Baker, 1969; James and Sampath, 1995; Cerquira and Fernandes, 2002; Fedeli et al., 2010; Witeska et al., 2010), interfere with the haem group of haemoglobin (Labieniec et al., 2009), decreases red blood counts (James and Sampath, 1995; Witeska et al., 2010; Nussey et al., 1995; Cerqueira and Fernandes, 2002; Fedeli et al., 2010), and decreases haematocrit (James and Sampath, 1995; Cerqueira and Fernandes, 2002; Witeska et al., 2010; Cyriac et al., 1989). All of these effects of Cu would cause hypoxaemia and interfere with oxygen delivery. Thus it is likely that the increased gill surface area did not fully compensate for the functional hypoxia in Cu-exposed fish.

An alternative explanation for the mismatch between increased gill surface area and increased hypoxia sensitivity is that the signaling pathway for emersion is activated separately regardless of the improved oxygen uptake from an enlarged surface area. For example, Cu has been observed to suppress production of important neurotransmitters in fish, including serotonin (Handy, 2003) and acetylcholine (Balambigai and Aruna, 2011), while effects on neurotransmitter degradation pathways have also been noted (e.g. inhibition of acetylcholinesterase; Frasco et al., 2005). Oxygen-sensing neuroepithelial cells (NECs) located within the gills release serotonin (Perry et al., 2009) and acetylcholine (Milsom and Burleson, 2007). In *K. marmoratus*, NECs containing serotonin and acetylcholine are found in the skin and gills and there is evidence that both neurotransmitters mediate, in part, the emersion response (Regan et al., 2011). In the present study, if Cu exposure interfered with the serotonin or acetylcholine-dependent hypoxia response, then we might have expected a reduced sensitivity to acute hypoxia. We found the opposite. Therefore, it is unlikely that oxygen sensing was impaired under these conditions.

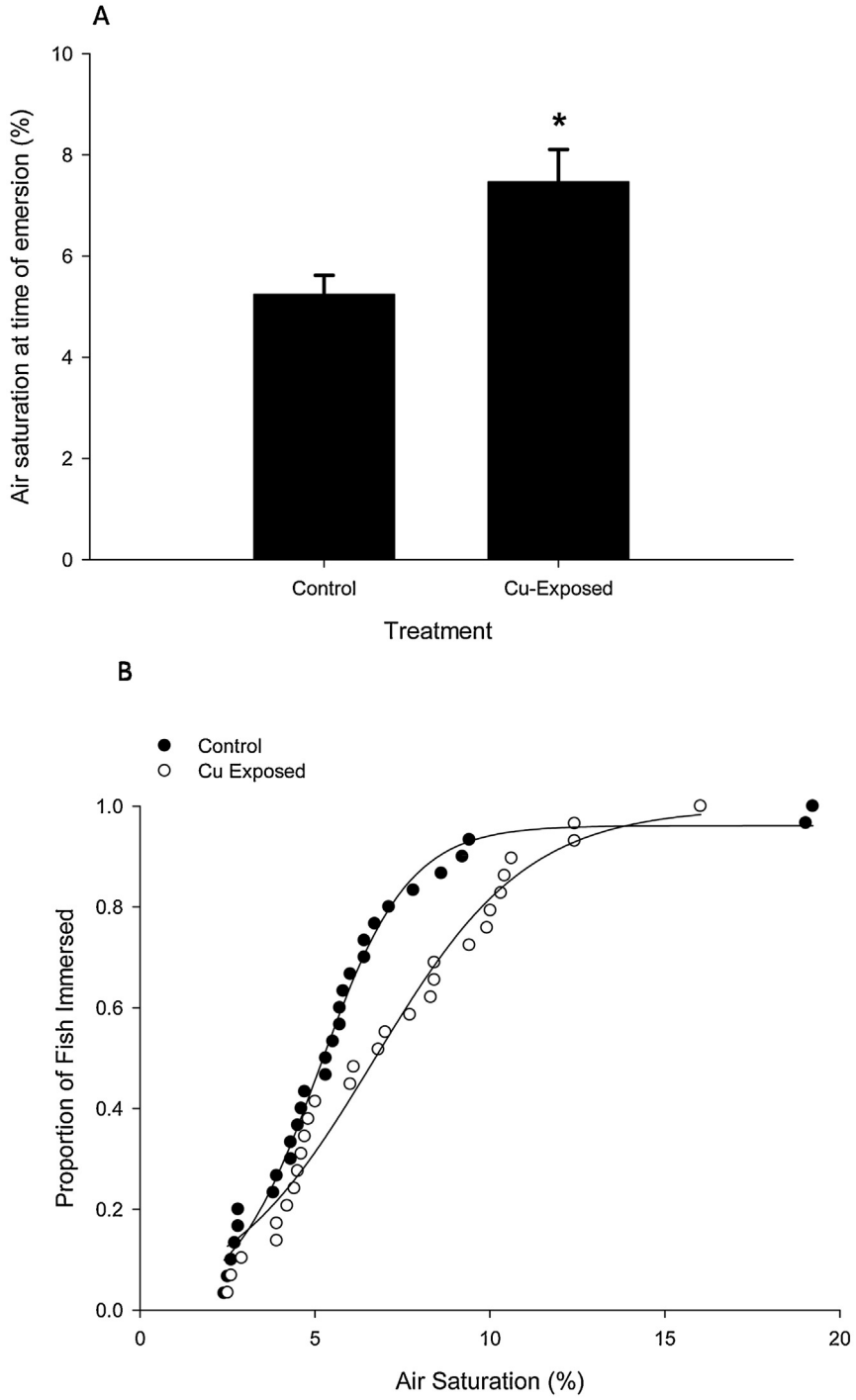

Fig. 1. A) Emersion DO level (%) for *K. marmoratus* exposed to either control (n = 30) or Cu (300 µg/L; n = 29) for 96 h and then subjected to an acute severe hypoxia challenge (progressive reduction of water DO to 0% in 15 min). Bars represent the mean (+S.E.M.). Asterisk indicates significant difference from control as determined by a 2-tailed students *t*-test (p = 0.001). **B**) The proportion of *K. marmoratus* (from n = 30, control and Cu n = 29) remaining immersed when subjected to an acute severe hypoxia challenge (progressive reduction of water DO to 0% in 15 min), following a 96 h exposure in the absence (black points, EC_{50} = 5.01% for control) or presence (300 µg/L; white points EC_{50} = 6.75% of Cu). Lines of best fit follows a logistic regression p < 0.001.

4.2. Water chemistry

In the Cu exposures in the current study, the dissolved measured concentrations of Cu were close to nominal (~300 µg/L, Table 1). While these concentrations are greater than those usually reported in mangrove waters (~110 µg L^{-1}), they are lower than those that have been previously shown to impact respiration in fish (Blanchard and Grosell, 2005), and lower than sediment values reported for mangrove areas (Bayen, 2012). The control values of Cu were 14.0–14.5 µg/L (Table 1). While comparatively low, the control Cu levels were higher than anticipated. This may have been a consequence of the artificial sea salt used, with some marine salts known to contain metal contaminants that increase exposure concentrations to levels above background (Arnold et al., 2007).

Another known issue with artificial salts is the addition of DOC. DOC concentrations were significantly increased in Cu exposures in the

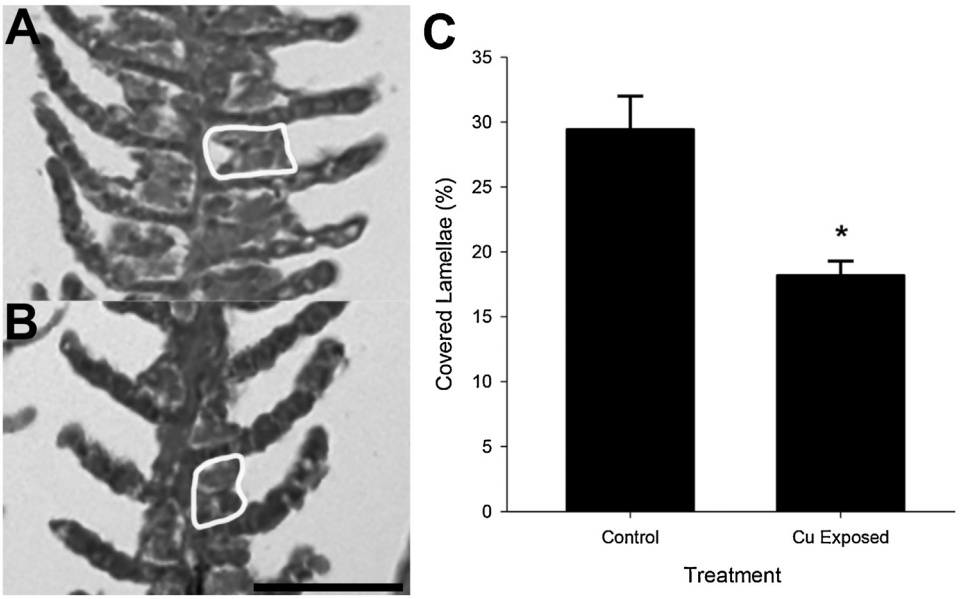

Fig. 2. Representative photomicrographs of gill sections from *K. marmoratus* exposed to **A**) control, **B**) Cu (300 μg/L) for 96 h. An exemplar interlamellar cell mass (ILCM) is outlined in white. Scale bar equals 50 μm. **C**) Coverage of the gill lamellae (%) in *K. marmoratus* exposed to either control (no added Cu) or Cu (300 μg/L) for 96 h and then subjected to an acute severe hypoxia challenge (progressive reduction of water DO to 0% in 15 min). Bars represent the mean (+S.E.M.) of 24–30 to replicates. Asterisk denotes significant difference from control, students paired *t*-test with significance accepted at $p < 0.05$.

current study. Arnold et al. (2007) found that the particular brand of artificial sea salt used in our experiments (Crystal Sea Marinemix) raised DOC, in some cases upwards of 3.5 mg/L. However, if this was true of the current study, then DOC would be high in both treatments, but DOC was found to be elevated only in Cu exposures. Consequently, it is likely that the increase in DOC is a stress response of the fish to Cu. Previous research has shown that fish release of mucus in response to environmental stress (Shephard, 1994), with metals in particular known to induce mucus hypersecretion (Handy and Eddy, 1990). In fact this response, which would add DOC to the water, may protect fish against the toxic impacts of metals. Binding of metals to body mucus, which is subsequently shed, will reduce metal bioavailability, and therefore reduce toxicological impact (Skidmore, 1970; Handy and Eddy, 1990).

Mucus may also explain the emersion of *K. marmoratus* at higher DO levels when pre-exposed to Cu for 96 h. A branchial covering of mucus, a commonly reported phenomenon in metal-exposed fish (Skidmore, 1970), would potentially impair diffusion, cause whole body hypoxaemia or HIF stabilization and lead to an earlier enacted behavioural emersion response. This would also explain the apparent contradiction between an increased surface area, but an earlier emersion response. Unfortunately, histological processing removes the gill mucus layer, but future work quantifying mucous cell size and number would be valuable.

5. Conclusions

Worldwide, mangroves are considered to be threatened environments (Bayen, 2012), with increased levels of contaminants being a major hazard to the integrity of these ecosystems (Bayen, 2012; Sadiq et al., 2003; Tam and Wong, 2000). The mangrove rivulus (*K. marmoratus*), a ubiquitous fish species of mangrove forests of the Western Atlantic, was shown in the current study to modify its behaviour and gill structure in response to acute hypoxia when pre-exposed to Cu. To our knowledge this is the first study to consider the behavioural impact of elevated Cu on a mangrove fish species.

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