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

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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

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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