Effects of copper on the acute ventilatory drive of killifish,
Fundulus heteroclitus

By

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Abstract

Organisms are often exposed to multiple stressors in their natural habitat. While the effects of individual stressors alone are generally well-studied, their combined interactions are often unknown. One such unknown interaction is that between copper, an essential micronutrient that is toxic in high levels, and hypoxia, a commonly experienced environmental stressor. This project sought to examine the effects that copper has on the hypoxic response as well as the combined effects of copper and hypoxia exposure using freshwater-acclimated adult killifish (*Fundulus heteroclitus*), a model euryhaline teleost. It was found that copper blunts the acute ventilatory response to hypoxia, ammonia, and combined hypoxia and ammonia exposure. Gill morphometry was also examined and it was found that while copper alone has no effect, when combined with hypoxia exposure it eliminates the increase in filament length and cross-sectional area seen when fish were exposed to hypoxia alone. Furthermore, when neuroepithelial cell size was examined, copper decreased cell projection area with or without the combined presence of hypoxia. Taken as a whole, this thesis indicates that copper exposure can significantly and negatively impact the ability of aquatic organisms to mount appropriate physiological responses to hypoxia.

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Thesis Organization and Format

This thesis is organized in a sandwich format approved by McMaster University and with the recommendation of the advisory committee. This thesis consists of three chapters. Chapter one consists of a literature review of the current knowledge in the field in order to provide context and significance for the research performed. Chapter two is a manuscript in preparation for submission to a peer-reviewed scientific journal. Finally, chapter three summarizes the effects of copper and hypoxia exposure on killifish and discusses future research directions.

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List of Abbreviations

96h – 96 hours

Ca – Calcium

CO_2 – Carbon Dioxide

COX – Cyclooxygenase

Cu – Copper

Cu+Hyp – Copper and hypoxia

FW – Fresh Water

HIF – Hypoxia Inducible Factor

HRE – Hypoxia Response Element

Hyp – Hypoxia

ILCM – Interlamellar Cell Mass

NEC – Neuroepithelial Cell

O_2 – Oxygen

PO_2 – Partial Pressure of Oxygen

SW – Seawater

Chapter 1: General Introduction

I. Aquatic Oxygen Depletion and Hypoxia

Aquatic vertebrates such as fishes respond to changes in both the biotic and abiotic characteristics of their environment on acute and chronic timescales. Appropriate levels of dissolved oxygen are essential for fishes to survive and grow. Due to this, low environmental oxygen levels can be a significant stressor to some fish species when experienced in their natural habitat (Farrell and Richards, 2009; Wu, 2002). Aquatic hypoxia affects millions of square metres of water around the world, leading to environments being less habitable for most aquatic organisms (Farrell and Richards, 2009; North et al., 2013; Wu, 2002). Hypoxia can have negative effects on an animal's ability to engage in aerobic metabolism and can also impact other oxygen-dependent physiological processes, such as gene expression patterns and proper enzyme function. This can lead to major problems of ecological concern, such as increased morbidity and mortality of fishes and other marine animals, leading to loss of ecological diversity (Diaz and Rosenberg, 1995; Farrell and Richards, 2009; Nikinmaa, 2013; Wu, 2002). In recent years, the leading cause of aquatic hypoxia has been eutrophication, an issue with anthropogenic origins that can significantly

impact ecosystem health (Farrell and Richards, 2009; North et al., 2013; Wu, 2002).

Eutrophication is defined as the production of organic material and biomass in excess of what the ecosystem is able to process adequately (Farrell and Richards, 2009; Nixon, 1995). Organic material entering the body of water leads to increased nutrient loads, resulting in an increase in microbial growth. As these microorganisms reproduce and increase in number, they consume more oxygen, which can lead to hypoxic or anoxic environments. This usually happens in enclosed bodies of water such as lakes, bays, and estuaries as these environments have limited water exchange and/or flow with other, non-eutrophied water bodies (Diaz and Rosenberg, 2008; Farrell and Richards, 2009; Joyce, 2000; Liu et al., 2013; Wu, 2002).

Recent increases in human-derived organic materials such as fertilizers pollute aquatic environments and cause plant and algae levels to increase, leading to eutrophication. This has become one of the most serious water pollution problems worldwide (Diaz and Rosenberg, 2008, 1995; Farrell and Richards, 2009; Joyce, 2000; Liu et al., 2013; North et al., 2013; Wu, 2002). It is expected that aquatic hypoxia will become an even more serious issue in the near future as the human population continues to grow, particularly near coastal areas (Farrell and Richards, 2009; North et

al., 2013; Rabalais et al., 2007; Wu, 2002). This will likely lead to increased nutrient loads entering nearby bodies of water, raising the probability of eutrophication and associated hypoxia. Additionally, if current climate change continues to lead to elevations in temperature, warmer water with decreased oxygen solubility and increased eutrophication will continue to increase in prevalence, overall leading to a higher occurrence of hypoxia in aquatic environments (Farrell and Richards, 2009; North et al., 2013; Rabalais et al., 2007; Wu, 2002).

II. The Multifunctional Fish Gill

Fish represent the largest and most diverse group of vertebrates (Evans et al., 2005). To survive in their aquatic environment, fishes possess a number of anatomical and physiological characteristics that enable them to thrive in an environment where abiotic characteristics can vary greatly. Of these, perhaps the most important for maintaining systemic homeostasis is the gill. In addition to being the main organ involved in oxygen and carbon dioxide transfer, the gill is used for osmoregulation, acid-base regulation, and the excretion of nitrogenous wastes, all of which are physiological processes crucial to survival (Evans et al., 2005; Hoar and Randall, 1984; Olson, 2002).

The gills of fishes are made up of four gill arches, which are further subdivided into gill filaments composed of many individual lamellae. Distributed on the epithelium of the lamellae are chemoreceptors (called neuroepithelial cells or NECs) which enable fishes to detect and respond to the concentrations of various substances in their environment. The primary receptor involved in the control of respiration is the O_2 sensor; however studies have shown that changes in ammonia, CO_2, pH, and cyanide levels all can evoke a respiratory response in fishes (Evans et al., 2005; Gilmour, 2001; Randall and Ip, 2006; Tzaneva and Perry, 2010; Wilson and Laurent, 2002; Wu, 2002; Zhang et al., 2011; Zhang and Wood, 2009). The majority of O_2 sensors are thought to be external, located on the gill to detect O_2 levels in water; however there are some internal sensors which can detect arterial blood PO_2 (Evans et al., 2005; Jonz and Nurse, 2006, 2003; Tzaneva and Perry, 2010; Vulesevic et al., 2006).

III. The Acute Ventilatory Drive and the Hypoxic Response

To maintain homeostasis and avoid negative effects on health and fitness, it is important for fishes to rapidly detect and respond to changes in environmental conditions (Farrell and Richards, 2009). As mentioned above, NECs are distributed across the gills to sense the physical and chemical properties of water, allowing fish to respond appropriately to

changes in water chemistry. NECs are particularly important when the fish is in a hypoxic environment, as when the fish senses a low oxygen level, they can mount an appropriate physiological response (Gilmour, 2001; Randall and Ip, 2006; Tzaneva and Perry, 2010; Vulesevic et al., 2006). In hypoxic conditions, NEC oxygen receptors are depolarized through inhibition of resting passive potassium current, which leads to neural signals that initiate the hypoxic response (Abdallah et al., 2015; Farrell and Richards, 2009; Jonz et al., 2004; Wu, 2002). Fishes are able to initiate hypoxic responses within seconds of the hypoxic water contacting the gills (Gilmour, 2001; Randall and Ip, 2006; Tzaneva and Perry, 2010; Vulesevic et al., 2006). While oxygen is the most important respiratory gas controlling ventilation in fishes, carbon dioxide and ammonia are also able to induce a ventilatory response (Gilmour, 2001; Zhang and Wood, 2009). High levels of ammonia and/or carbon dioxide lead to hyperventilation in fish (Randall and Ip, 2006; Zhang and Wood, 2009). For this reason, increases in the concentration of ammonia in the water can be used as a stimulant of ventilation (Randall and Ip, 2006; Vulesevic et al., 2006; Wu, 2002).

The typical response of a fish to acute hypoxia is very similar to those seen in high ammonia or hypercapnia (high CO_2 concentration). The first response of a fish is to try to move away from the low oxygen environment into a normoxic one (Farrell and Richards, 2009; Wu, 2002). Other

immediate responses to hypoxia include increasing ventilatory frequency and/or volume to increase water flow over the gills to provide sufficient oxygen uptake and transfer to the tissues of the fish in order to maintain a constant metabolic rate when experiencing decreasing water PO_2 (Evans et al., 2005; Randall and Ip, 2006; Tzaneva and Perry, 2010; Wilson and Laurent, 2002; Zhang and Wood, 2009).

Responses to Chronic Hypoxia

Chronic hypoxia exposure can lead to remodelling of the gill to increase exchange surface area so that the fish is able to uptake the maximum possible amount of oxygen from the limited amount found in the water. However, this increase in surface area can lead to ion loss across the gills in freshwater, or unwanted ion uptake in seawater. This is known as the ionoregulatory compromise (Randall and Ip, 2006; Tzaneva et al., 2011; Tzaneva and Perry, 2010; Wilson and Laurent, 2002; Wood and Grosell, 2015).

In many animals, genes involved in the hypoxic response are regulated by a DNA-binding protein known as hypoxia-inducible factor 1 (HIF-1). HIF-1 is made up of an α and a β subunit. HIF-1α is expressed and subsequently degraded in the cytoplasm of cells when O_2 levels are high; whereas HIF-1β is found in the nucleus of cells under both normoxic and

hypoxic conditions. Because of this, formation of the HIF-1 complex is dependent on the levels of the O_2-labile HIF-1α 1 isoform (Feng et al., 2009; Giaccia et al., 2003; Nikinmaa and Rees, 2005; Salceda and Caro, 1997; van Heerden et al., 2004). The HIF-1α is rapidly ubiquinated by prolyl hydroxylase in the presence of oxygen for subsequent degradation; however in hypoxic or anoxic conditions hydroxylation reactions are inhibited, causing HIF-1α to rapidly accumulate, localize to the nucleus, dimerize with HIF-1β in the nucleus, and induce transcription and translation of hypoxic response genes (Feng et al., 2009; Guillemin and Krproasnow, 1997; Nikinmaa and Rees, 2005; Salceda and Caro, 1997; Semenza, 1999; Wu, 2002). These genes include vascular endothelial growth factor, glucose transporters, and enolase which respectively allow for blood vessel proliferation, glucose transport, and increased glycolysis, all of which are important for a fish to cope with hypoxia (Arany et al., 1996; Ebert et al., 1995; Guillemin and Krasnow, 1997; Okino et al., 1998; Semenza et al., 1996; Wu, 2002). In addition, signal transduction is initiated in response to hypoxia due to changes in the levels of cytosolic O_2. The signals can lead to downregulation of energy demand and energy supply pathways, such as protein synthesis and degradation, glucose formation, and maintenance of electrochemical gradients to maintain energy balance under anaerobic conditions (Guillemin and Krasnow, 1997; Lushchak, 2011; Wu, 2002).

IV. Copper

The effect of eutrophication on aquatic organisms can be exacerbated by the presence of other pollutants such as metals, which can be toxic at high levels. These pollutants can enter the water through mechanisms such as leaching or as part of effluent due to mining or similar industrial processes and can be found both dissolved in water in various ionic forms as well as deposited in sediment beds (Malekpouri et al., 2016; Moiseenko and Kudryavtseva, 2001). A decline in the level of PO_2 in water is the main factor that leads to the release of metal ions from sediment beds. One such metal that may pose an ecological problem is copper, a pollutant becoming increasingly prevalent due to increased industrial and agricultural activities near to aquatic environments. Copper typically enters water bodies as runoff from mining or agrochemicals (Malekpouri et al., 2016; Moiseenko and Kudryavtseva, 2001; Mustafa et al., 2012). The two species of copper thought to contribute the most to copper toxicity are the unliganded free copper ion Cu^{2+} and the positively charged $Cu(OH)^+$ cation (United States Environmental Protection Agency, 2003; Wang et al., 2016). Many factors can contribute to the speciation of copper, including water temperature, pH, and salinity (Blanchard and Grosell, 2006).

Copper is an essential micronutrient for all aerobic organisms due to its role in the structure and activity of cytochrome *c* oxidase; it is also involved in numerous redox reactions (such as a component of superoxide dismutase), bone formation, connective tissue development, and cardiac function (Feng et al., 2009; Grosell and Wood, 2002; Hartz and Deutsch, 1972; Monteiro et al., 2012; van Heerden et al., 2004). However, it has toxic effects at high concentrations as its redox properties can generate harmful reactive oxygen species (Carvalho and Fernandes, 2006; Feng et al., 2009; Grosell and Wood, 2002; van Heerden et al., 2004). Additionally, copper can bind to histidine, cysteine, and methionine protein residues, which can lead to protein dysfunction (Grosell and Wood, 2002).

Gills play a role in essential metal ion uptake from the surrounding water; however in high concentrations, copper ions can cause gill damage and local tissue hypoxia (Cerqueira and Fernandes, 2002; Grosell and Wood, 2002; van Heerden et al., 2004). Copper has been seen to inhibit gill Na^+/K^+ ATPase activity by competitive inhibition with sodium, which can have deleterious effects on the affected fish (Craig et al., 2009; Grosell and Wood, 2002; Lee et al., 2010; van Heerden et al., 2004). Copper has also been seen to block ion channels, damage mitochondria, and suppress Na+/K+ activity in rainbow trout and killifish, leading to ion imbalance (Blanchard and Grosell, 2005). Additionally, in high concentrations, copper

has been seen to induce a response similar to that caused by hypoxia in killifish (*Fundulus heteroclitus*); namely an increase in gill surface area in addition to expression of hypoxic-response genes (Jabari and McClelland, Unpublished; Li and McClelland, Unpublished; Martin et al., 2005).

V. Copper and Hypoxia: Common and Divergent Effects in Fishes

Copper is involved in the normal hypoxic response, as it is required for HIF-1α and HIF-1β to complex together and for HIF-1 to bind to target genes via the hypoxia-responsive element (HRE) (Feng et al., 2009; Qiu et al., 2012). Not surprisingly, copper exposure has been seen to increase the relative concentration of HIF-1α in the cytosol of gills in rainbow trout under normoxic conditions. (Martin et al., 2005; van Heerden et al., 2004). It is believed that this is due to the fact that copper not only increases binding activity of HIF-1 but also, like other transition metals, has the ability to stabilize and block degradation of the HIF-1α protein (degraded in normoxic conditions) (Feng et al., 2009; Martin et al., 2005; Nikinmaa and Rees, 2005; Qiu et al., 2012; van Heerden et al., 2004). It has been proposed that copper affects the activity of prolyl hydroxylases, such as HIF-1α prolyl-4 hydroxylase, which typically ubiquitinate the protein for degradation in normoxic conditions (Feng et al., 2009; Martin et al., 2005; Qiu et al., 2012). When stabilized by copper, HIF-1α accumulates and has been shown to

initiate a hypoxic-like response in some fishes which consists of gill remodeling to increase surface area (Martin et al., 2005; Nikinmaa and Rees, 2005; Qiu et al., 2012; Sollid et al., 2006; van Heerden et al., 2004).

Previous work in our lab on killifish has shown that exposure to chronic simultaneous hypoxia and copper blunts the hypoxic-ventilatory response, indicating that copper may be reducing the ability of the fish to detect hypoxia, thus reducing the ventilatory response (Baker and McClelland, Unpublished; Jabari and McClelland, Unpublished; Li and McClelland, Unpublished). Additionally, while both hypoxia and copper can induce oxidative stress, when fish were exposed to the combination of these stressors, no synergistic effect was seen (Ransberry et al., 2016). Cu and hypoxia have also been seen to have both synergistic and antagonistic effects in cyclooxygenase enzymes in rainbow trout with variation based upon tissue type and specific COX enzyme (Sappal et al., 2016).

Additionally, it is possible that copper may interact with NECs of the gills of fishes and affect the ability of the fish to detect ventilatory stimuli. For example, one study found that chronic sublethal copper exposure led to a decrease in the number of functional NECs on the gills of Nile Tilapia (*Oreochromis niloticus*) (Monteiro et al., 2012). The basal portions of the lamellae were seen to be physically damaged, while the external portion was less damaged, suggesting a higher resistance and/or compensatory

response to toxins such as copper in the apical portion of the lamellae (Monteiro et al., 2012). Additionally, it has been seen that Cu^{2+} ions can inhibit Ca^{2+} ion channels due to the similar charge and size of the ions (Lacinová, 2012; Lu et al., 2009).

Due to the various ways in which copper can interact with the hypoxic response in fish, it is important to understand how it can play a role in the control of respiration in fish, as this is one of the most important aspects of the hypoxic response. This thesis will seek to further the understanding of the interactions of the environmental stressors copper and hypoxia and how their interactions can affect the control of ventilation in fish.

Objectives and Hypotheses:

The objective of this thesis was to investigate the effects of aquatic copper exposure on the ventilatory system in killifish. Specifically,

Objective 1) Examine the acute ventilatory response to known ventilatory stimuli and changes in response to both chronic and acute copper exposure.

Objective 2) Examine the changes in gross gill morphometry in response to either chronic copper or hypoxia or a combination of the two stressors.

Objective 3) Examine the changes in chemosensory cell morphometry after chronic exposure to either copper or hypoxia or a combination of the two stressors.

Hypothesis 1) The acute ventilatory response of killifish to a variety of respiratory stimuli will be blunted after exposure to copper, whether it be acute or chronic exposure.

Hypothesis 2) Chronic hypoxia will lead to an increase in gill surface area, while copper will block this response. Combined hypoxia and copper exposure will be similar to copper exposure alone.

Hypothesis 3) Hypoxia will lead to an increase in NEC projection area, while copper will lead to a decrease in NEC projection area due to previously observed NEC toxicity. Combined hypoxia and copper exposure will be similar to copper exposure alone.

Chapter 2: The effects of copper on the acute ventilatory drive of killifish

Abstract

Aquatic organisms are exposed to multiple stressors in their natural habitats. The effects of copper on the acute ventilatory drive of killifish were examined. It was found that copper blunts the acute ventilatory drive of killifish, reducing their ability to respond to stimulants of ventilation, namely hypoxia, ammonia, and the combination of the two stimulants. Additionally, various morphometric parameters of the gill were examined. Filament length was seen to increase after hypoxia exposure; however when combined with copper exposure this result was not seen. Finally, neuroepithelial cell projection area was examined, and it was seen that copper causes a reduction in cell projection area, an effect which hypoxia does not have a protective effect over. Overall, it was seen that copper prevents killifish from mounting appropriate physiological responses to environmental stressors such as high ammonia and/or low oxygen.

Introduction

In recent years, nutrient runoff into aquatic ecosystems has become an increasing issue as increased nutrient load can lead to microbial overgrowth and ensuing consumption of oxygen by these bacteria. This is known as eutrophication (Diaz and Rosenberg, 1995; Farrell and Richards, 2009; Nikinmaa, 2013; Wu, 2002). Anthropogenic inputs can also cause increased levels of metals, such as copper, in these same ecosystems due to the fact that population density, and therefore industrial activity, is highest near to coastal areas (Moiseenko and Kudryavtseva, 2001; Mustafa et al., 2012; Nikinmaa, 2013; Nixon, 1995). Although copper is an essential micronutrient, it is toxic at high levels. Copper can enter aquatic habitats through a variety of routes, most notably as industrial effluent or mining and farming runoff (Grosell and Wood, 2002; Moiseenko and Kudryavtseva, 2001; Mustafa et al., 2012). While the effects of these stressors alone are relatively well characterised, what is less well studied is the toxicological effects of combined stressors such as copper and hypoxia (McBryan et al., 2016).

Fish that experience hypoxia have numerous methods of coping with this environmental stressor when experiencing it both acutely and chronically. Neuroepithelial cells (NECs), located on the filament tips of the

gill, are chemosensory cells that allow for the detection of changes in water chemistry, such as low oxygen (Jonz and Nurse, 2003; Zhang et al., 2011). If a fish is not able to simply relocate to an area of appropriate oxygen level, they will begin to hyperventilate to increase water flow over the gills to provide sufficient oxygen in order to maintain aerobic metabolism (Evans et al., 2005; Randall and Ip, 2006; Tzaneva et al., 2011). Prolonged hypoxia exposure can lead to remodelling of the gill to increase surface area to facilitate elevated gas exchange (Randall and Ip, 2006; Tzaneva et al., 2011; Tzaneva and Perry, 2010; Wilson and Laurent, 2002).

High levels of waterborne copper can have negative effects on fishes, such as generating reactive oxygen species, initiating protein dysfunction, and suppressing Na+/K+ ATPase activity leading to ion imbalance (Blanchard and Grosell, 2005; Cerqueira and Fernandes, 2002; Craig et al., 2009; Grosell and Wood, 2002; van Heerden et al., 2004). Copper is involved in the normal hypoxic response due to its requirement for the complexation of the hypoxic response proteins HIF-1α and HIF-1β. Copper has been seen previously to instigate gill remodelling due to its role in the HIF pathway (Qiu et al., 2012; van Heerden et al., 2004). It has also been shown that sublethal copper exposure can damage NECs of Nile Tilapia (*Oreochromis niloticus*) and lead to NEC loss (Monteiro et al., 2012). Thus, there are likely multiple effects of interacting hypoxia and copper

exposure on the structure and function of the ventilatory system in fishes. Negative impacts of copper on NECs could lead to dysfunction of the hypoxic ventilatory response due to improper oxygen sensing in fish with damaged NECs.

This study investigates the effects of copper on the structure and function of the ventilatory system of killifish (*Fundulus heteroclitus*). I also examined the response to chronic Cu, hypoxia, and combined Cu and hypoxia on gill morphometry and changes in NECs. To do this, *F. heteroclitus,* a euryhaline teleost found along the east coast of North America that is tolerant to changes in salinity and hypoxia was used as a test organism (Burnett et al., 2007; Ransberry et al., 2016). Due to their intertidal habitat, often near to highly populated areas, killifish are likely to experience combined stressors such as copper and hypoxia as a result of anthropogenic activity (Burnett et al., 2007). Killifish were exposed to copper and hypoxia, as well as a combination of the two stressors. Breathing rates, gill morphometry, and neuroepithelial cell (NEC) size were quantified to assess the toxic effects of copper on ventilation in killifish.

Methods

Exposures

Adult killifish of mixed sexes were collected from the wild saltwater environment (Aquatic Research Organisms, Hampton, New Hampshire) and acclimated to fresh water for a minimum of two weeks before experimental exposures. Killifish were transferred from 35 ppt to 0 ppt by gradually diluting the sea water with dechlorinated Hamilton, ON tap water (moderately hard: $[Na^+]$=0.6 mequiv/L, $[Cl^-]$=0.8 mequiv/L, $[Ca^{2+}]$=1.8 mequiv/L, $[Mg^{2+}]$=0.3 mequiv/L mM, $[K^+]$=0.05 mequiv/L, titration alkalinity 2.1 mequiv/L, pH ~8.3, hardness, ~140 mg/L as $CaCO_3$ equivalents) (Ransberry et al., 2015). During acclimation, fish were fed daily with commercial tropical fish food (Big Al's Aquarium Supercenter, Woodbridge, Ontario). Killifish were then exposed to either normoxia, (9.3 mg/L O_2), hypoxia (1.8 mg/LO_2), 118±4.5 µg/L copper, or both hypoxia and copper combined for 96 hours at 19°C. Aquatic hypoxia was achieved by bubbling nitrogen through water and maintained using an O_2 regulation system (Qubit Systems, Kingston, Ontario). Water was 100% renewed every 24 hours. All fish were fasted for 24 hours prior to and for the duration of the 96 hour exposures.

In a separate experiment, killifish were acclimated over 2 weeks to the calcium equivalents of 100% seawater (9.3 mM $CaCl_2$). These fish were

then exposed to either chronic or acute copper and then acute hypoxia to investigate the protective effects of calcium ions against copper. After measurement of ventilation, fish were immediately sacrificed for measurement of gill copper accumulation.

Measurement of breathing rate

Fish were exposed to one of three conditions known to stimulate increased ventilation – 1.9 mg/L O_2 (hypoxia), 100 mM aquatic ammonia, or both ammonia and hypoxia combined and compared to controls measured in normoxic conditions with no added ammonia. Ventilation was quantified prior to and after 5, 15, and 30 minutes of acute exposure. The greatest change in ventilatory rate was seen after 15 minutes and so only those values and resting values are presented.

Opercular movements were quantified electronically using a breathing recording chamber constructed at McMaster University used to detect changes in impedance associated with movements of the operculum as described previously (Drummond, 1977; Vulesevic et al., 2006). To measure breathing rate, fish were placed individually in a cylindrical glass tube (5 by 15 cm; volume=295 mL) submerged in a tank filled with 8L of fresh, dechlorinated water. The apparatus was sealed at each end with rubber stoppers. Standard copper electrodes protruded on the internal side

of the stoppers and plastic mesh was used to prevent the fish from directly contacting the electrodes. Water flow was maintained through the tube using an Eheim Universal Pump (Deizisau, Germany) to maintain constant oxygen levels within the tube. To minimize interference of electrical recordings, the pump was shut off during recording periods. Measurements were recorded at 10 Hz and passed through a signal conditioner. Data was recorded using an analog to digital converter and data acquisition software (Expedata, Sable Systems, North Las Vegas, Nevada). Each peak of the electronic trace was taken to represent a single breath. Relative ventilatory amplitude was quantified electronically by finding the ratio of amplitude after acute exposure to the amplitude prior to any acute exposure to ventilatory stimuli.

Light microscopy of gill morphometry

In a separate experiment, fish were stunned with a blow to the head and then decapitated. Whole gill baskets were dissected from the fish and fixed at 4°C using a 2% paraformaldehyde, 2% glutaraldehyde solution in 0.2M PBS for 24 hours. Images were taken of whole gill arches and individual filaments and analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland) to quantify filament length, lamellar density and lamellar area. Gill filament length was measured from filament tip to gill

arch and multiplied by 1.15 to account for curling as recommended previously (Hughes, 1966). Several filaments were measured on both sides of all four gill arches to obtain measurements representative of the entire gill. Lamellar density was calculated by measuring the length of filament containing ten lamellae three times on the first gill arch. Five transverse sections through three filaments were used to quantify lamellar area, which was then found by measuring the area of each lamellar pair and multiplying by two to account for each lamellae having two sides.

Confocal microscopy of NECs

In a separate experiment, fish were stunned with a blow to the head and then decapitated. Procedures for immunohistochemical labelling were modified from (Jonz and Nurse, 2003). Whole gill baskets were dissected from the fish and fixed for 24 hours at 4°C using 4% paraformaldehyde in 0.1M phosphate-buffered solution (PBS) containing (in mM) NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7, KH_2PO_4, 1.5; pH 7.8. Gill baskets were then subsequently permeabilized for 24 hours in 1% fetal bovine serum and 0.5% Triton X-100 in 0.1 M PBS. Single gill arches were separated and labelled with anti-serotonin (Sigma-Aldrich, St. Louis, Missouri), anti-synaptic vesicle protein 2 (Developmental Studies Hybriodoma Bank, Iowa City, Iowa), and anti-zn-12 (Developmental Studies Hybridoma Bank, Iowa City,

Iowa) antibodies before being rinsed in PBS and further labelled with secondary antibodies for one hour in darkness (FITC from Jackson Laboratories, Bar Harbour, Maine and Alexa594 from Life Technologies, Burlington, Ontario). Gill arches were then rinsed in PBS and mounted on glass microscope slides in Vectashield (Vector Laboratores, Burlingame, California) to prevent photobleaching. Images were taken using a confocal microscope (Nikon, Tokyo, Japan) in 1 μm thick sections. Maximum intensity projections and average intensity projection images were analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland) to quantify individual NEC surface area.

Measurement of Gill Copper Accumulation

After quantification of breathing rate, fish were stunned with a blow to the head and then decapitated. Gill baskets were removed and placed into pre-weighed tubes containing 2N trace metal grade nitric acid (Fisher Scientific, Ottawa, Ontario). The tubes containing the gills were placed into a 60°C water bath for 48 hours, with vigorous vortexing after 24 hours. Samples were then appropriately diluted and analyzed using a Graphite Furnace Atomic Absorption Spectroscopy (GFAAS, Spectra AA 220Z, Varian Palo Alto, California). A 40 μg/L Cu standard was used for

comparison, as well as an Environment Canada standard (TM 15-2, Gatineau, Quebec).

Statistical analysis

All data are expressed as means ± standard error of the mean (SEM). Analyses of variance (ANOVA) or Analyses of Covariance (ANCOVA) followed by post-hoc Tukey's tests were performed to evaluate potential differences between treatment groups as appropriate (control, copper, hypoxia, and combined copper and hypoxia). For all tests, a p-value ≤0.05 was considered statistically significant. All statistical analyses were performed using Minitab 16 (State College, PA, USA).

Results

Fish weights

The body weights of fish used in the gill copper accumulation experiment did not significantly between treatments (p=0.408). In the gill morphometry experiment, fish weights did differ between treatment groups (p=0.034). Control fish were found to have a mass of 6.11±0.87 g, fish exposed to 96h of copper had a mass of 5.15±0.57 g, fish exposed to 96h of hypoxia had a mass of 4.06±0.36 g, and fish exposed to 96h of combined copper and hypoxia had a mass of 4.74±0.49 g. In the NEC experiment, fish weights also differed between treatment groups (p=0.022) – control fish weighed 1.51±0.20 g, 96h copper exposed fish weighed 2.52±0.45 g, 96h hypoxia exposed fish weighed 1.73±0.28 g, and 96h combined copper and hypoxia exposed fish weighed 3.10±0.58 g.

Water chemistry and gill copper accumulation

For the 96 hour exposures, the average measured dissolved Cu concentrations in exposure water were found to be 0.41±0.02 µg/L for control (9.3 mg/L O_2) and hypoxia (1.9 mg/L O_2) treatments and 118±4.5 µg/L for copper treatments.

Gill copper loads after 96 hour exposures (**Figure 2.1**) were found to be 0.89±0.05 µg of copper per gram of tissue in control fish and 1.03±0.07 µg of copper per gram of tissue in hypoxia exposed fish. These values were not significantly different. Fish chronically exposed to copper alone were found to have 2.42±0.08 µg/g tissue, while fish exposed chronically to both copper and hypoxia had a similar value of 3.22±0.29 µg of copper per gram of tissue. Both copper and combined copper and hypoxia exposed fish had significantly higher copper accumulation than either controls or fish exposed to hypoxia alone (p=0.000). Finally, calcium had a protective effect against copper accumulation in the gills – fish exposed to 96h of calcium were found to have 1.57±0.18 µg/g tissue. This value is significantly less than either copper or combined copper and hypoxia exposed fish (p=0.014) though it is significantly greater than either control fish or fish exposed to 96h of hypoxia (p=0.014).

Breathing Rates

Resting breathing rates

Ventilatory frequency was determined in all fish in water with no added Cu before any acute exposures to elicit ventilatory drive (**Figure 2.2**). Resting ventilatory frequency was not significantly different between control

fish, at 112±3 breaths/min (N=19), compared to fish exposed to copper for 96 hours, fish exposed to both copper and hypoxia for 96 hours, or fish exposed to calcium for 96 hours (117±2 breaths/min (N=15), 107±2 breaths/min (N=20), and 113±5 breaths/min (N=5)). However, 96 hours of chronic hypoxia elevated resting breathing rate (p=0.000) to 153±6 breaths/min (N=19) even in normoxic copper-free water.

Acute hypoxia changes in ventilatory frequencies

Control killifish exposed to acute hypoxia markedly and significantly increased their breathing rate (**Figure 2.3**). When compared to ventilation of 104±1 breaths/min at rest in the period before acute hypoxia was added to the test water, at 15 minutes of hypoxia breathing increased to 152±8 breaths/min (p=0.001). However, when tested in water containing 100 µg/L copper, control fish showed no significant increase in ventilation from 108±6 breaths/min prior to and 131±11 breaths/min after 15 minutes of hypoxia. In fish chronically exposed to 96 hours of 100 µg/L copper, hypoxia did not induce an increase in ventilation, neither when tested in water without added Cu (105±5 breaths/min at rest and 112 4 breaths/min after hypoxia) nor when tested in water with 100 µg/L copper added (105±4 breaths/min at rest and 119±5 breaths/min after 15 minutes of hypoxia). The

96 hour hypoxia exposed group had a significantly higher resting breathing rate compared to the same chronic treatment tested in acute Cu, with breathing rates of 163±7 and 101±2 breaths/min respectively (p=0.000).

In the 96h hypoxia exposed fish, ventilatory rates did not increase significantly in response to 15 minutes of acute hypoxia (183±8 breaths/min) when tested in water with no added Cu. These ventilatory rates were 45% higher than fish tested in water with added Cu (p=0.000) However, acute Cu exposure reduced resting ventilatory rates by 38% compared to rates in water with no added Cu (163±7 versus 101±2 breaths/min). In acute Cu, this group significantly increased ventilation by 25% when exposed to 15 minutes of acute hypoxia to 126±6 breaths/min (p=0.005), indicating that chronic hypoxia exposure allows the fish to overcome the acute effects of copper. Finally, when fish were exposed for 96h to both hypoxia and 100 µg/L copper combined, resting rates were unaffected, and there was no significant elevation in breathing rate when they were subsequently exposed to acute hypoxia – fish tested in water with no added Cu had a resting breathing rate of 108±6 breaths/min and a breathing rate of 121±1 breaths/min after 15 minutes of acute hypoxia, while those fish tested in copper-containing water had a resting breathing rate of 109±7 breaths/min and a breathing rate of 120±11 breaths/min after 15 minutes of acute hypoxia.

Fish exposed to calcium for 96h saw no change in breathing rate when subsequently exposed to acute hypoxia in water with 100 µg/L Cu added (**Figure 2.4**), with breathing rates of 114±5 breaths/min before and 130±8 after hypoxia exposure. Fish exposed to both calcium and copper for 96h also saw no change in breathing rate when exposed to acute hypoxia, with breathing rates of 102±6 breaths/min before and 96±7 breaths/min after.

Changes in ventilatory frequency in response to acute ammonia

Acute exposure to 100 mM ammonia was used to stimulate ventilation and tested in water with and without added Cu (**Figure 2.5**). Control fish tested in water with no added Cu were exposed to aquatic ammonia and showed a significant 15% increase in their breathing rate, from 109±2 breaths/min at rest to 125±3 after 15 minutes of acute ammonia exposure (p=0.002). However, control fish tested in water with 100 µg/L Cu added did not significantly elevate their breathing rate, with a resting breathing rate of 116±3 breaths/min and a breathing rate of 127±4 breaths/min after ammonia exposure. Fish chronically exposed to 100 µg/L copper that were tested water with no added Cu were also able to significantly elevate their breathing rate by 9% in response to acute

ammonia exposure, from 119±2 breaths/min at rest to 130±4 breaths/min after 15 minutes of ammonia exposure (p=0.039). However, 96h Cu-exposed fish showed no change in ventilation with 15 minutes of acute ammonia when tested in acute Cu (113±4 breaths/min before and 112±5 breaths/min after 15 minutes of acute ammonia exposure). Fish chronically exposed to hypoxia for 96h did not significantly elevate their breathing rate either when tested in water with no added Cu (116±6 before and 138±14 breath/min after 15 minutes of ammonia exposure) or with 100µg/L Cu (108±5 before and 159±8 breath/min after 15 minutes of ammonia exposure). However there was a 13% difference in breathing rate with ammonia between the fish tested under conditions with and without added Cu. Finally, fish chronically exposed to both copper and hypoxia combined showed no significant change in their breathing rates after 15 minutes of ammonia exposure whether they were tested water with or without Cu. Those fish tested in clean water had a resting breathing rate of 108±5 breaths/min and a breathing rate of 107±5 after 15 minutes of ammonia exposure, while those tested in copper-containing water had a resting breathing rate of 108±5 breaths/min and a breathing rate of 108±6 after 15 minutes of ammonia.

Changes in ventilatory frequency with combined acute hypoxia and ammonia

In control fish, combined acute exposure to hypoxia and ammonia leads to a dramatic and significant 40% elevation in breathing rate, from 126±9 breaths/min at rest to 175±10 after hypoxia and ammonia exposure (**Figure 2.6**). Absolute ventilatory rates with acute combined hypoxia and ammonia were 15% higher than hypoxia alone and 40% higher than ammonia alone. When control fish were water with Cu added, the increase in ventilation was blunted to 30% from 114±15 breaths/min at rest to 148±6 breaths/min (p=0.052). In contrast, fish chronically exposed for 96h to copper did not significantly change their breathing rate in response to combined hypoxia and ammonia in either water with no copper added (117±7 breaths/min versus 128±6 breaths/min and 97±13 breaths/min versus 126±2 breaths/min, for no added Cu and 100 μg/L Cu, respectively). Fish chronically exposed to 96h of hypoxia and tested in water with no Cu added did not alter their breathing rate in response to combined hypoxia and ammonia (166±15 breaths/min before and 157±14 breaths/min after 15 minutes of hypoxia and ammonia exposure). These ventilatory rates were significantly higher than their counterparts in fish tested in copper-containing water by 58% before and 35% after 15 minutes of combined hypoxia and ammonia exposure (p=0.010 and p=0.031 respectively).

Finally, fish chronically exposed for 96h to combined copper and hypoxia did not change breathing rates in response to acute hypoxia and ammonia. Fish tested in water with no added Cu had a breathing rate of 102±5 breaths/min before and 107±5breaths/min after 15 minutes of hypoxia and ammonia exposure; while fish tested in copper-containing water had a breathing rate of 105±5 breaths/min at rest and 115±6 breaths/min after hypoxia and ammonia exposure.

Relative breathing amplitude

Changes in the ventilatory amplitude of each ventilatory movement from rest to after stimulated ventilation were assessed for each experimental group (**Figures 2.7-2.9**). Breathing amplitudes did not change significantly across any treatment or exposure, with the exception of acute hypoxia and ammonia in fish chronically exposed to hypoxia and copper tested in water with no copper added. This group had a significant increase in relative breathing amplitude. (Relative amplitude at 15 minutes after acute hypoxia and ammonia compared to resting=2.6±0.40; p=0.023).

Gill Morphometry

Results (**Table 2.1**) were analyzed both weight-independently and –dependently. Gill filament length did not change with 96h exposures (2213±112 µm for controls, 2105±67 µm for copper exposed, 2050±77 µm for hypoxia exposed, and 1997±74 µm for fish exposed to both copper and hypoxia); however when these data were normalized to body mass significant differences were seen (**Figure 2.10**). Control fish (387.5±48.1 µm/g), copper exposed fish 421.9±31.4 µm/g), and copper and hypoxia exposed fish 399.8±41.2 µm/g) did not differ in their filament length; however hypoxia exposed fish had significantly longer filaments at 514.6.2±27.7 µm/g (p=0.043, p=0.046, and p=0.017 respectively).

Total filament number did not differ significantly between treatments when analyzed weight-independently or –dependently (**Figure 2.11**) with the exception of fish exposed to hypoxia for 96h having significantly more filaments per gram of body mass than control fish (167±11 filaments/g and 120±15 filaments/g respectively, p=0.026). The values for fish exposed to copper for 96h (139±13 filaments/g) and fish exposed to combined copper and hypoxia for 96h (130±11 filaments/g) did not differ significantly from either of these values.

Lamellar density did not significantly change between treatments whether analyzed weight-independently or weight-dependently (**Figure 2.12**). Functional lamellar surface area did not change when examined weight independently, though it did follow the same pattern as weight-dependent filament length (**Figure 2.13**), with an insignificant trend (p=0.089) of hypoxia exposed fish possessing a greater functional lamellar area (13744±1532 μm^2/g) than controls, copper exposed fish, and fish exposed to both copper and hypoxia (11524±804 μm^2/g, 11538±1532 μm^2/g, and 11775±1543 μm^2/g respectively). The same trend was true of non-functional lamellar cross-sectional area, with no differences observed independent of weight, and an insignificant trend (p=0.085) of hypoxia possessing a greater non-functional cross-sectional area when weight normalized (8223±617 μm^2/g) compared to controls, copper exposed fish, and fish exposed to both copper and hypoxia, which had non-functional cross-sectional areas of 7273±703 μm^2/g, 7345±390 μm^2/g, and 6304±1022 μm^2/g respectively (**Figure 2.14**).

Finally, neither weight-independent nor weight-normalized total filament functional area were seen to change across treatments, with the exception of fish exposed to hypoxia for 96h having a significantly greater total surface area than fish exposed to both copper and hypoxia for 96h (266±24 mm^2/g and 185±21 mm^2/g respectively, p=0.030) (**Figure 2.15**).

Weight normalized total surface area for control fish (237±12 mm^2/g) and fish exposed to copper for 96h (224±26 mm^2/g) did not differ significantly from either of these values.

Neuroepithelial Cells

No changes were observed in NEC shape or distribution after 96h of Cu, hypoxia, or Cu and hypoxia combined. The number of NECs (**Figure 2.16**) did not differ significantly between control fish or fish exposed to either copper or hypoxia for 96h (65.8±5.4, 58.8±12.9, and 49.1±9.6 cells per filament respectively); though fish exposed to both copper and hypoxia for 96h had significantly fewer NECs per filament than controls (45.4±3.2 cells per filament, p=0.014). The number of cells per filament did not differ significantly between fish exposed to copper or hypoxia for 96h and fish exposed to both copper and hypoxia for 96h. NEC projection area did not differ between groups; however when weight was added as a cofactor and an ANCOVA was performed, it was found that the average projection area per cell still does not differ between control and hypoxia fish (12.34±1.12 µm^2, mass=1.51±0.19 g and 15.70±1.53 µm^2, mass=2.52±0.45 g respectively). However, the average projection area of fish chronically exposed to copper and fish exposed chronically to both copper and hypoxia

were both significantly smaller than control fish, with projection areas of 9.59±2.16 µm^2 (p=0.002, mass=1.73±0.28 g) and 9.25±1.40 µm^2 (p=0.001, mass=3.10±0.58 g) respectively (**Figure 2.17**). When normalized to weight, these values become 8.64±1.03 µm^2/g for control fish, 10.39±2.06 µm^2/g for hypoxia exposed fish, 4.06±0.398 µm^2/g for copper exposed fish, and 3.40±0.47 µm^2/g for fish exposed simultaneously to copper and hypoxia. Representative images of NECs from all exposure groups can be found in **Figure 2.18**.

Discussion

In these experiments I found that copper exposure, whether it be chronic or acute, can impact the ability of fish to mount appropriate physiological responses to ventilatory stimuli. Fish exposed to 100 µg/L Cu for 96h showed no change in resting ventilatory rate when compared to control fish, nor did fish exposed to calcium for 96h. Fish exposed to 96h of hypoxia had a significantly higher ventilatory rate than controls at rest; however combined 96h exposure to hypoxia and copper resulted in a resting breathing rate that was not different from controls. While chronic copper exposure had no impact on resting breathing rates, when combined with chronic hypoxia exposure, copper did not allow the fish to increase resting breathing rate like they did when exposed to chronic hypoxia alone. Additionally, the presence of water-borne Cu significantly reduced the hyperventilatory response to known stimulants of ventilation – hypoxia, ammonia, and hypoxia and ammonia combined. These results all support the hypothesis that copper blunts the acute ventilatory drive. Gill morphometry was also impacted by chronic Cu and hypoxia exposure. Hypoxia increased filament length and lamellar area at a level approaching significance. Copper alone had no significant effect on gill structure, but when combined with hypoxia, copper dampened the hypoxia-induced gill remodelling, supporting the proposed hypothesis. Finally, it was observed

that chronic copper exposure led to a reduction in neuroepithelial cell projection area as hypothesized. This response was not altered when coupled with chronic hypoxia exposure. Contrary to the hypothesis however, 96h copper exposure did not result in an increase in NEC area.

Copper Accumulation

The gills of killifish exposed to Cu alone or Cu and hypoxia combined for 96h took up significant amounts of copper. This is consistent with previous studies that have shown that the gills are the primary target of Cu toxicity in teleosts (Blanchard and Grosell, 2006; Daglish and Nowak, 2002). As gills are the main site for ionoregulation in fish, it follows that they will also be the tissue most affected by metal toxicity (Evans et al., 2005; Lee et al., 2010). In one study, it was found that different levels of waterborne copper do not affect copper accumulation in the gills – gills accumulate the same levels of copper at both high and low water Cu concentrations. However, at high levels of copper, Cu begins to accumulate in the liver, indicating that there is a threshold level of copper that can be sequestered in the gills before it must be stored elsewhere in the body (Ransberry et al., 2015). It was also found that combined calcium and copper exposure led to a significant reduction in Cu accumulation in the gills compared to fish

exposed to Cu in water with no calcium added. Seawater has been seen previously to have a protective effect against copper exposure. It is thought that this is due to competition between ions (Blanchard and Grosell, 2006; Ransberry et al., 2015). It is evident from the results of this study that Ca^{2+} ions play an important role in this protective effect, though Ca^{2+} levels equivalent to those found in SW are not enough to fully protect the gills against Cu and reduce the level of Cu in the gill to that which is seen in control fish.

Breathing Rates

Control fish responded to acute hypoxia, ammonia, and combined ammonia and hypoxia exposure with hyperventilation as shown previously for multiple fish species (Randall and Ip, 2006; Tzaneva et al., 2011, 2011; Vulesevic et al., 2006; Zhang and Wood, 2009). However, when exposed to Cu acutely or for 96h, killifish were unable to mount the same increase in ventilation in response to these acute stimulants of ventilation. Both acute and chronic copper exposure reduced the ability of fish to hyperventilate in response to hypoxia, ammonia, and combined hypoxia and ammonia. The exception to this was in fish chronically exposed to hypoxia, who retained the ability to hyperventilate in response to subsequent acute hypoxia.

However, this only occurred when ventilation was tested in water with added Cu. This suggests that these fish have increased sensitivity to hypoxia that allows them to overcome the inhibitory effects of acute copper exposure. Finally, when fish were exposed chronically to calcium with or without added copper and then tested in water with copper added, they did not hyperventilate in response to subsequent acute hypoxia. This indicates that any protective effect of calcium is not potent enough to overcome the inhibitory effects of hyperventilation of copper.

Additionally, fish chronically exposed to hypoxia have an elevated breathing rate compared to all other groups. This suggests that copper exposure prevents the fish from elevating resting breathing rate when experiencing combined chronic copper and hypoxia exposure. Chronic sublethal copper exposure has been seen previously to cause hypoventilation in carp and killifish (De Boeck et al., 2007; Li and McClelland, Unpublished). The suppressive effects of copper on ventilation may explain why fish do not hyperventilate when exposed to stimulants of ventilation or chronic hypoxia in the presence of copper. Additionally, if fish were to hyperventilate while exposed to copper, this would increase water flow over the gills and could increase copper uptake. One study found that carp have higher sensitivity to the toxicity of cadmium when exposed to

hypoxia compared to normoxia, likely due to a higher breathing rate in hypoxia (Hattink et al., 2005).

Gill Morphometry

Plasticity of gill surface area is a response to hypoxia seen in many fish species, such as goldfish and carp, in order to increase surface area for gas exchange (Sollid et al., 2003; Tzaneva et al., 2011; Tzaneva and Perry, 2010). When exposed to moderate levels of hypoxia (1.9 mg/L O_2), it was found that 96h of hypoxia increases filament length and non-functional lamellar surface area. However, this change is not seen when chronic hypoxia is coupled with chronic copper exposure. The length of exposure may not have been long enough to elicit significant responses to any of the test conditions; previous studies investigating gill remodelling have lasted longer (Borowiec et al., 2015; Tzaneva et al., 2011; Tzaneva and Perry, 2010). While some species such as goldfish and carp adjust functional gill surface area by changing ILCM (Sollid et al., 2003; Tzaneva et al., 2011; Tzaneva and Perry, 2010), some species are able to increase functional SA through gill hypertrophy in response to hypoxia either by increasing filament length or functional lamellar surface area (Chapman et al., 2000; Sollid et al., 2003). Fishes adapted to hypoxic environments and

different modes of breathing can have similar strategies to cope with hypoxia, as it has been seen that air breathing fish can also increase lamellar size in response to hypoxia (Blank and Burggren, 2014). The length and degree of hypoxic exposure appears to play an important role in the remodelling that occurs. Our results suggest that killifish may adjust surface area in hypoxia by changing filament length. This is associated with a non-significant increase of non-functional cross sectional area of the filament as a larger tissue diameter is required to support the increased filament length. However, a longer exposure time would likely show more dramatic changes, as it has been seen previously that 28 days of hypoxia exposure at a similar level to this study caused a significant reduction in filament length, possibly to reduce long-term metabolic costs of ion transport (Borowiec et al., 2015). It is possible that in the short term, killifish increase surface area in order to facilitate gas exchange; however over time their response shifts to energy conservation leading to hypotrophy of gills.

Though fish can increase the surface area of their gills to facilitate increased functional surface area for gas exchange, this increased surface area also leads to greater ion transfer and uptake, which can have deleterious effects when fish are exposed to aquatic metals such as copper (Blanchard and Grosell, 2006; Mustafa et al., 2012; Sollid et al., 2003; Wood and Grosell, 2015). This may explain why fish are not increasing surface

area when exposed to combined chronic copper and hypoxia. Since gills serve as the major ionoregulatory organ in fishes, there is a balance between increasing surface area for gas exchange and increased ion loss to the hypoosmotic FW environment. Since copper is known to cause ionoregulatory disruptions in the gills of fishes through inhibition of Na+/K+ ATPase activity (Blanchard and Grosell, 2005; Craig et al., 2009; Grosell and Wood, 2002; van Heerden et al., 2004), it is possible that copper-induced dysregulation in ion transport overrides hypoxia-induced gill remodelling to increase surface area, rendering the fish unable to increase surface area in response to hypoxia. Furthermore, as killifish have a very high hypoxic tolerance (Borowiec et al., 2015; Burnett et al., 2007), it may be the case that killifish are able to not increase gill surface area in response to hypoxia in the presence of copper in order to maintain ionoregulatory balance and avoid oxidative stress associated with copper exposure.

Neuroepithelial Cells

NECs are thought to be the primary external oxygen sensor in fish, therefore their proper function is essential for fish to maintain homeostasis in challenging environments (Jonz and Nurse, 2006, 2003; Tzaneva and Perry, 2010). Since changes in ventilation are thought to be regulated at

least in part by NEC sensing of water chemistry, I hypothesized that these cells would change in morphology with Cu exposure after I found that copper blunts the acute ventilatory response of killifish. Additionally, since NEC size increases in zebrafish with 60 days of hypoxia (Jonz et al., 2004), I also hypothesized that hypoxia may mitigate the effects of copper exposure. Since cell projection area is thought to play a role in NEC ability to detect changes in water chemistry and/or transmit neural signals, increases or decreases in this measure may indicate an increase or decrease in sensitivity to water chemistry.

In this study, it was found that combined exposure to copper and hypoxia significantly reduces the number of neuroepithelial cells per gill filament compared to control fish. Additionally, it was found that copper exposure significantly reduces the projection area of NECs, even when combined with chronic hypoxia exposure. The hypotrophic effects of copper overrode any effects of hypoxia and reduced cell size to the levels of copper exposure alone when fish were exposed to 96h hypoxia and copper combined. Reduction in NEC size could be the cause of reduced sensitivity to stimulants of ventilation as the cells have less functional surface area available for the detection of changes in water chemistry. Additionally, it is possible that NEC size is correlated with neurotransmitter release onto

postsynaptic fibres, meaning that smaller cells are not able to initiate neural signalling as well as large cells (Jonz et al., 2004).

Since NECs use calcium as a signalling molecule to initiate neural transmission in response to hypoxia (Abdallah et al., 2015, 2012; Jonz et al., 2004; Zachar and Jonz, 2012), it is possible that copper may be interfering with this pathway. It has been seen previously that elemental Cu^{2+} ions can inhibit Ca^{2+} ion channels due their similar size and charge (Lacinová, 2012; Lu et al., 2009). This would explain why fish do not respond to stimulants of ventilation in the presence of copper, as proper calcium ion channel function is essential to NEC signalling (Abdallah et al., 2015, 2012; Jonz et al., 2004). The acute ventilatory response may be blunted by copper due to Cu^{2+} ions competitively blocking Ca^{2+} channels and thus not allowing Ca^{2+} signalling essential for proper NEC neural communication.

Conclusions

In this research I found that copper significantly affected acute ventilatory drive of killifish to ventilatory stimuli. Copper blunts the acute ventilatory response, prevents increases in filament length due to hypoxia, and causes hypotrophy of neuroepithelial cells. From this information, it can

be seen that copper has effects at many levels of biological organization, from the whole organism to the cellular levels, though direct links between responses at the cellular level to those at the whole animal level remain to be demonstrated. The inability to respond adequately to changes in water chemistry likely exacerbates the toxic effect of Cu previously defined. These data highlight the importance of examining the physiological responses to multiple stressors in order to predict and preserve ecosystem health.

Figure 2.1: Gill copper loads in response to 96h chronic exposures to control conditions or to Cu and hypoxia alone or in combination. Bars represent µg of copper per gram of wet weight of gill tissue. Values presented are means ± SEM. Values that do not share the same letter indicate significant difference (p≤0.05, N=6-10).
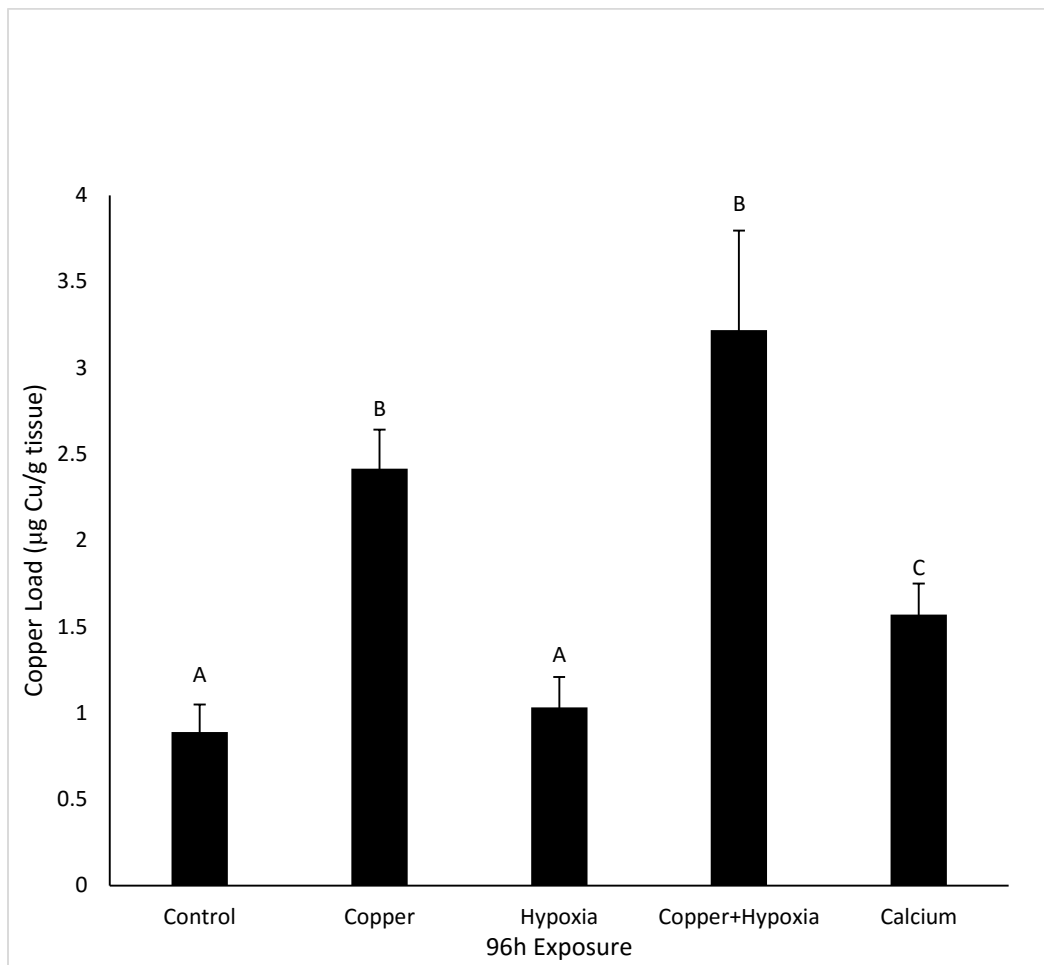
Figure 2.2: Resting ventilatory frequencies in killifish after 96h exposures to control conditions or Cu and hypoxia alone or in combination, or Ca. These breathing rates were determined in clean Cu-free water. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=18-20).
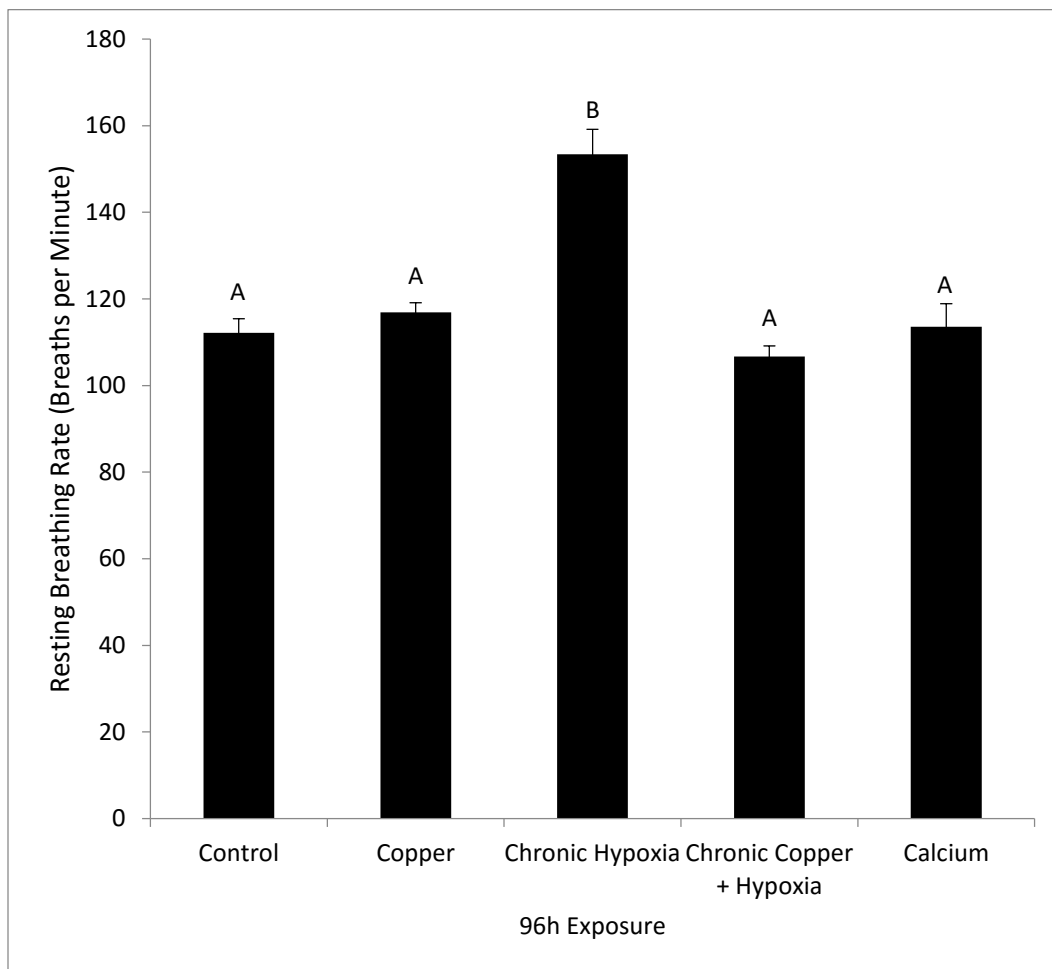
Figure 2.3: Breathing rates at rest and after 15 minutes of acute hypoxia for killifish exposed to acute copper, chronic copper, chronic hypoxia, or a combination of chronic copper and hypoxia. Values shown are means ± SEM. A * is used to indicate a significant difference between resting breathing rate and after 15 minutes of hypoxia, while a † is used to indicate a significant difference between groups at the same time point (p≤0.05, N=5 for all treatments).
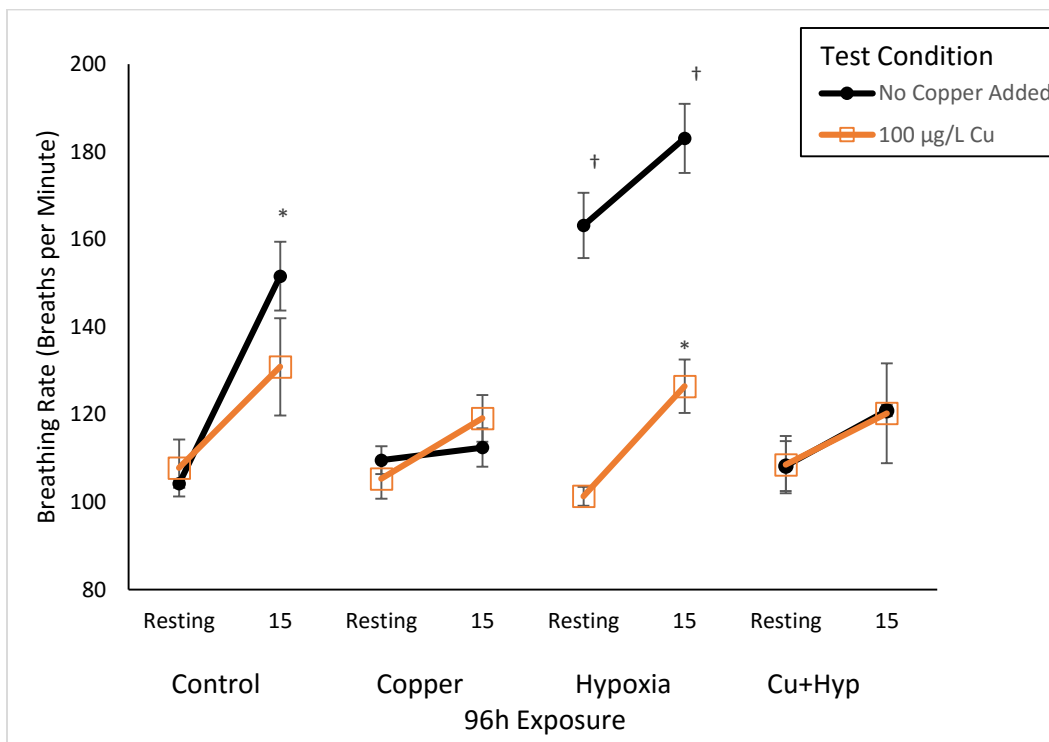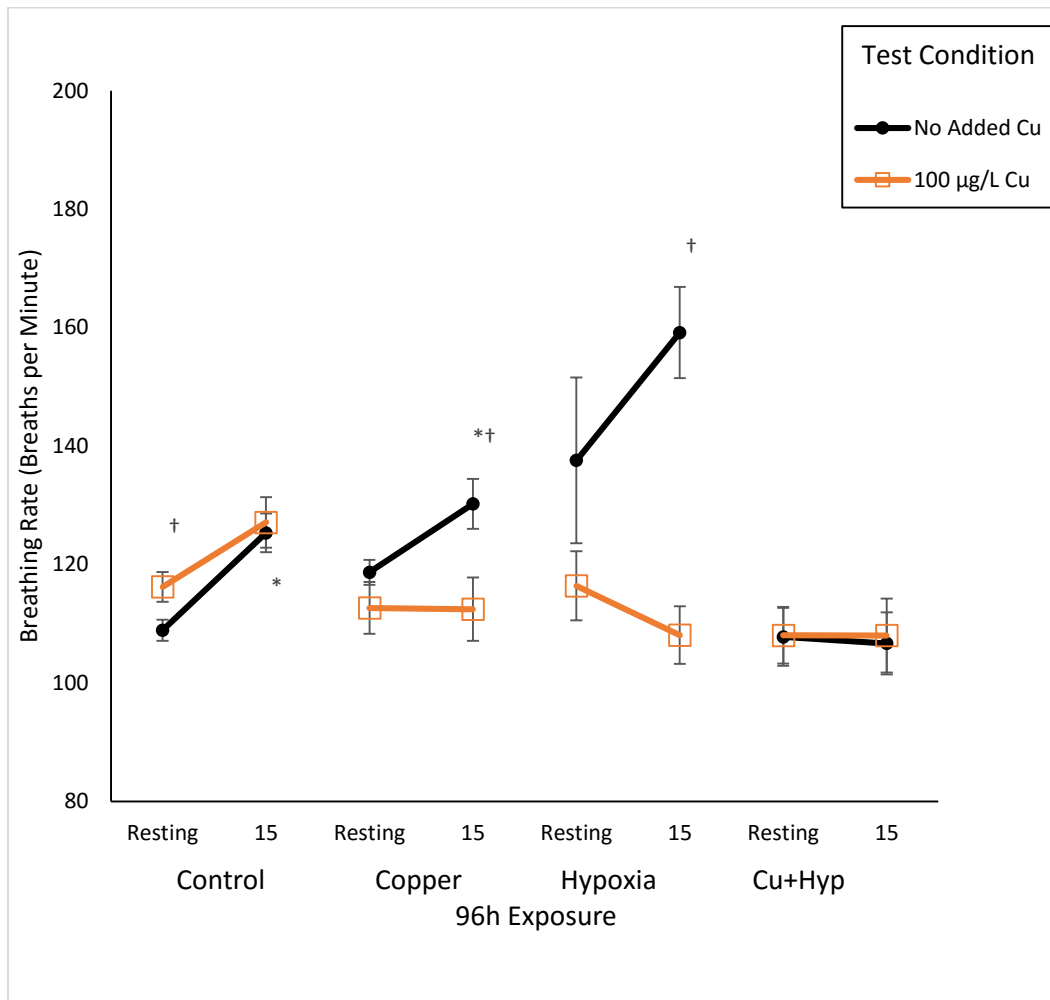
Figure 2.4: Breathing rates at rest and after 15 minutes of acute hypoxia for killifish exposed to 96h of calcium or 96h of combined calcium and copper. All fish were tested in 100 µg/L Cu. Values shown are means ± SEM. No significant differences were observed (p≤0.05, N=5 for all treatments).
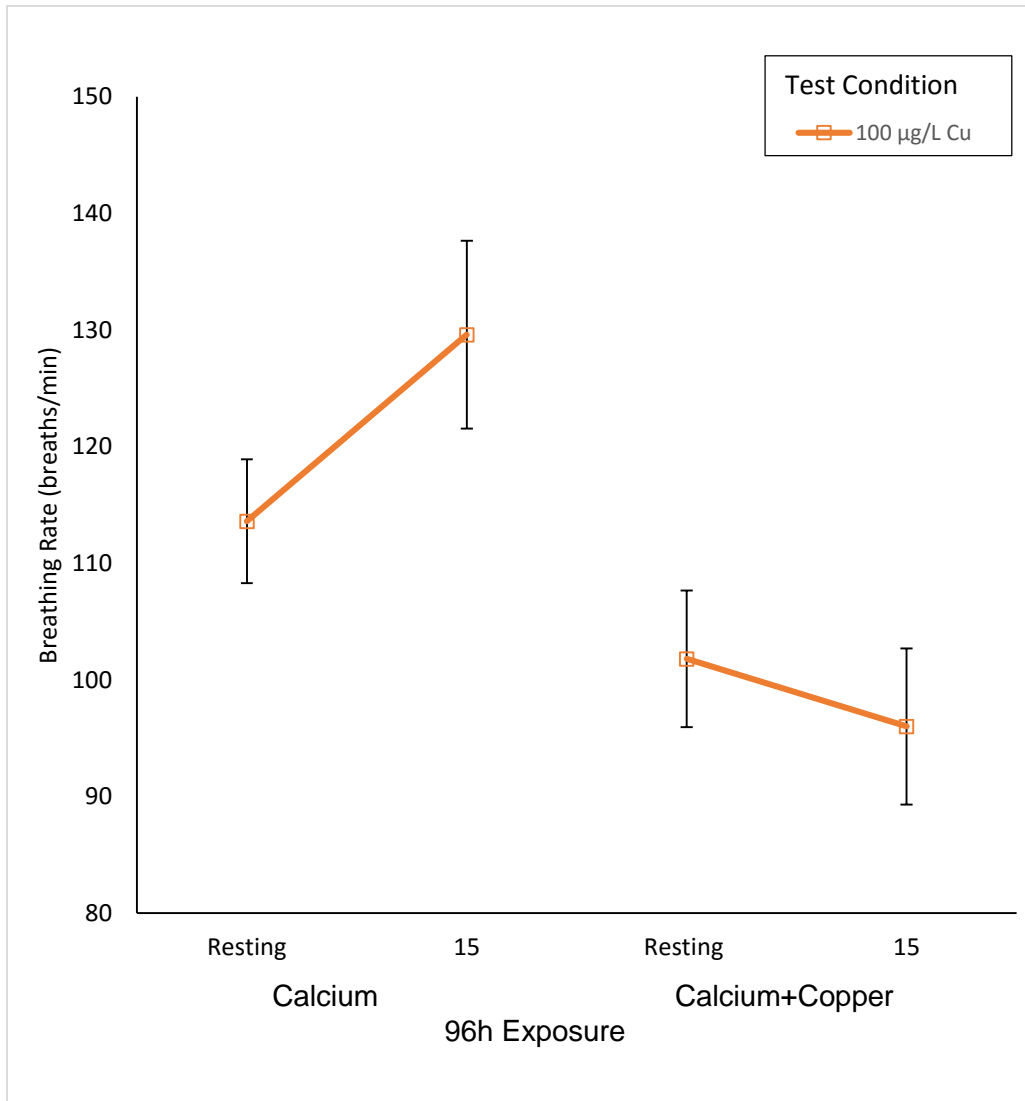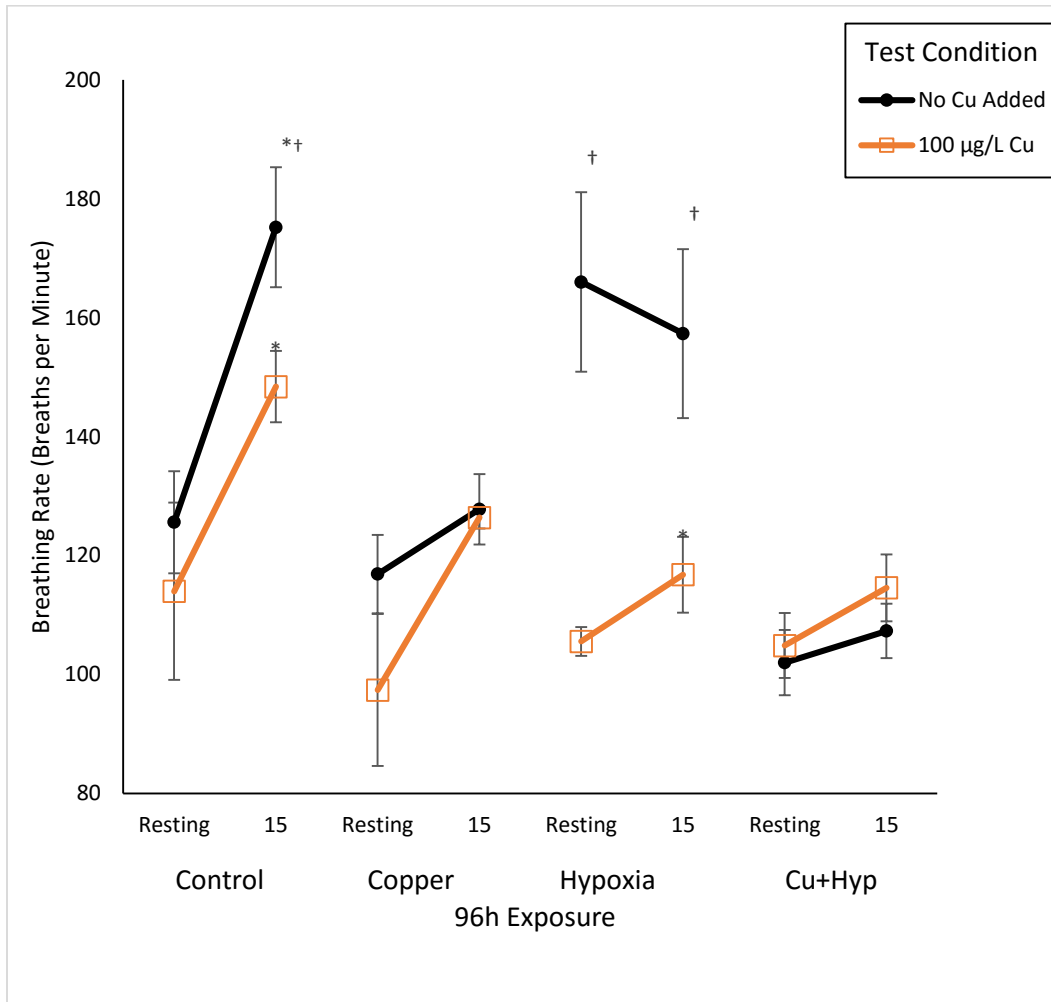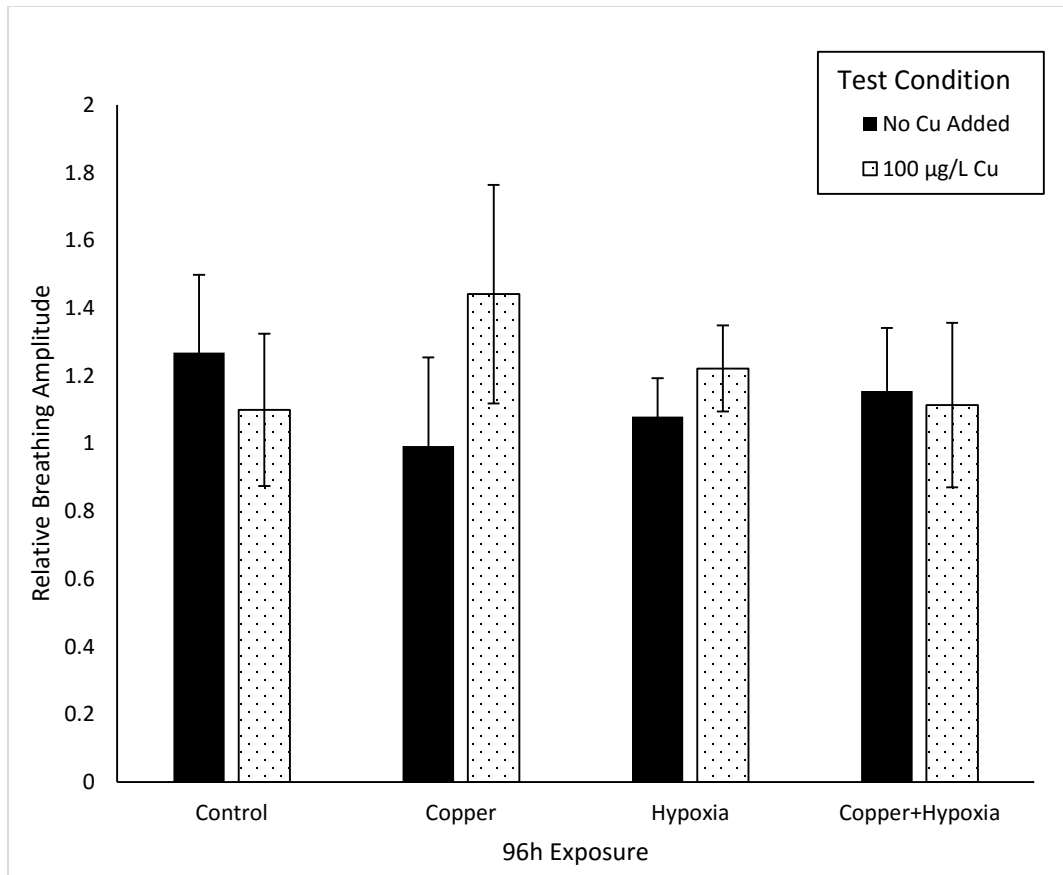
Figure 2.5: Breathing rates at rest and after 15 minutes of acute ammonia for killifish exposed to acute copper, chronic copper, chronic hypoxia, or a combination of chronic copper and hypoxia. Values shown are means ± SEM. A * is used to indicate a significant difference between resting breathing rate and after 15 minutes of hypoxia, while a † is used to indicate a significant difference between groups at the same time point (p≤0.05, N=5 for all treatments).

Figure 2.6: Breathing rates at rest and after 15 minutes of acute ammonia and hypoxia for killifish exposed to acute copper, chronic copper, chronic hypoxia, or a combination of chronic copper and hypoxia. Values shown are means ± SEM. A * is used to indicate a significant difference between resting breathing rate and after 15 minutes of hypoxia, while a † is used to indicate a significant difference between groups at the same time point (p≤0.05, N=5 for all treatments).
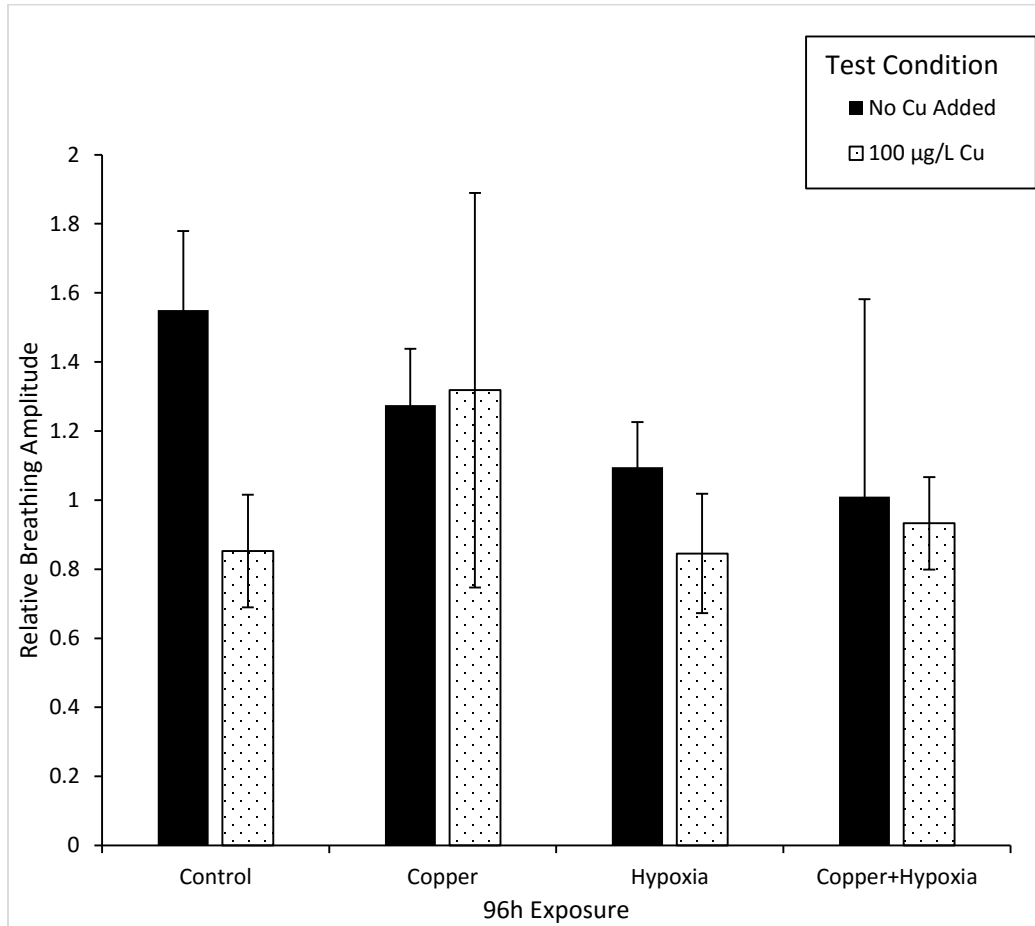
Figure 2.7: Relative changes in breathing amplitude between 15 minutes of acute hypoxia and at rest for killifish exposed to acute copper, chronic copper, chronic hypoxia, or a combination of chronic copper and hypoxia. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5 for all treatments).

Figure 2.8: Relative changes in breathing amplitude between 15 minutes of acute ammonia and at rest for killifish exposed to acute copper, chronic copper, chronic hypoxia, or a combination of chronic copper and hypoxia. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5 for all treatments).
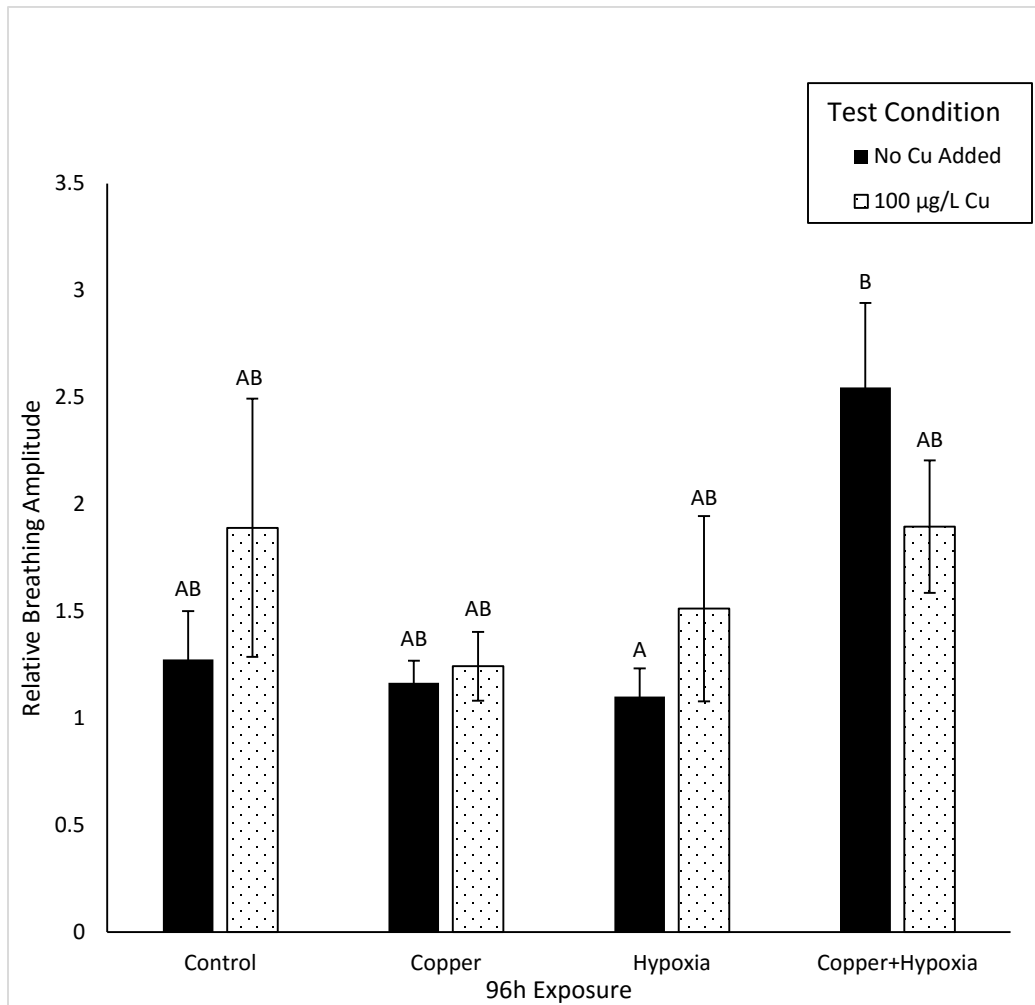
Figure 2.9: Relative changes in breathing amplitude between 15 minutes of acute ammonia and at rest for killifish exposed to acute copper, chronic copper, chronic hypoxia, or a combination of chronic copper and hypoxia. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5 for all treatments).

Table 2.1: Various gill morphological parameters after chronic exposure to copper, hypoxia, or both copper and hypoxia. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different ($p \leq 0.05$, N=5-7).

	Control	Copper	Hypoxia	Copper+Hypoxia
Filament Length (µm)	2213±112	2105±67	2050±77	1997±74
Lamellar Density (lamellae/mm)	27.6±0.3	26.8±0.6	28.0±1.1	27.3±0.7
Functional Lamellar Area (µm²)	67994±6993	59251±8650	54026±5884	57557±2673
Non-functional Lamellar Cross-sectional area (µm²)	42403±3903	37132±2470	32212±2452	32306±2751
Total Number of Filaments	686±36	690±14	667±16	685±32
Total Functional Area (mm²)	1472±264	1156±185	1071±108	970±130
Fish mass (g)	6.11±0.87[a]	5.15±0.57[ab]	4.06±0.36[b]	4.74±0.49[ab]

Figure 2.10: Gill filament lengths of killifish exposed to copper, hypoxia, or a combination of copper and hypoxia, normalized to body mass. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5-7).
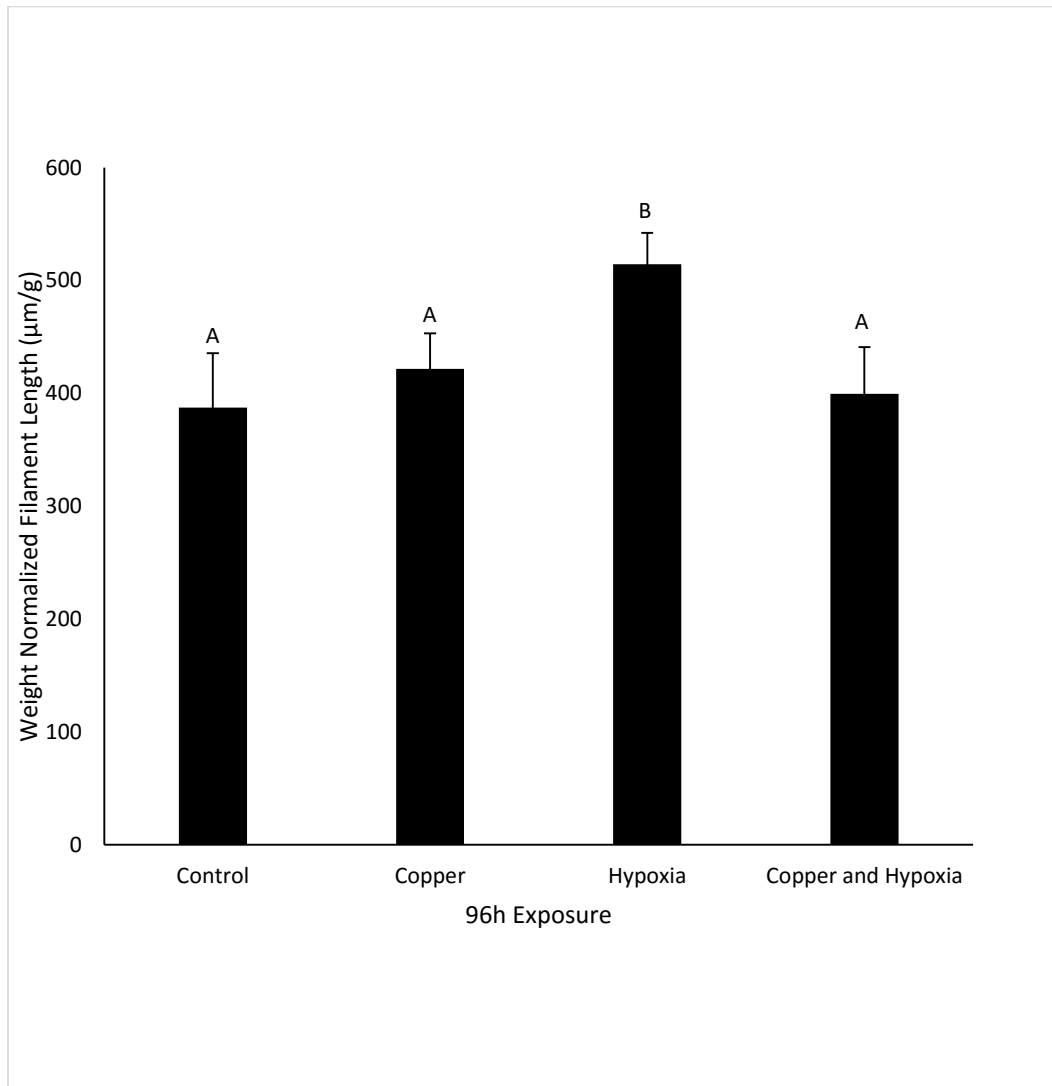
Figure 2.11: Total filament number of killifish exposed to copper, hypoxia, or a combination of copper and hypoxia, normalized to body mass. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5-7).
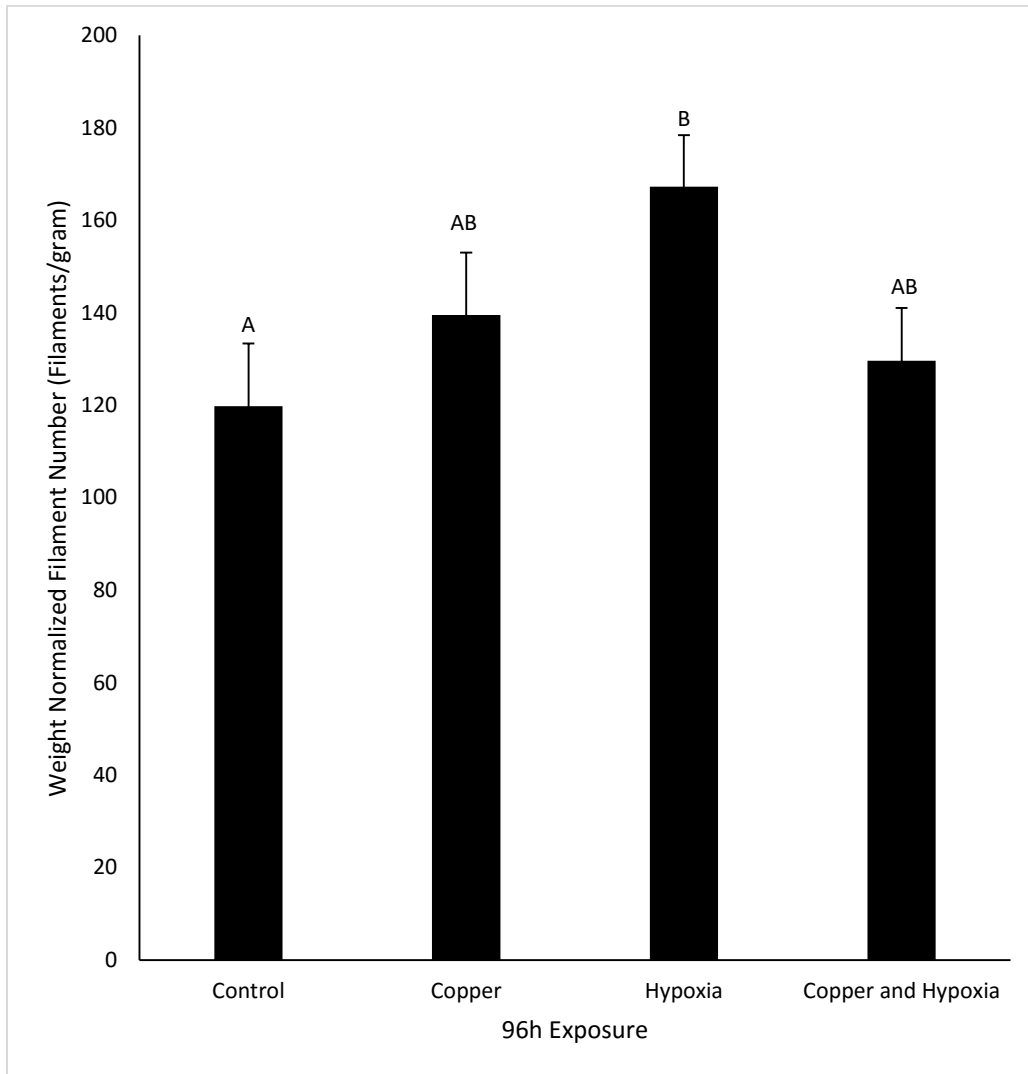
Figure 2.12: Lamellar densities of killifish exposed to copper, hypoxia, or a combination of copper and hypoxia, normalized to body mass. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5-7).

Figure 2.13: Functional lamellar surface area of killifish exposed to copper, hypoxia, or a combination of copper and hypoxia, normalized to body mass. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5-7).

Figure 2.14: Nonfunctional lamellar surface area of killifish exposed to copper, hypoxia, or a combination of copper and hypoxia, normalized to body mass. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5-7).

Figure 2.15: Total functional gill surface area of killifish exposed to copper, hypoxia, or a combination of copper and hypoxia, normalized to body mass. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different (p≤0.05, N=5-7).
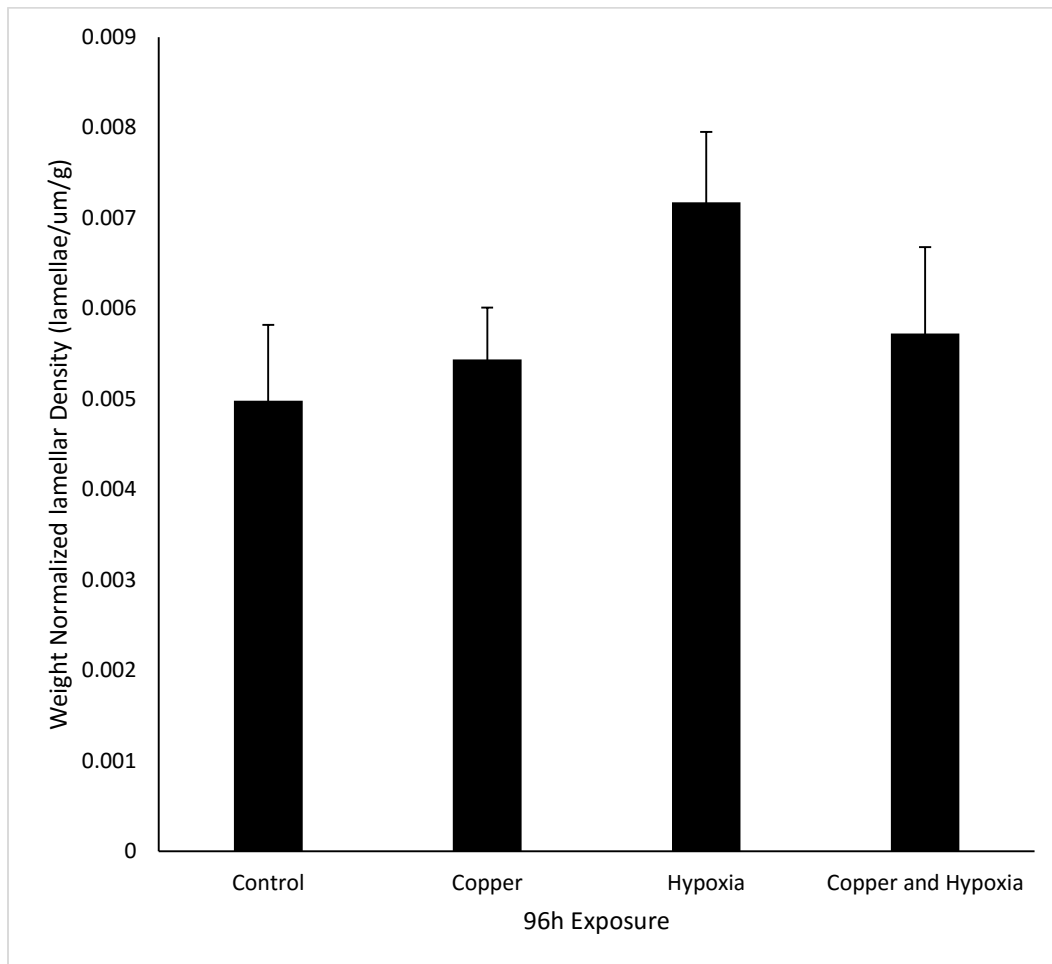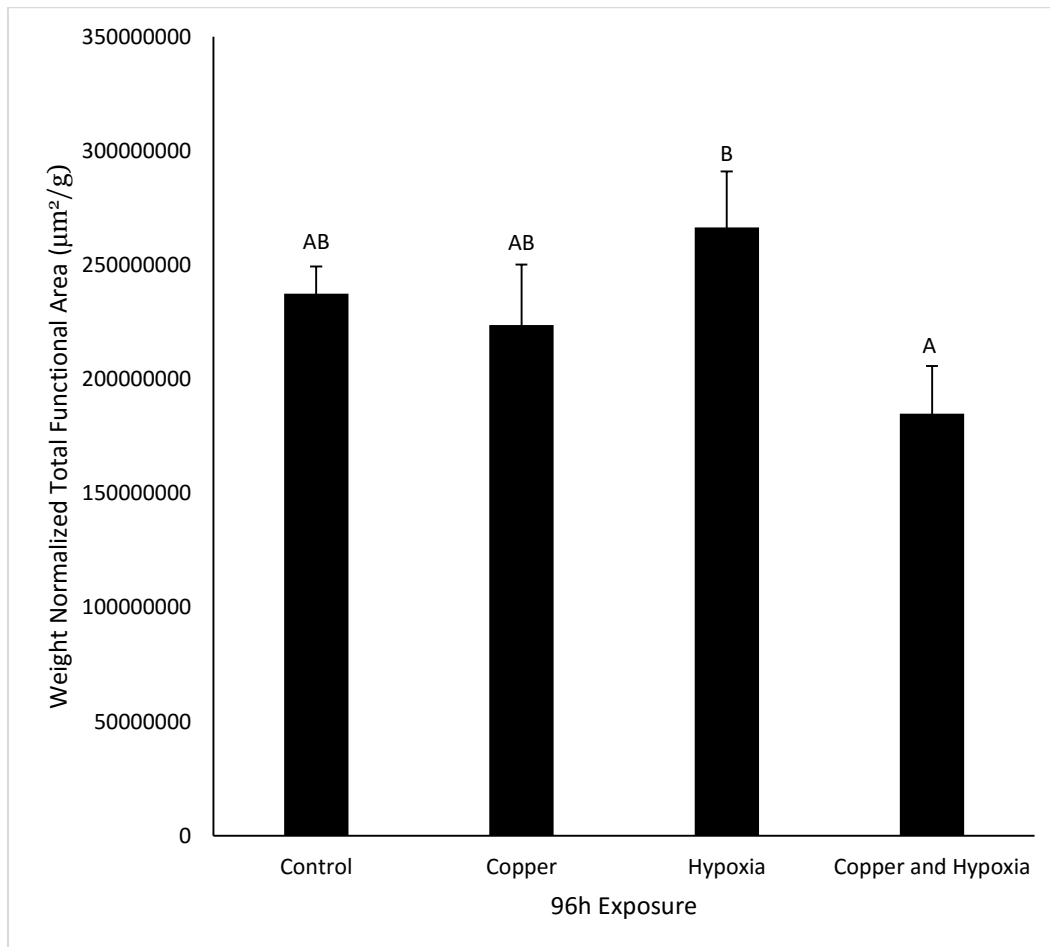
Figure 2.16: Number of neuroepithelial cells per filament of killifish exposed to copper, hypoxia, or a combination of copper and hypoxia, normalized to body mass. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different ($p \leq 0.05$, N=6-9).
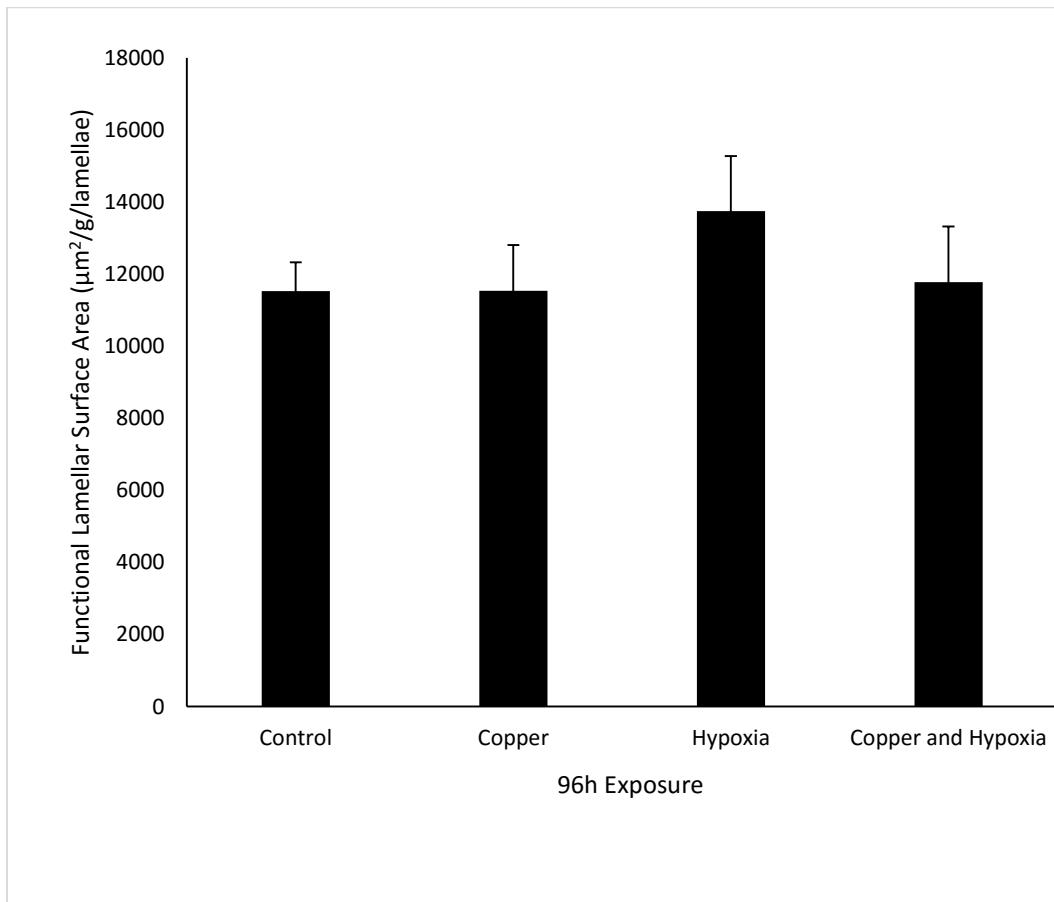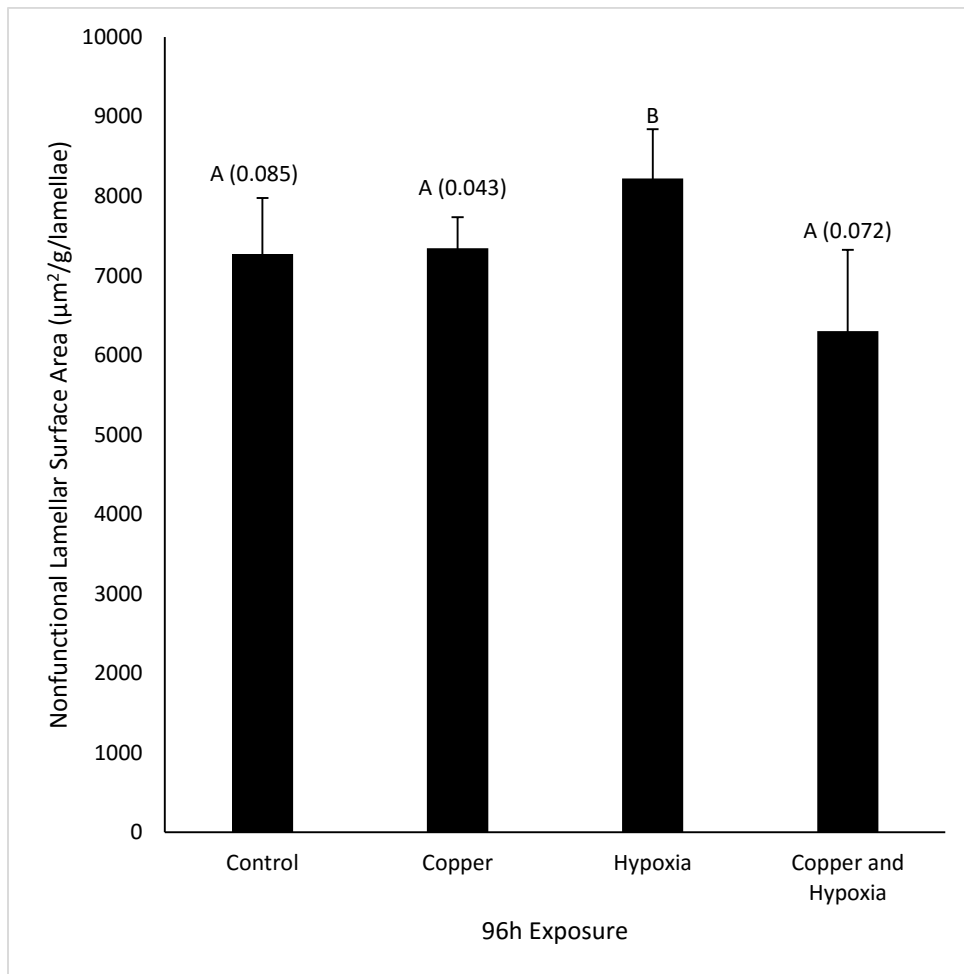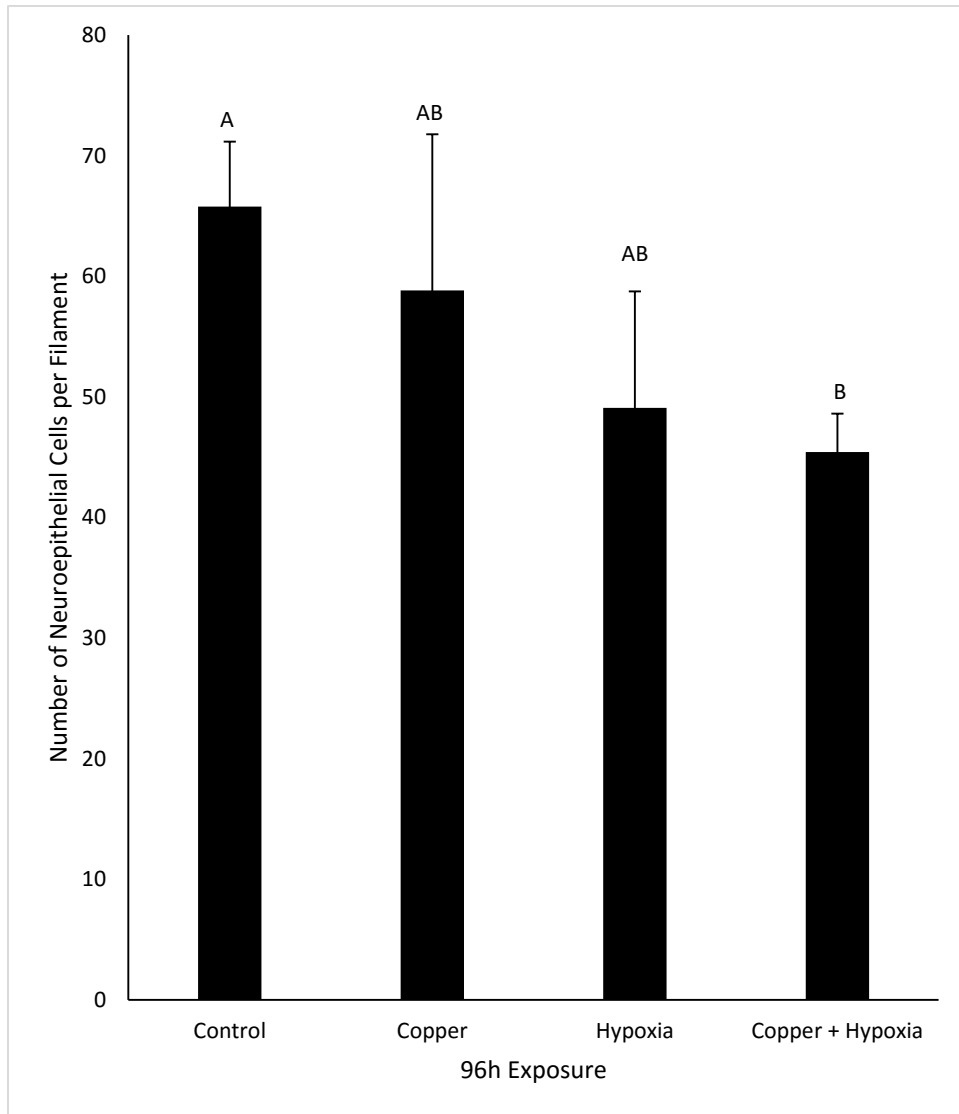
Figure 2.17: Neuroepithelial cell projection area of killifish exposed to copper, hypoxia, or a combination of copper and hypoxia, normalized to body mass. Values shown are means ± SEM. Groups that do not share a letter are considered significantly different after ANCOVA with weight as a covariate (p≤0.05, N=7-10).
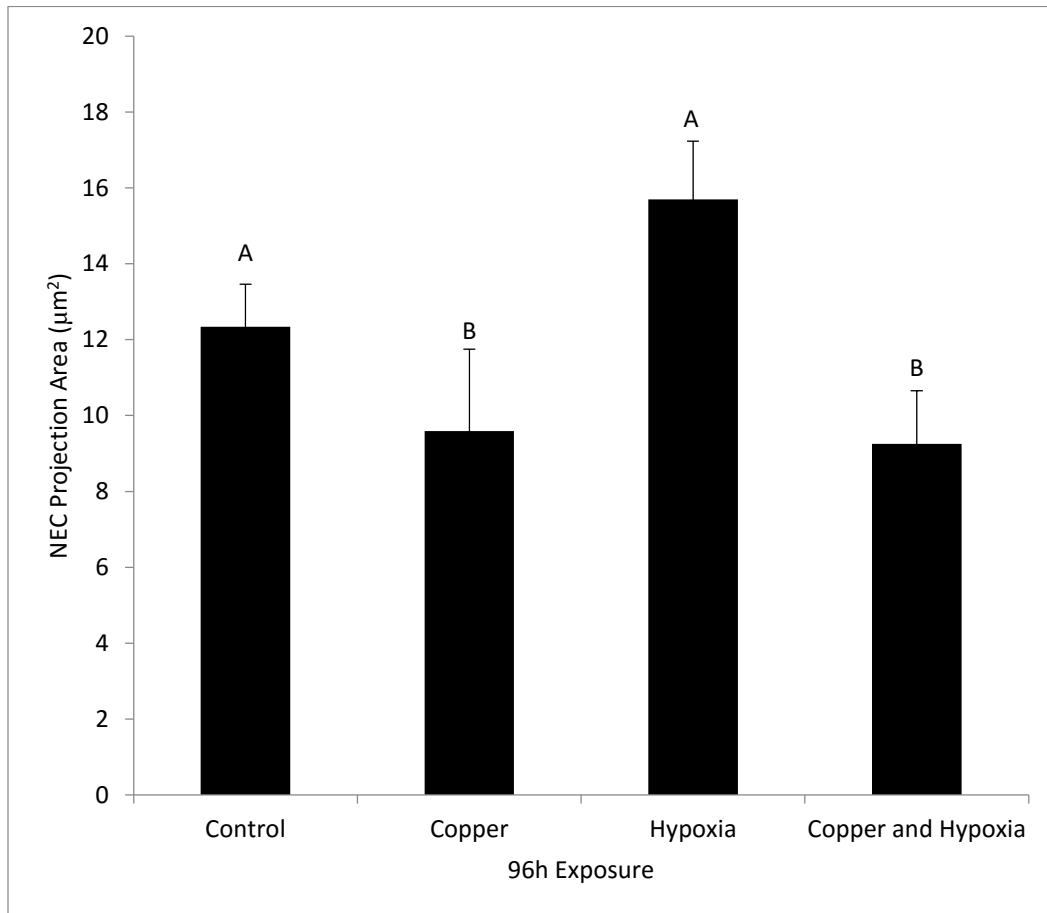
Figure 2.18: Immunohistochemical labelling of neuroepithelial cells of killifish exposed to control conditions (A) or 96h of copper (B), hypoxia (C), or combined copper and hypoxia (D). NECs (shown in green) were stained with anti-serotonin antibodies and then labelled with FITC.
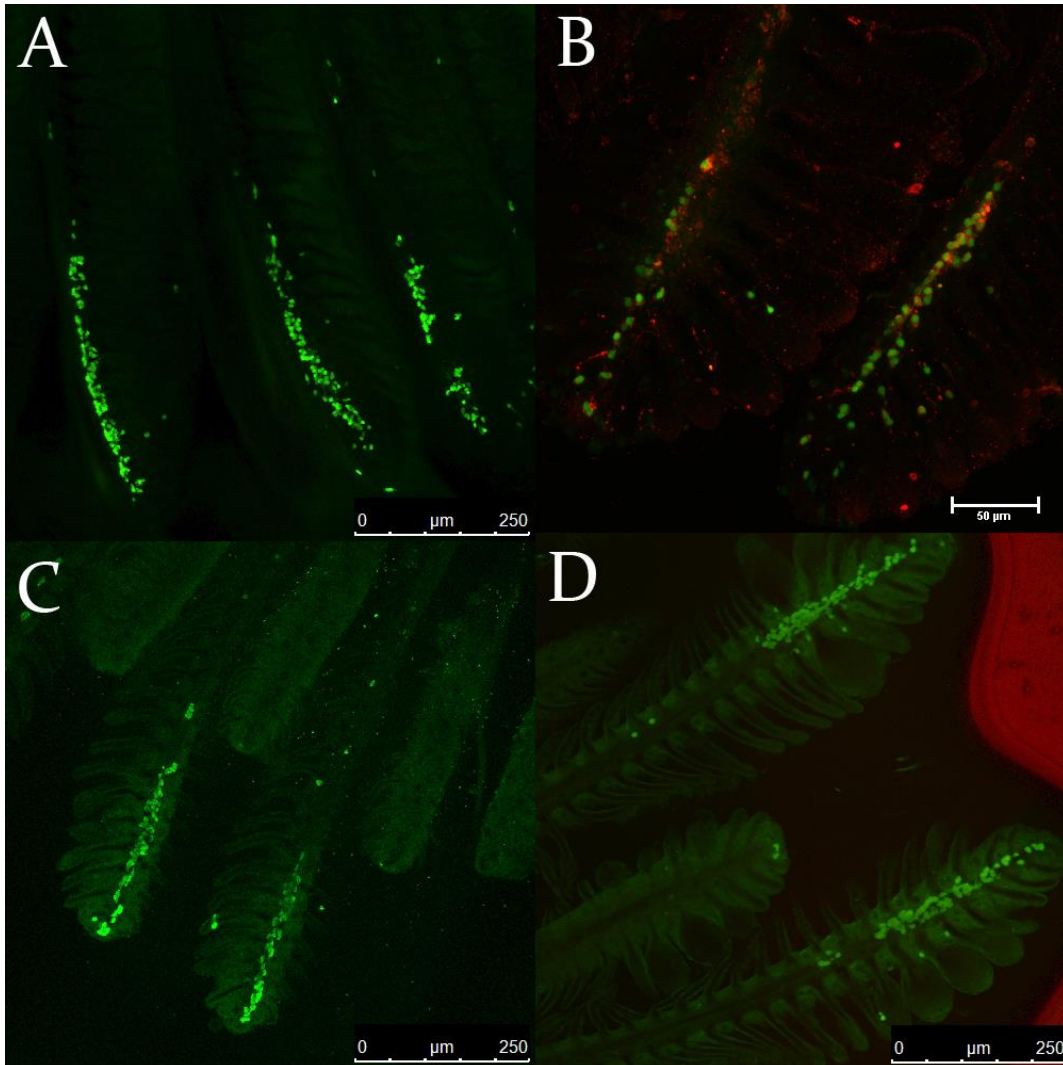
Chapter 3: General Summary and Conclusions

In natural habitats, animals are often exposed to multiple stressors simultaneously, but to date, few studies have examined the potential combined effects of environmental stressors such as copper and hypoxia. This study has investigated the combined effects of these stressors on various parts of the ventilatory pathway in killifish in attempts to better understand the interactions between these stressors.

The combined effects of environmental stressors is an important area of research as these studies more accurately reflect what organisms experience in nature. Unlike responses to single stressors most often examined in lab studies, response to multiple stressors can have vastly different effects, from additive or synergistic to antagonistic or ameliorating the effect of the other. In this study, I have shown that copper had antagonistic effects on the hypoxic response in killifish. Copper negatively impacts the acute ventilatory drive of killifish, as well as impacting gill remodelling and NEC size. I found that while hypoxia, ammonia, and ammonia and hypoxia together all cause immediate increases in breathing rate in control fish, this response is almost entirely inhibited in the presence of copper, whether it be experienced on an acute or chronic timescale. The exception to this is in fish chronically exposed to hypoxia, who have the

ability to respond to subsequent acute hypoxia in the presence of acute copper. This may indicate that prior exposure to hypoxia renders the fish more sensitive to subsequent hypoxia, so that the stimulatory effects of hypoxia overcome the inhibitory effects of acute copper exposure.

While measurement of ventilation is one method of assessing Cu toxicity, it is important to investigate other aspects of the ventilatory system as there are multiple ways to cope with hypoxia additional to hyperventilation. It was seen that gill filament length and non-functional lamellar area increases after 96h hypoxia exposure, although when fish were exposed to combined hypoxia and copper for 96h, this increase was not seen. Copper seems to be playing a similar role in gill morphometry as with resting breathing rates, where it reduces the ability of fish to increase a particular physiological parameter in response to hypoxia, in this case non-functional lamellar area and filament length. When combined with the fact that fish do not hyperventilate in response to typical stimulants of ventilation, this means that fish are likely not able to acquire enough oxygen in environments where they are exposed to both hypoxia and copper. The trade-off to this however is that fish are more able to maintain ion balance with smaller gill surface area, as copper is known to disrupt ion balance by interfering with Na+/K+ ATPase (Grosell and Wood, 2002; van Heerden et al., 2004)

Furthermore, it was found that chronic copper exposure leads to a reduction in NEC size, even when combined with chronic hypoxia exposure. Hypoxia has been seen previously to increase NEC size (Jonz et al., 2004); however hypoxia did not have a protective effect against copper in this study. It is possible that the smaller NECs found in copper-exposed fish contributes to their disability to hyperventilate in response to stimulants of ventilation. Smaller cells may mean less ion channels and ability to signal through neurotransmitter release and therefore less ability to detect changes in water chemistry and relay these changes to the brain. This likely warrants further study through techniques such as patch clamp recording to determine if the sensitivity of the cells to changes in water chemistry changes after chronic copper exposure. Additionally, copper has been seen to block calcium channels due to the similar size and charge of the ions (Lacinová, 2012). This could also explain why fish do not hyperventilate in response to hypoxia after copper exposure – the copper ions may be blocking the calcium channels involved in neural signalling by NECs.

Calcium was seen to significantly reduce the amount of copper accumulation in the gills. However, combined exposure of calcium and copper, whether acute or chronic, did not allow the fish to overcome the inhibitory effects of copper on ventilation in response to hypoxia. Copper accumulation in the gills was reduced in chronic copper exposure coupled

with copper likely due to competition of copper and calcium ions due to their similar size and charge. However, this ameliorating effect of calcium on copper uptake was not of great enough magnitude to allow fish to hyperventilate in response to hypoxia when coupled with copper exposure.

The results of this thesis indicate that in nature, organisms subjected to both copper and hypoxia may have reduced fitness due to not being able to acquire enough oxygen. While it remains unclear whether fishes exposed chronically to copper are unable to detect low oxygen or whether they are unable to mount the appropriate physiological responses, what is clear is that fish are not mounting these responses and unless they are compensating in another way that was not investigated in this thesis, then they are likely not acquiring enough oxygen. This can lead to a lowering of metabolic rate and ecological fitness and have significant ecological impacts. While killifish are not evolved to be Cu-tolerant, they are very tolerant of hypoxia. For this reason, minimizing ionoregulatory disturbances and oxidative stress associated with copper exposure appears to be the more important physiological response in killifish compared to mounting responses to hypoxia. It is therefore likely that similar responses would be seen in other hypoxia tolerant species. It remains unclear what the response would be in fish species that are not as hypoxia tolerant, as they may not

be able to simply stop hypoxic responses when concurrently exposed to copper.

Overall, the results of this thesis indicate that investigation of combined stressors is essential to proper understanding of the impacts of anthropogenic influences on aquatic ecosystems. Additionally, it should be emphasized that it is important to also examine multiple physiological endpoints as there can be many differential and compensatory responses to combined stressors. The results of this thesis can hopefully be used to create better predictive models and water quality guidelines in the future in order to preserve both ecological diversity and economically important species.

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