OXIDATIVE STRESS AND METABOLIC RESPONSES OF ACUTE

WATERBORNE COPPER EXPOSURE IN KILLIFISH, *FUNDULUS*

HETEROCLITUS

By

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ABSTRACT

Copper (Cu) is an essential trace metal, but many aspects of its toxicity remain unclear, particularly in seawater (SW) and little is known regarding the interaction effects of Cu and hypoxia. Few studies have examined the effects of excessive waterborne metals, like Cu, on fish species under saline environment conditions and in combination with other natural stressors, like hypoxia. I first examined the acute effects of sublethal waterborne Cu and hypoxia on the oxidative stress response in freshwater (FW)-acclimated adult killifish (*Fundulus heteroclitus*), a model euryhaline teleost, which highlighted the need to investigate metabolic responses including oxygen consumption rates (MO_2). I found that hypoxia had no effect on Cu accumulation and that Cu induced antioxidant protection pathways and reduced oxidative capacity in a tissue-specific manner. I found that hypoxia may have an antagonistic effect on Cu-induced lipid peroxidation, although this pattern was not observed in all tissues.

I then examined the acute effects of sublethal waterborne Cu on oxidative stress and metabolic responses in FW- and SW-acclimated adult killifish. I found that the oxidative stress and metabolic responses induced by Cu in killifish acclimated to SW differed only slightly from those in FW. I found that Cu had no effect on oxygen consumption rates after 96-h exposure in both FW and SW-acclimated fish, however Cu greatly reduced opercular frequency. In addition, we found that Cu-induced antioxidant protection pathways, although the response differed depending on the specific enzyme. This thesis has advanced our understanding of Cu toxicology in terms of oxidative stress and metabolic responses in freshwater and marine environments and emphasized the importance of utilizing multiple physiological endpoints. Hopefully, this work will contribute to the future development of Cu water quality criteria and building more accurate predictive and regulatory models.

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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. The thesis consists of four chapters. Chapter one consists of an introduction and summary of the major findings and significance of the research performed. Chapters 2-3 comprise manuscripts in preparation for submission to peer-reviewed scientific journals. Finally, Chapter four summarizes the effects of acute copper exposure in killifish and discusses future research directions.

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LIST OF ABBREVIATIONS

BLM – Biotic Ligand Model
BSA – Bovine Serum Albumin
CAT – Catalase
COX – Cytochrome *c* Oxidase
CS – Citrate Synthase
CTR1 – Copper-Specific Transporter 1
CTRL – Control
CU – Copper
CU+HYP – Cu and Hypoxia
DMT1 – Divalent Metal Transporter 1
DNPH - 2,4-Dinitrophenylhydrazine
DO – Dissolved Oxygen
DOC – Dissolved Organic Carbon
DOM – Dissolved Organic Matter
ETC – Electron Transport Chain
$FADH_2$ - Reduced Form of Flavin Adenine Dinucleotide
FIH – Factor Inhibiting Hypoxia Inducible Factor
FW – Freshwater
HB – Hemoglobin
HC – High Copper
HCT – Hematocrit
HIF – Hypoxia Inducible Factor
HK – Hexokinase
HRE – Hypoxia Responsive Element
HYP – Hypoxia
LC – Low Copper
LC50 – Median Lethal Concentration
LDH – Lactate Dehydrogenase
MCH – Mean Cell Hemoglobin
MCHC - Mean Cell Hemoglobin Concentration
MCV – Mean Cell Volume
MDA – Malondialdehyde
MO_2 – Oxygen Consumption Rate
MT – Metallothionein
NADH – Nicotinamide Adenine dinucleotide
P_{crit} – Critical Oxygen Pressure
PEP – Phosphoenol Pyruvate
PHD – Prolyl Hydroxylase Domains
PK – Pyruvate Kinase
PMSF - Phenylmethylsulphonyl Fluoride
PO_2 – Partial Pressure of Oxygen

ROS – Reactive Oxygen Species
SDS - Sodium Dodecyl Sulfate
SOD – Superoxide Dismutase
SW – Seawater
TBA - Thiobarbituric Acid
TBARS – Thiobarbituric Acid Reactive Substances
TCA – Tricarboxylic Acid Cycle
VHL – Von Hippel Lindau
Vmax – Maximal Enzyme Activity

CHAPTER 1

GENERAL INTRODUCTION

Copper in the Environment

Copper (Cu), a trace metal is an essential micronutrient, but at high concentrations Cu can be toxic to organisms, including fish, due to its highly reactive nature that can cause damage to cells. Because of Cu's essentiality and toxicity, all organisms have evolved elaborate homeostatic controls to balance between Cu deficiency and excess, to avoid decreased overall fitness (Pane et al. 2003; Prohaska and Gybina, 2004). Naturally occurring trace levels of Cu are present in aquatic environments ranging from 0.2 to 30 μg/L in freshwater ecosystems (USEPA, 2007), 0.06 to 17 μg/L in coastal ecosystems (Klinkhammer and Bender, 1981; van Geen and Luoma, 1993; Kozelka and Bruland, 1998) and 0.001 to 0.1 μg/L in oceanic waters (Bruland, 1980; Coale and Bruland, 1988; Sherrell and Boyle, 1992). Due to a combination of anthropogenic sources (e.g. mining, industrial and domestic discharge, and agriculture practices) the amount of Cu introduced into marine environments has considerably increased causing stress on organisms and the ecosystem, with the potential of ambient Cu concentrations rising from 100 μg/L to 200 mg/L in mining areas (USEPA, 2007).

Copper Uptake

In aquatic organisms Cu uptake can occur through two pathways: from dietary Cu that is absorbed through the intestine, and waterborne Cu that enters through the gills primarily (Grosell and Wood, 2002; Kamunde et al. 2002b). Interactions between the two uptake pathways appear to exist, with dietary and overall Cu availability regulating branchial

waterborne Cu uptake (Kamunde et al. 2002b). This thesis focuses solely on the effects of waterborne Cu.

Water salinity plays a significant role on a fish's osmoregulatory and ionoregulatory physiology, and, therefore, can affect waterborne Cu uptake (Loretz, 1995; Marshall and Grosell, 2005). In freshwater (FW), fish gain water and lose ions to their hypotonic external environment, and to eliminate the excess water gained they excrete high amounts of dilute urine, while compensating for ion loss by uptake at the gill. Waterborne Cu is readily taken up at the gill in FW via the apical, high-affinity Cu specific transporter 1 (CTR1) and Na^+ channels, such as the H^+-ATPase coupled Na^+ channel, which involves Cu outcompeting Na^+ (Wood, 2001; Grosell and Wood, 2002). CTR1 is a highly conserved transmembrane protein, expressed in all tissues and used as the principal means for essential Cu uptake (Dancis et al. 1994). In addition, Cu internalization from the extracellular medium is also mediated by CTR1, as it transports essential Cu facilitating Cu-dependent activities, such as mitochondrial respiration, resistance to oxidative stress and high affinity iron uptake (reviewed in Leary et al. 2009). When excessive levels of Cu enter cells, Na^+/K^+-ATPase on the basolateral membrane is inhibited, effectively disrupting normal active Na^+ uptake, which leads to decreased levels of whole body and plasma Na^+ (Lauren and McDonald, 1987; Grosell and Wood 2002).

In seawater (SW), fish lose water and gain ions from their hypertonic external environment. Fish compensate for water loss to the environment by elevating their drinking rates, as a consequence more ions are taken up and absorbed by the intestine than the gills, which facilitates the absorption of water and the resulting Na^+ and Cl^- gained is excreted via the gill (Loretz, 1995; Marshall and Grosell, 2005). Because of elevated drinking rates, SW organisms ingest increased waterborne Cu that can accumulate and interact with the intestinal epithelium and lead to

impaired osmoregulation (Grosell et al. 2004; Blanchard and Grosell, 2005; Marshall and Grosell, 2005). In SW, it appears that excess Cu disturbs the process of excretion of Na^+, since a net gain of whole body and plasma Na^+ is observed (Stagg and Shuttleworth, 1982; Wilson and Taylor, 1993; Larsen et al. 1997; Grosell et al. 2004; Blanchard and Grosell, 2005). However, in SW inhibition of Na^+/K^+-ATPase has not been observed during waterborne Cu exposure in the gill or intestine, and as such, cannot account for the increase of plasma Na^+ (Stagg and Shuttleworth, 1982; Grosell et al. 2004). Intestinal transport of Cu uptake primarily occurs in the mid/posterior region of the intestinal tract (Clearwater et al. 2000; Kamunde et al. 2002b; Nadella et al. 2006). Cu uptake is through passive diffusion across the apical membrane and biologically mediated across the basolateral membrane of enterocytes, which is controlled by a regulated transport mechanism (Clearwater et al. 2000; Nadella et al. 2006). Two major candidates implicated for mediating Cu uptake are the divalent metal transporter (DMT1), which is located on the apical surface of intestinal epithelial cells (Nadella et al. 2007) and CTR1, which is highly expressed in the intestinal region (Mackenzie et al. 2004; Minghetti et al. 2008). Because intestinal uptake of Cu predominates in SW fish, this suggests CTR1 and DMT1 may be responsible in uptake of dietary and waterborne ingested Cu, as in mammals (Kamunde et al. 2002b; Nadella et al. 2007).

Once Cu enters the cell, it is bound to various proteins, such as metallothionein (MT), which play a vital role in storage and delivery of essential Cu (Selvaraj et al. 2005; Carpene et al. 2007). There has been evidence to suggest MT is also vital in reactive oxygen species (ROS) scavenging activity and sequestrating toxic levels of Cu, however the exact reaction remains unknown, but nonetheless has gained popularity

as a biomarker for environmental metal exposure (Hylland et al. 1992; Carpene et al. 2007).

Copper-induced Oxidative Stress

Due to Cu's highly reactive nature, Cu has the ability to readily catalyze reactions that produce reactive oxygen species (ROS) via the Fenton and Haber-Weiss reactions (Halliwell and Gutteridge, 1990; Harris and Gitlin, 1996). In excess concentrations Cu can disturb the balance between production and elimination of ROS, leading to elevated ROS levels inducing oxidative stress, which can damage cellular components (reviewed in Lushchak, 2011). ROS can lead to lipid peroxidation, oxidation of proteins and DNA damage, all of which have been suggested to be effective biomarkers indicating oxidative stress (Halliwell and Gutteridge, 1990). Antioxidant responses can also indicate the presence of oxidative stress, as increased ROS levels induce the upregulation of ROS scavenging enzymes, such as superoxide dismutases, catalase and glutathione peroxidases. In fishes, waterborne Cu exposure has been shown to induce both the production of ROS and antioxidant response pathways (reviewed in Lushchak, 2011). Oxidative stress can also be measured directly by the production of ROS in cell culture, however this latter method is difficult to the extremely unstable nature and short-lived intermediates of many ROS (Kobayashi et al. 2001). Therefore, this thesis utilized, by-products of lipid peroxidation and protein carbonylation and activities of key antioxidant enzymes.

Hypoxia in the Environment

Many coastal ecosystems currently suffer from eutrophication, an increase in nutrients leading to increased algae and plant growth, resulting in increased hypoxic conditions due to an increased biological oxygen

demand stimulated by high rates of primary productivity. Natural causes can trigger the eutrophication process resulting in organic enrichment in isolated water bodies; however, eutrophication has become much more widespread due to anthropogenic influences (reviewed in Breitburg, 2002). Anthropogenic influences not only cause eutrophication, but also contaminate marine environments with metals (e.g. Cu), and as such, a combination of multiple stressors is likely to be experienced by aquatic organisms. It is important to consider metal exposure in areas experiencing hypoxia, since the interaction of more than one stressor may affect physiological and toxicological responses.

Under hypoxic conditions, fish initially respond by attempting to maintain oxygen delivery, followed by utilizing two metabolic strategies to conserve energy: 1) overall reduction in metabolic rate and 2) a shift in total metabolism from aerobic to anaerobic metabolism (Hochachka et al. 1996, 1997; Storey 1998; Virani and Rees, 2000). For example, in the hypoxia tolerant killifish, *Fundulus heteroclitus*, an increase in hematocrit and hemoglobin oxygen affinity was observed, increasing oxygen-carrying capacity of blood during chronic hypoxic exposure near their critical oxygen pressure (P_{crit}) 1.6 mg O_2/L when exposed to hypoxia (Cochran and Burnett, 1996). In addition, killifish also increased lactate dehydrogenase activity, an enzyme involved in anaerobic glycolysis after exposure to hypoxia, indicating a shift from aerobic to anerobic metabolism (Greaney et al. 1980). Hypoxia-inducible factor (HIF) regulatory proteins are crucially involved in maintaining oxygen homeostasis, including adaptive responses, such as decreased metabolic rate and increased anaerobic metabolism as mentioned previously, against hypoxic stress (Semenza, 2001). HIF-1 consists of two subunits, a O_2 labile HIF-1α and a constitutively expressed HIF-1β subunit (Wang et al. 1995). Oxygen level is not directly sensed by HIF-1α, but by two iron

dependent enzymes, called prolyl hydroxylase domain proteins (PHD) and factor inhibiting HIF (FIH). Under normoxic conditions, prolyl hydroxylases (PHD 1, 2 and 3) hydroxylate HIF-1α, which increases the affinity of HIF-1α for the von Hippel-Lindau (VHL) protein, targeting HIF-1α for proteosomal degradation (Huang et al. 1998; Ivan et al. 2001; Jaakkola et al. 2001; Maxwell et al. 1999). Degradation of the α subunit disrupts HIF-1, thus inactivating any cellular response. Under hypoxic conditions, hydroxylation reactions are inhibited due to substrate limitations, and HIF-1α is stabilized and translocated to the nucleus, where it dimerizes with HIF-1β assembling the HIF-1 transcriptional complex. In the nucleus, HIF-1α is hydroxylated by FIH, similar to prolyl hydroxylation, this modification requires oxygen and is inhibited during hypoxia; FIH prevents the interaction of HIF-1α and its cofactors, inhibiting the transcription activity of HIF-1 (Mahon et al. 2001; Dames et al. 2002). Active HIF-1 transcription factor binds specifically to regulatory regions of over 70 target genes, known as the hypoxia-responsive element (HRE), altering rates of hypoxia-inducible genes and activities of their protein products to help sustain supply of O_2 to tissues (Bunn and Poyton, 1996; Semenza, 1999; Wenger, 2002; Rocha, 2007; Kaluz et al. 2008).

During hypoxia cells become highly reduced (Kehrer, 2000), caused by an increase and build up of reducing equivalents in the mitochondria, such as nicotinamide adenine dinucleotide (NADH) and reduced form of flavin adenine dinucleotide (FADH$_2$), when an insufficient amount of O_2 is available for their reduction by the electron transport chain (ETC), mainly at complex III (Guzy and Schumacker 2006). As a consequence, availability of electrons for reduction reactions increases, which can result in the increased production of ROS and if not combated with protective antioxidant systems, such as ROS-scavenging enzymes superoxide dismutase (SOD) and catalase (CAT), to alleviate the effects

of ROS, elevated ROS may lead to cellular oxidative damage (DiGiulio et al. 1989; Halliwell and Gutteridge, 1990; Sies, 1991). Aquatic hypoxia encountered by fish can affect the balance of ROS production and elimination within a cell, which can lead to oxidative stress. In the common carp, hypoxia exposure was shown to alter levels of oxidative damage (e.g. protein carbonyls, thiobarbituric reactive substances and lipid peroxides; Lushchak et al. 2005). In addition to oxidative damage, hypoxia exposure can stimulate changes in antioxidant enzyme activities, such as superoxide dismustase (SOD) and catalase (CAT), as seen in tissues of goldfish (Lushchak et al. 2001) and the common carp (Vig and Nemcsok, 1989; Lushchak et al. 2005).

Although ROS can have harmful effects on all cellular macromolecules, it also appears to play a role in the regulation of HIF-1 activity. During severe hypoxia or anoxia PHDs are completely inhibited, while under moderate hypoxia, ROS are needed for complete inhibition of hydroxylation, which leads to the stabilization of HIF-1α and induction of a hypoxic adaptive response (Brunelle et al. 2005; Aragones et al. 2009).

Interaction of Copper and Hypoxia

Exposure to waterborne metals, including excess Cu, has been shown to induce a hypoxic-like response in fish under normoxic conditions (Duyndam et al. 2001; Gao et al. 2002; Costa et al. 2003; Salnikow et al. 2003). Cu appears to mimic hypoxia by; 1) stabilizing HIF-1 (van Heerden et al. 2004; Martin et al. 2005) and 2) facilitating DNA binding of HIF-1 to HRE on target genes (Feng et al. 2009). Cu plays an essential role in hypoxia-induced HIF-1 activation; yet, the effects of excess copper enhancing HIF-1 transcription activity are quite different (Feng et al. 2009). Studies have suggested Cu is required at different steps of HIF-1α activation; Cu appears to be essential in the process of HIF-1 binding to

the HRE region on target genes and regulating HIF-1 transcriptional complex formation by inhibiting FIH activity, which enables HIF-1 to bind to its cofactors (Feng et al. 2009). In comparison, excess copper, like other transition metals, may enhance HIF-1 transcriptional activity by increasing HIF-1α protein stability involving increased accumulation of HIF-1α and inhibition of the degradation process (Yuan et al. 2003; Maxwell and Salnikow, 2004; Salnikow et al. 2004; van Heerden et al. 2004; Hirsila et al. 2005; Martin et al. 2005). While, Cu appears to play an essential role in HIF-1 activation, in excess concentrations Cu's effects are altered in this activation process. Therefore, excess Cu may elicit similar hypoxia induced-responses and when combined with hypoxic conditions, may interact additively or synergistically to produce a greater hypoxic response.

Protective Effects of Salinity

The presence of dissolved cations in the environment impact Cu uptake and ameliorate the effects of Cu toxicity. At increasing salinities Cu accumulation in the gill decreases, most likely as a result of competing cations at the gill and a change in the physiology of the gill at high salinities, as seen in killifish, *F. heteroclitus* (Blanchard and Grosell, 2006; Scott et al. 2008). In freshwater, Na^+ and Ca^{2+} ions have been observed to have a protective effect against acute Cu toxicity (48-h exposure) reducing gill and liver Cu load and attenuating oxidative damage in softwater-acclimated zebrafish (Craig et al. 2007). However, Na^+ had a more protective effect than Ca^{2+}, particularly against lethality (Craig et al. 2007). This trend was also apparent when zebrafish were exposed to Cu chronically for 21-days, with Na^+ reducing Cu load in the gill, liver and gut, while Ca^{2+} only reduced branchial accumulation (Craig et al. 2010). In contrast to these results, Grosell and Wood (2002) observed no protective effect against acute Cu toxicity (8-h exposure) as indicated by Cu uptake

concentrations in the presence of Ca^{2+} in rainbow trout. Therefore, the protective mechanism of Ca^{2+} has been suggested to be related more to physiology, such as intestinal and branchial ion transport and ammonia excretion disturbances, rather than reduced uptake (e.g. Grosell et al. 2007). In contrast, clear evidence exists for the protective effects of Na^+ against acute and chronic Cu toxicity. For example, reduced branchial Cu uptake observed in rainbow trout (Grosell and Wood, 2002) and decreased Cu-induced oxidative stress and gene expression in zebrafish (Craig et al. 2007, 2010), demonstrating competitive interactions exist reducing Cu uptake and other physiological mechanisms of protection.

In addition to the protective effects dissolved cations provide to aquatic organisms, dissolved organic carbon (DOC) shares a similar relationship with Cu that it diminishes Cu toxicity (Playle et al. 1992, 1993; Richards et al. 2001; Sciera et al. 2004). Copper binds to DOC with a high affinity, preventing and reducing Cu binding and uptake (Playle et al. 1992, 1993; Richards et al. 2001; Sciera et al. 2004), and therefore, should be taken into account when interpreting results, especially when manipulating water composition, such as salinity.

Copper Speciation

While, anthropogenic inputs affect the concentrations of Cu, pollutants are not the sole factor contributing to Cu toxicity. The speciation of Cu controls the type and prevalence of Cu complexes available within an aquatic environment, affecting the toxicity of the metal and Cu uptake. The biotic ligand model (BLM) is a predictive tool that quantitatively accounts for water chemistry affecting metal speciation and other ions present when examining toxicological effects in aquatic environments (Paquin et al. 2002). Water chemistry parameters that alter metal speciation include DOC, pH, alkalinity, hardness, salinity and temperature.

Speciation of Cu and the level of toxicity are also affected by salinity (e.g. Chakoumakos et al. 1979; Blanchard and Grosell, 2006). For example, in SW Cu is mainly in the form of $CuCO_3$ and $Cu(CO3)_2^{2-}$, which is generally thought not to exert toxicity but may be available for accumulation (Grosell et al., 2004; Blanchard and Grosell, 2006). Only a small percentage of Cu is present in the main toxic form, ionic Cu^{2+}, with CuOH- and $Cu(OH)_2$ also present and able to exert toxicity (Chakoumakos et al. 1979; Erickson et al. 1996; Paquin et al. 2002). Similar inorganic Cu speciation can apply to freshwater of high alkalinity and pH (Blanchard and Grosell, 2006), however with changing alkalinity and pH, speciation of Cu can vastly differ with ionic Cu^{2+} becoming the dominant form of Cu at low alkalinity and neutral or acidic pH (Chakoumakos et al. 1979; Cusimano et al. 1986). Therefore, it is very important to take into account water chemistry to predict metal toxicity not only for its protective effects but effect on Cu speciation. For my thesis, both FW and 100% SW conditions were examined to better understand the mechanisms of Cu toxicity at the extremes of a salinity gradient.

Objectives

The objective of this thesis was to examine the changes in oxidative stress of killifish exposed to acute waterborne Cu, under conditions of FW and SW and in combination with hypoxia. The ultimate goal is to improve predictive and regulatory models, such as the BLM incorporating novel physiological data that also takes into account additional stressors, such as hypoxia to better mimic real-world conditions that can better predict acute and chronic toxicity levels of Cu. To fulfill this goal, the specific objectives and hypotheses of this thesis were to:

Objective 1) Examine the potential interaction effects of two common stressors, waterborne Cu+Hyp, and whether the combined effects of these stressors induce changes in the oxidative stress response in killifish.

Objective 2) Examine the effects of salinity on the copper-induced oxidative stress and metabolic responses in killifish.

Hypothesis 1) Combined exposure to sublethal waterborne Cu+Hyp will synergistically induce oxidative stress.

Hypothesis 2) Increased salinity will protect against Cu-induced oxidative stress and minimize changes in aerobic metabolism.

CHAPTER 2

The Oxidative Stress Response in Freshwater-Adapted Killifish, *Fundulus heteroclitus* to Acute Copper and Hypoxia Exposure

Abstract

Aquatic organisms face multiple stressors in natural ecosystems. Here we examine the effects of moderate hypoxia and low level waterborne copper (Cu) concentrations on freshwater-acclimated killifish. Both Cu and hypoxia can affect oxidative stress in fish, but it is unclear if in combination these two stressors would act synergistically. We exposed freshwater-acclimated adult killifish (*Fundulus heteroclitus*) to either Cu alone (Cu, $23.4 \pm 0.9 \mu g/L$ Cu), hypoxia alone (Hyp, $2.33 \pm 0.01 mg/L$ O_2), or Cu and hypoxia combined (Cu+Hyp, $23.6 \pm 0.8 \mu g/L$ Cu and $2.51 \pm 0.04 mg/L$ O_2) and compared them to normoxic controls (Ctrl, $0.7 \pm 0.1 \mu g/L$ Cu and $9.10 \pm 0.00 mg/L$ O_2). There were significant accumulations of Cu in gill tissues with both Cu and Cu+Hyp exposed fish. This was accompanied by significant increases in catalase (CAT) maximal enzyme activity (Vmax) and no significant change in either protein carbonyls or thiobarbituric acid reactive substances (TBARS) relative to controls. Hypoxia alone resulted in a decrease in protein carbonyls in the gills. Liver showed no significant Cu load, but showed a significant decline in CAT activity in Cu and Cu+Hyp fish. Liver showed no significant changes in either indices of oxidative damage or superoxide dismutase (SOD) activity, but Cu+Hyp caused a significant decline in the activity of cytochrome *c* oxidase. Both low level [Cu] and moderate hypoxia affected gill and liver phenotypes. However, none of the variables measured showed a synergistic response to combined Cu+Hyp compared to each stressor alone.

Introduction

Many coastal ecosystems currently suffer from eutrophication, resulting in hypoxic conditions. Natural causes can trigger the eutrophication by organic enrichment; however, eutrophication has become much more widespread due to anthropogenic influences. Anthropogenic influences can also lead to increased levels of metals, such as copper (Cu), in marine environments. Thus, marine organisms are likely to experience a combination of metal and hypoxic stress in their natural environments. However, few studies have addressed physiological and toxicological effects of combined stressors, such as Cu and hypoxia (Cu+Hyp), in aquatic organisms. The potential of multiple stressors to invoke synergistic or antagonistic physiological and/or toxicological effects is an area of study that has received little attention, despite the increasing prevalence of anthropogenic sources contaminating environments with both organic matter and metals.

Copper, a trace metal is an essential micronutrient, but at high concentrations Cu can be toxic to aquatic organisms due to its highly reactive nature (Harris and Gitlin, 1996). Naturally occurring levels of Cu resulting from weathering and land drainage, range from 0.2 to 30 μg/L in freshwater ecosystems (USEPA, 2007) and 0.06 to 17 μg/L in coastal marine ecosystems (Klinkhammer and Bender, 1981; van Geen and Luoma, 1993; Kozelka and Bruland, 1998). However, anthropogenic sources (e.g. mining, industrial and domestic discharge, and agriculture practices) have increased the amount of Cu introduced into marine environments and represent a potential stress to marine organisms.

In freshwater (FW), the major uptake route of waterborne metals is through the gills, as fish are hyperosmotic regulators they have an abundance of active transport pumps to maintain ions at levels higher than their external environment, which can also facilitate metal uptake

(reviewed in Wood, 2011). One key mechanism of Cu toxicity in freshwater is associated with disturbances at the gills, specifically the disruption of Na^+ and Cl^- regulation, leading to decreases in plasma concentrations of Na^+ and Cl^-, resulting in haemoconcentration and ultimately leading to cardiac failure (Wilson and Taylor, 1993; Grosell et al. 2002). However, at high concentrations, Cu can also exert toxicity by inducing oxidative stress (Luschak, 2011).

Cu is highly reactive with hydrogen peroxide (H_2O_2) that can cause rapid generation of reactive oxygen species (ROS) (Harris and Gitlin, 1996). If ROS production is not combated with protective antioxidant systems, such as ROS-scavenging enzymes superoxide dismutase (SOD) and catalase (CAT), and exceeds ROS degradation, then oxidative damage to lipids, protein and nucleic acids can ensue (DiGiulio et al. 1989; Halliwell and Gutteridge, 1990; Sies, 1991; Sies, 1993; Filho et al. 1993, Hermes-Lima et al. 1998, Kelly et al. 1998, Chandel et al. 2000). Interestingly, SOD, an important enzyme involved in the oxidative stress response, also requires Cu to function. The catalytic function of Cu, Zn-SOD is quickly impaired in tissues when Cu levels are deficient (Harris, 1992). In addition to the generation of ROS, Cu can also result in oxidative stress by inhibition of antioxidant enzymes, alterations in the mitochondrial electron-transport chain and depletion of cellular glutathione (Pruell and Engelhardt, 1980; Shukla et al. 1987; Freedman et al. 1989; Stohs and Bagchi, 1995; Rau et al. 2004; Wang et al. 2004). Alterations in mitochondrial respiration may be in part due to Cu's essential role in the formation of cytochrome *c* oxidase (COX), the terminal enzyme of the electron-transport chain (Uauy et al. 1998).

While it is clear increased oxygen levels increase ROS generation due to enhanced probability of free electrons escaping from the electron-transport chain and reacting with oxygen to form superoxide, decreased

oxygen concentrations may also induced oxidative stress (reviewed in Lushchak, 2011). Hypoxia has been observed to increase SOD and CAT activities in numerous fish, including goldfish, common carp and rotan (Lushchak et al. 2001, 2005; Lushchak and Bagnyukova, 2007).

Interestingly exposure to waterborne metals, such as Cu, can induce a hypoxic response in normoxia (e.g. Duyndam et al. 2001; Gao et al. 2002; Salnikow et al. 2003). Copper appears to mimic hypoxia in two ways; 1) by stabilizing HIF-1 (van Heerden et al. 2004; Martin et al. 2005) and 2) by facilitating DNA binding of HIF-1 to HRE on target genes (Feng et al. 2009). In addition, mortality of fish exposed to lethal concentrations of Cu may be due to disruption of gill function, resulting in internal hypoxia (Rankin et al. 1982, Mallatt 1985, Evans 1987).

The killifish, *F. heteroclitus*, is a small, euryhaline, non-migratory teleost, inhabiting intertidal marshes along the east coast of North America (reviewed in Burnett et al. 2007). This abundant species plays an important role as both piscivore and prey for a variety of birds, fishes and invertebrates (reviewed in Able, 2002; Able et al. 2007; Kimball and Able, 2007). *F. heteroclitus* is an excellent model for biological and ecological responses to natural environment changes, due to its ability to tolerate wide changes in salinity, oxygen, temperature and pH. As coastal areas are highly populated resulting in increased anthropogenic sources, killifish face a wide range of environmental stressors, including Cu+Hyp. Due to killifish's abundance, accessibility and adaptability to a wide range of environmental conditions, killifish are an ideal model for laboratory studies (Burnett et al. 2007).

The goal of this study was to assess the potential interaction effects of two common stressors, Cu+Hyp, currently facing aquatic organisms, and whether the combined effects of these stressors at environmentally relevant concentrations would act antagonistically or synergistically to

induced changes in the oxidative stress response and quantitative and qualitative mitochondrial characteristics. Killifish were acclimated to freshwater to eliminate potential salinity interactions, as salinity has been observed to ameliorate Cu toxicity (e.g. Bianchini et al. 2004), in an effort to better understand the interaction between Cu+Hyp. We hypothesized that the combination of sublethal Cu+Hyp would synergistically induce oxidative stress, as well as decrease quantity and quality of mitochondria in killifish. We exposed freshwater-acclimated adult killifish to waterborne Cu, hypoxia and a combination of Cu+Hyp. After 96-h exposure, we measured indicators of oxidative damage (protein carbonyl content and lipid peroxidation, assessed as thiobarbituric acid reactive substances (TBARS)), as an indication of oxidative stress. We examined the oxidative stress defenses in killifish by measuring the maximal activities of ROS detoxifying enzymes (CAT, SOD) and indices of mitochondrial quantity and quality (citrate synthase (CS) and COX, respectively). These results will allow us to begin to understand the potential interaction effects of multiple stressors on fish and aid in developing environmental regulations and guidelines that better protect aquatic organisms.

Materials and Methods

Experimental Animals

Adult killifish of mixed sex (body mass: 1.11-6.08 g) were collected from the wild (Aquatic Research Organisms, Hampton, NH, USA) and gradually acclimated to decreasing salinity levels from 35 parts per thousand (ppt) to 10 ppt in an aerated 300-liter aquarium with carbon filtration for a minimum of two weeks. Approximately 200 killifish were then acclimated to freshwater (FW) conditions in carbon-filtered, aerated, 45-liter tanks, with 20% water-renewed daily for a minimum of 2-weeks prior to experimentation. During the acclimation period, fish were fed daily with a commercial tropical fish flake (Big Al's Aquarium Supercenter, Woodbridge, ON, CA) and maintained under natural photoperiod (12:12-h light:dark) at approximately 20 °C. Fish were fasted for 48 hours prior to the start of experiments and throughout the 96-hour exposure period.

Copper and Hypoxia Exposure

Killifish (n = 192) were removed from their freshwater acclimation tanks and placed into 8-liter experimental tanks (n = 12 per tank, 4 treatments and 4 replicates per treatment). The four experimental conditions (n = 48 each) used were: Controls (Ctrl; 9.10±0.00mg/L O_2 and 0.7±0.1μg/L Cu), Cu (Cu; 9.10±0.00mg/L O_2 and 23.4±0.09μg/L Cu), Hypoxia (Hyp; 2.33±0.03mg/L O_2 and 1.1±0.4μg/L Cu) and Cu+Hypoxia (Cu+Hyp; 2.51±0.04mg/L O_2 and 23.6±0.1μg/L Cu) with each treatment performed in 4 replicates, using a stock Cu solution made from $CuSO_4$ dissolved in 1% HNO_3, which had no significant effect on water pH (Table 2.1). Tank water was renewed daily by 80%, with thoroughly mixed renewal water prepared 24-h in advance. Control and Cu treatment water was fully oxygen-saturated by means of an airstone. For hypoxia and Cu+Hyp treatments, tanks were covered with plastic wrap and dissolved

oxygen (DO) concentration of the water was lowered by bubbling a mixture of compressed nitrogen (N_2) gas and air in the correct combination using mass flow controllers (Sierra Instruments, Moterey, CA, US) to achieve approximately 25% O_2 saturation, delivered by means of an airstone as described previously (Virani and Rees, 2000). DO was monitored three times daily using a microcathode oxygen electrode and oxygen meter (Strathkelvin Instruments Ltd., Glasgow, Scotland). The oxygen sensor was calibrated with air-saturated water (100% of air saturation). Flow rate of N_2 gas and air was adjusted as needed to maintain the desired O_2 saturation.

Sampling

Water samples were taken prior to each water replacement, filtered through a 0.45 μm filtration disc (Pall Life Sciences, East Hills, NY, USA) to measure water chemistry parameters. After 96-h exposures, killifish were quickly euthanized by cephalic concussion and gill, liver, muscle, intestine and remaining carcass were removed and weighed and quickly frozen in liquid N_2.

Tissue and Water Analyses

Cu concentration in tissue and water samples were measured by Graphite Furnace Atomic Absorption Spectroscopy (GFAAS, Spectra AA 220Z, Varian Palo Alto, CA) as described previously (Craig et al. 2007). Tissue samples were digested in a volume of 2N nitric acid equivalent to five times their volume (Trace metal grade, Fisher Scientific, Ottawa, ON, CA) at 60 °C for 48 hours, and vortexed after 24 hours. Tissue digests were then diluted as necessary and dissolved Cu concentrations determined using a 40 μg/L Cu standard for comparison (Fisher Scientific, Ottawa, ON, CA). Tissue and water ion compositions were measured by

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Flame Atomic Absorption Spectroscopy (Spectra AA 220FS, Varian, Inc., Mulgrave, Victoria, Australia) as previously described (Craig et al. 2007) and verified with appropriate standards (Fisher Scientific, Ottawa, ON, CA). Tissue samples were diluted as necessary with 1% HNO_3 (Na^+), 1% $LaCl_3$ in 1% HNO_3 (Ca^{2+}, Mg^+) or 0.1% $CsCl_2$ in 1% HNO_3 (K^+). Total DOC concentration of water samples was measured using a Shimadzu TOC-VCPH/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan) as described previously (Al-Reasi et al. 2012).

Oxidative Damage

Protein carbonyls. Protein carbonyl content was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI) as previously described (Craig et al. 2007). Tissues were homogenized in 1 ml of homogenization buffer (50 mM phosphate buffer, 1 mM EDTA, pH 6.7) and centrifuged at 10,000 *g* for 15 min at 4 ℃ and the supernatant was removed for analysis. Samples and controls were prepared with 200 μl of supernatant added plus 800 μl of 2,4-dinitrophenylhydrazine (DNPH) to the sample tube and 800 μl of 2.5 M HCl to the control tube. Sample and control tubes were then incubated in the dark for 1 h. After incubation, 1 ml of 20% TCA was added, tubes were vortexed and then centrifuged at 10,000 *g* for 10 min at 4 ℃. The supernatant was removed, 1 ml of 10% TCA was added, and tubes were vortexed and centrifuged again. The supernatant was removed, and the pellet was washed with three sequential washes of 1 ml 1:1 ethanol:ethyl acetate followed by centrifugation. After the third wash, the pellet was resuspended in 500 μl of 2.5 M guanidine hydrochloride. Then, 220 μl from each sample and control tube was placed in a 96-well plate, and the absorbance measured at 375 nm in duplicate. Peak absorbance from the control value was subtracted from the sample value, and this corrected absorbance was used to calculate protein carbonyl content.

Protein concentration of the tissue homogenate was assayed according to Bradford (1976), using bovine serum albumin (BSA) to construct standard curve at 565 nm. Protein carbonyls are expressed in nanomoles per milligram total protein.

Thiobarbituric Acid Reactive Substances (TBARS). Malondialdehyde (MDA) concentrations, a product of lipid peroxidation, were determined using a commercial TBARS assay kit (Cayman Chemical, Ann Arbor, MI, USA). Briefly, tissues (~25 mg) were sonicated for 15 seconds at 40V over ice in 250 μl of homogenization buffer (100 mM Tris-HCl, 2 mM EDTA, 5 mM $MgCl_2 \bullet 6H_2O$, pH 7.75) containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor, and then centrifuged at 1,600 g for 10 minutes at 4°C. To 100 μl of sample and MDA standard curve solutions (0 – 5 μM), 100 μl of sodium dodecyl sulfate (SDS) solution was added, followed by 4 ml of the colour reagent (thiobarbituric acid (TBA), acetic acid and sodium hydroxide). The colour reagent was made fresh daily. Sample and standards were placed into boiling water for 1 h, immediately removed and incubated on ice for 10 minutes, then centrifuged at 1,600 g for 10 minutes at 4 °C. Then, 150 μl of supernatant was loaded into a black 96-well plate, and fluorescence was measured at an excitation and emission wavelength of 530 and 550 nm, respectively, using a Spectramax fluorescence microplate reader (Molecular Devices, Menlo Park, CA, USA). Protein concentration of the tissue homogenate was assayed as previously described and TBARS levels are presented in micromolar MDA per milligram total protein.

Enzyme Activity

Frozen gill and liver tissues were powdered in a liquid nitrogen-cooled mortar and pestle, then diluted 1:20 (mg tissue:μL) in ice-cold

homogenization buffer (20mM HEPES, 1 mM EDTA, 0.1% Triton X-100, pH 7.2) using a cooled glass on glass homogenizer. All enzyme activity levels were assayed in 96-well format using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Menlo Park, CA, USA). Assays were performed in triplicate, with an additional negative control well lacking substrate, to correct for any background activity, as previously described (McClelland et al. 2006; Craig et al. 2007). All chemicals used were purchased from Sigma Aldrich (Oakville, ON, CA) and reaction buffers were prepared fresh daily. All enzyme activity data are reported as units per milligram protein, where 1 U = μmol/min.

Catalase (CAT) activity was measured according to Clairborne (1985) by observing the decomposition of H_2O_2 into oxygen and water at 240 nm over 1 min. The reaction mixture consisted of 20 mM K-phosphate, pH 7.0, and 20 mM H_2O_2 as described previously (Craig et al. 2007).

Superoxide dismutase (SOD) activity (CuZn-SOD and Mn-SOD) was determined by using a commercial kit (Fluka, Sigma Aldrich, Oakville, ON). The assay measures the formation of formazan dye, utilizing the reduction of tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo, Kumamoto, Japan) with superoxide radicals at 450 nm. The reaction of xanthine oxidase generates the source of superoxide radicals. One unit of SOD is defined as the amount required to inhibit the reduction of WST-1 to WST-1 formazan by 50%. The reaction mixture comprised 200 μl WST working solution, 20 μl enzyme working solution and 20 μl of supernatant.

Cytochrome oxidase (COX) activity was measured by the addition of tissue homogenate to a reaction buffer containing 50 mM Tris-Hcl, pH 8.0, and 50 μM reduced cytochrome c, as described previously (McClelland et al. 2006; Craig et al. 2007). After mixing, the absorbance was measured at 550 nm and the reaction was followed for 90 s.

Homogenate volumes were chosen to ensure cytochrome c was at saturating concentrations.

Citrate synthase (CS) activity was measured after tissue homogenates were frozen and rethawed three times. CS activity, measured at 412 nm, was assayed in 20 mM Tris-HCl, pH 8.0, 0.1 mM 2,2'-nitro-5,5'dithiobenzoic acid (DTNB) and 0.3 mM acetyl-CoA, with 0.5 mM oxaloacetate added as substrate, as described previously (McClelland *et* al. 2006; Craig et al. 2007).

Statistical Analysis

All data have been expressed as means ± SEM. One-way analysis of variance (ANOVA) followed by a post hoc Fisher's least significant difference test were performed to evaluate potential differences between treatment groups. Gill protein carbonyl content data were normalized by log transformation prior to ANOVA. If conditions to perform an ANOVA were not fulfilled (i.e., data were not normally distributed or did not have equal variances), the Kruskal-Wallis ANOVA on ranks and Dunn's tests were performed. For all tests, a *p-value* ≤ 0.05 was considered statistically significant. All statistical analysis was performed using SigmaStat 3.5 (Chicago, IL, USA).

Results

Fish Survival and Weight

After 96-h Cu exposure either alone or in combination with hypoxia, we observed no mortalities in experimental treatment tanks, with only one death among control tanks. Single and combined exposure to Cu+Hyp had no effect on mean weight of adult killifish compared to controls. Mean weights of fish were 3.34 ± 0.16 g (Ctrl), 3.22 ± 0.20 g (Cu), 3.26 ± 0.15 g (Hyp) and 2.99 ± 0.16 g (Cu+Hyp).

Tissue and Water Chemistry

The average measured dissolved Cu concentrations (Table 2.1) were 0.65 ± 0.11 μg/L (Ctrl), 23.38 ± 0.94 μg/L (Cu), 1.09 ± 0.37 μg/L (Hyp) and 23.59 ± 0.78 μg/L (Cu+Hyp). Normoxic dissolved oxygen concentrations (Table 2.1) were maintained at 9.10 ± 0.00 mg/L (100% air saturation) for controls and Cu treatment tanks, compared to 2.33 ± 0.03 mg/L and 2.51 ± 0.04 mg/L for hypoxia alone and Cu+Hyp treatment tanks, respectively. Water temperature (19.9 ± 0.12 °C) did not vary over the course of the experiments and average water ion concentrations and pH did not significantly fluctuate over time or among treatments (Table 2.1). Dissolved organic carbon (DOC) ranged from 2.03 to 3.09 mg/L across treatment tanks (Table 2.1).

Exposure to Cu, hypoxia alone or in combination caused only limited changes in tissue ion levels. Copper alone caused a significant increase of Ca^{2+} in muscle tissue (44%, p=0.023, Figure 2.1A) and K^+ in the carcass (15%, p=0.016, Figure 2.1B) compared to controls. However, this effect was absent when killifish were exposed to Cu in combination with hypoxia (p>0.05). Whole carcass samples (whole animal minus tissues sampled, see Methods) showed a significant decrease in Mg^{2+} when exposed to hypoxia alone and when combined with Cu (14%, p=0.035 and 13%, p=0.042, respectively, Figure 2.1C) relative to controls.

In comparison, Cu in combination with hypoxia caused a 27% increase of Mg^{2+} in the intestine (p=0.048, Figure 2.1C). There were no significant changes in ion levels observed in either the gill or liver with the experimental treatments.

Copper Accumulation

In the gill, Cu concentrations were significantly elevated when fish were exposed to Cu alone (p<0.001) and in combination with hypoxia (p<0.001, Figure 2.2) compared to controls. While the concentration of Cu was greatly increased by as much as 23-fold in the liver compared to the gill, intestine and muscle, there were no significant differences among treatment groups. In the intestine, muscle and carcass, no changes in Cu concentrations were observed between controls and experimental treatment groups (Figure 2.2).

Oxidative Damage

Despite increased concentrations of Cu in the gill associated with Cu exposure alone and in combination with hypoxia, no significant increases in gill protein carbonyl content were observed (Figure 2.3A). Hypoxia alone significantly decreased gill protein carbonyl content in fish exposed to hypoxia alone (~-61%, $p = 0.018$) compared to controls. When exposed to hypoxia in combination with Cu gill protein carbonyl content tended to decrease (~-38%) compared to controls, but was not statistically significant. Results generally showed protein carbonyl content was higher in the gill, ranging from 0.37 to 7.93 nmol per mg protein compared to 0.09 to 2.41 nmol per mg protein in the liver. Neither copper nor hypoxia exposure had any effect on protein carbonyl content in the liver (Figure 2.3B). In contrast to protein carbonyl content results, TBARS increased by as much as 2.8-fold in response to single and combined Cu+Hyp exposure in the gill and liver of killifish, but high variation within each

experimental group resulted in these changes being not significantly different than controls (Figure 2.3). TBARS showed a trend to increase within the gill with exposure to Cu (2.8-fold) and hypoxia (2.5-fold) alone, as well as in combination (2.9-fold) (Figure 2.3C). However, the same trend was not observed in the liver; although Cu exposure alone caused a non-significant increase in TBARS levels by 2.1-fold, hypoxia alone and in combination with Cu showed TBARS levels similar to controls (Figure 2.3D).

Enzyme Activities

Catalase

In the gill, Cu alone and in combination with hypoxia induced a significant 2-fold (p=0.004) and 2.2-fold (p=0.002) increase in catalase activity, respectively, compared to controls (Figure 2.4A). In contrast, there was a significant decrease in catalase activity in the liver of killifish exposed to Cu (0.27-fold, p=0.05) and a combination of Cu+Hyp (0.36-fold, p=0.006, Figure 2.4B). We observed that hypoxia decreased liver catalase activity by 0.21-fold but the decrease was not significantly different from controls (p=0.10). Liver catalase activity was approximately 10-fold higher than in the gill.

Superoxide Dismutase

Neither copper, hypoxia nor the combination of the two had any effect on SOD activity in the gill and liver of killifish (Figure 2.4C,D).

Cytochrome c Oxidase and Citrate Synthase

Copper and hypoxia alone and in combination, did not induce changes in COX or CS activity in the gill compared to controls (Figure 2.5A,C). Similarly, there was no change in the gill COX-to-CS ratio

between treatment groups (data not shown). Exposure to hypoxia alone tended to increase liver CS activity, but was not significant (Figure 2.5D). However, we observed a significant decrease in liver COX activity in fish exposed to combined Cu+Hyp relative to controls (*p=0.003,* Figure 2.5C). Despite decreased liver COX activity of killifish exposed to Cu in combination with hypoxia, there was no significant change in the COX-to-CS ratio in the liver between treatment groups (data not shown).

Discussion

My findings suggest that the combined effects of waterborne Cu+Hyp do not work synergistically in terms of oxidative stress. Levels of oxidative stress markers protein carbonyls and TBARS did not increase with Cu or with Cu+Hyp. The combination of Cu+Hyp did however reduce the maximal activity of COX in the liver suggesting that together Cu+Hyp reduced this tissues oxidative capacity. Acute Cu exposure alone and in combination with hypoxia also induced significant increases in catalase activity in the gill. In addition, hypoxia alone significantly decreased gill protein carbonyl content, while single and combined Cu+Hyp exposure resulted in a non-significant increase in gill lipid peroxidation. We observed that hypoxia had an antagonistic effect on copper-induced lipid peroxidation in the liver, however this trend was not substantiated across our tests. Therefore, it remains inconclusive whether Cu+Hyp work antagonistically or synergistically to induce changes in the oxidative stress response and oxidative capacity in killifish. However, the results of our study provide important insight into the combined effects of two stressors that commonly co-occur in marine environments (Cu+Hyp) and the biochemical and physiological responses they elicit in an euryhaline species.

Antioxidant Responses

Copper-induced ROS formation is well documented among fish species, most notably occurring in the gill (Bopp et al. 2008) and liver cells (Manzl et al. 2004). While it may seem illogical since the probability of ROS generation increases as O_2 levels increase, hypoxia has been observed to increase ROS production (Chandel et al. 2000; Lushchak et al. 2005, 2011). Fish and other vertebrates utilize enzymatic antioxidant defense mechanisms, such as detoxifying enzymes, CAT and SOD, to

combat ROS production, reducing excess ROS, thus minimizing oxidative damage. Superoxide dismutase acts as the primary preventative inhibitor converting superoxide anions into H_2O_2, but is intrinsically linked to CAT, which then is responsible for converting H_2O_2 into H_2O and O_2 (Kono and Fridovich, 1982). The activities of SOD and CAT are mutually dependent upon one another (Cooper *et al.,* 2002); however, in the present study we did not observe parallel changes in the activity of SOD and CAT (Figure 2.4). Sanchez et al. (2005) showed that exposure to Cu initially induced SOD activity after 4-days, followed by a transitory decrease after 21-days. Therefore, it is possible that the initial increase may have occurred prior to 96-h in the present study and the subsequent H_2O_2 produced by SOD, induced CAT activity (Sanchez et al. 2005).

We found that gill CAT activity increased in response to Cu alone and in combination with hypoxia, indicating the activation of defensive mechanisms presumably to prevent oxidative damage. Although it appears that combined Cu+Hyp did not elicit a synergistic effect. Exposure to Cu and hypoxia alone increased gill CAT activity 2- and 1.5-fold, respectively, however, in combination an additive effect was absent, with activity remaining similar to Cu alone (2.2-fold, Figure 2.4). We suggest that Cu and hypoxia may induce CAT by the same mechanism that has a finite capacity for increased CAT activity, as opposed to Cu+Hyp acting by separate pathways inducing activity. In contrast, we observed significant decreases in CAT activity in the gill of killifish exposed to hypoxia and in the liver of killifish exposed to Cu alone and in combination with hypoxia. Although, hypoxia can induce ROS production, hypoxia also tends to suppress metabolism, which has been suggested to affect both protein synthesis and xenobiotic processing, such as reducing CAT activity (Lushchak et al. 2005). Reduced CAT activity in killifish exposed to Cu alone and in combination with hypoxia may be due to excessive ROS, as it

has been shown that ROS can inactivate CAT (Halliwell and Gutteridge, 1989).

There were no changes in gill and liver SOD activity observed, however, conserved constitutive levels of SOD activity could explain this, since these levels appear to be sufficient to protect against Cu+Hyp induced oxidative stress (Figure 2.4). Sampaio et al. (2008) observed significant increases in SOD activity in the liver of *Piaractus mesopotamicus*, while here in killifish CAT activity significant decreased in response to Cu exposure alone and in combination with hypoxia. It appears that the relationship between the antioxidant defenses of SOD and CAT may be more complicated; therefore the authors would caution against the use of single biomarkers as an indicator of oxidative stress. Excess Cu may also interfere with oxygen transport and energy metabolism (e.g. Depledge, 1984), thus we suggest future studies should measure indicators of oxidative stress combined with metabolic rate and key enzymes of metabolism as physiological endpoints to assess Cu toxicity.

Oxidative Damage

Gills are the primary target organ of Cu toxicity, particularly in freshwater, due to the direct contact with the external environment (Brungs et al. 1973; Buckley et al. 1982; Stagg and Shuttleworth, 1982a). As expected, we found significant Cu accumulation in the gill of killifish exposed to Cu alone and in combination with hypoxia (Figure 2.2). The addition of hypoxia had no effect on accumulation, in agreement with results previously reported by Pilgaard et al. (1994). Our TBARS data showed no statistically significant changes, however, Cu alone and Cu+Hyp tended to induce lipid peroxidation in the gill (Figure 2.3), despite the activation of CAT (Figure 2.4) to combat H_2O_2 derived from O_2^-. In

contrast, Cu on its own and combined with hypoxia was insufficient to induce gill protein carbonylation. The accumulation of protein carbonyls in response to Cu appears to be both time- and dose-dependent, as demonstrated in zebrafish (*Danio rerio*) by Craig et al. (2007, 2010) who observed increased protein carbonyls in softwater-acclimated zebrafish acutely and chronically exposed to high Cu concentrations. Consequently, the exposure time and level of copper used in the present study may not have been sufficient to induce oxidative stress via protein carbonylation. The results of our study suggest lipid peroxidation is a more sensitive indicator of acute Cu-induced oxidative stress than are protein carbonyls. In agreement with this observation are the results of Loro et al. (2012), in which gill lipid peroxidation showed the greatest change in response to another metal that induces oxidative stress (zinc) compared to protein carbonyl levels and any other antioxidant measurement.

Surprisingly, we found that hypoxia had opposite effects to Cu on protein carbonylation and lipid peroxidation in the gill of killifish. Hypoxia significantly decreased gill protein carbonyls, while gill lipid peroxidation tended to increase (Figure 2.3). Similarly, Lushchak et al. (2005) observed lipid peroxidation increased accompanied with decreased or unchanged protein carbonyl content during hypoxia, and only after normoxic recovery did protein carbonyls accumulate in the liver of common carp, *Cyrinus carpio*. Oxygen tension has been shown to have variable effects on the oxidative stress response; hyperoxia clearly induces oxidative stress in different fish species, while hypoxia is known to induce oxidative stress as well as decrease ROS production (reviewed in Lushchak et al. 2011). The mechanisms responsible for hypoxia-induced oxidative stress may include electron transport chains becoming more reduced, increasing electron availability to yield partially reduced oxygen intermediates or the ability of xanthine reductase to be converted to xanthine oxidase, which can act as

ROS producer (Chandel and Shumacker, 2000; Lushchak et al. 2012). However, it seems that some fish species are not directly responding to the stress of hypoxia, but more so preparing to cope with oxidative stress upon recovery, known as the "preparation to oxidative stress" idea (Hermes-Lima et al. 1998). When Cu exposure was combined with hypoxia, gill protein carbonyls also tended to decrease compared to controls. It would be interesting to examine the combined effects of Cu+Hyp on the recovery response of killifish, to determine whether their ability to cope with reintroduction to normoxia is impaired.

The liver is the main detoxifying organ in the fish, as in mammals, and as such is equipped with high antioxidant capacity. However, the liver also possesses a high metabolic rate, which makes it a target for oxidative damage (Ji et al. 1988). Despite observing no changes in Cu accumulation in the liver, single Cu exposure tended to increase liver lipid peroxidation (Figure 2.3). However, when killifish were exposed to Cu in combination with hypoxia or hypoxia alone we did not observe the same trend. These results may suggest that hypoxia and the physiological responses that hypoxia induces, such as metabolic suppression and increased antioxidant defense (Hochachka, 1986), may protect against lipid peroxidation. There are numerous antioxidant enzymes and proteins that were not measured; it is possible that other antioxidant defenses were induced to combat lipid peroxidation, such as glutathione and glutathione peroxidases (Lushchak et al. 2001; Hermes-Lima and Zenteno-Savin, 2002), which could explain why hypoxia alone and in combination with Cu did not induce lipid peroxidation changes in the present study.

Mitochondrial Quantity and Quality

Despite Cu's essential role in mitochondrial function, the mitochondrion is a major target for Cu-toxicity, with potential targets

including oxidative mitochondrial membrane damage and poisoning of enzymes of the tricarboxylic acid cycle (TCA) and energy metabolism (Sheline and Choi, 2004; Arciello et al. 2005). The enzyme activities of CS, a key enzyme in TCA cycle, and COX, a key enzyme in aerobic metabolism, can be used as indicators of mitochondrial density and mitochondrial inner membrane, respectively (Capkova et al. 2002). In our study, no significant differences in the activities of gill CS and COX were found between Cu alone and combined Cu+Hyp exposure (Figure 2.5), which was consistent with the results of Cooper *et al.* (2002) where they observed no change in gill CS induced by hypoxia in spot fish (*Leiostomus xanthurus*) and Lauer et al. (2012) who found Cu exposure did not induce changes in gill COX activity in crab (*Neohelice granulate*).

We observed a significant decrease in liver COX activity in killifish exposed to Cu in combination with hypoxia (Figure 2.5)**.** A reduction in COX activity after exposure to Cu+Hyp may suggest a decrease in COX protein**.** Proper COX assembly is dependent on the presence of Cu; an important step of this pathway involves COX-17, a protein that aids in the assembly of the Cu center of COX (Glerum et al. 1996; Carr and Winge, 2003). Therefore, excess Cu may affect this pathway impacting COX assembly and ultimately protein abundance, as suggested previously (Craig et al. 2007). In addition, molecular oxygen (or lack of oxygen) may directly modify the catalytic activity of COX, which would be consistent with an observed decease in COX enzyme activity (Chandel and Budinger, 1995). However, neither Cu or hypoxia alone induced changes in COX, thus, both stressors may act via different mechanisms to depress COX activity. We expected CS activity to decrease in killifish exposed to hypoxia, as a reduction in activity has been observed to correlate with a reduction in oxygen consumption rates in several fish species (Yang and Somero, 1993), as well as the tendencies of hypoxia to increase the usage

of glycolytic pathways while decreasing the use of aerobic pathways (Cooper et al. 2002). Conversely, we observed hypoxia had no significant effects on liver CS activity. However, no change in liver CS activity has been observed in spot fish (Cooper et al. 2002), gilthead sea bream (Perez-Jimenez et al. 2012) or carp (Zho et al. 2000). Therefore, it appears the effect of hypoxia on the CS activity may be quite variable and dependent on the degree of hypoxia.

A decrease in the ratio of COX/CS can also be used to indicate a biochemical marker of mitochondrial dysfunction (Capkova et al. 2002). In the gill, no change in ratio was observed. While in the liver the combination of Cu+Hyp induced a significant decrease in COX activity, it did not have a large enough impact to significantly affect the COX/CS ratio compared to control fish (Figure 2.5). Therefore, it suggests that neither Cu singly nor combined with hypoxia exposure induced mitochondrial dysfunction in adult killifish.

Perspectives

We show here that combined exposure to environmentally relevant levels of Cu+Hyp induced antioxidant protection pathways and reduced oxidative capacity in a tissue-specific manner, and that hypoxia may have an antagonist effect on Cu-induced lipid peroxidation in freshwater-acclimated killifish. It is important to note, however, that the effect of combined Cu+Hyp on the oxidative stress response may have been masked by individual biological variation in our data. It is clear from this study along with many others that variation in antioxidant enzyme activity is common in fish (e.g. Virani and Rees, 2000; Lushchak, 2011). Therefore, we suggest future studies will need to expose fish chronically to environmentally relevant Cu concentrations versus acutely or increase Cu+Hyp exposure levels that surpass environmentally relevant

concentrations to better elucidate the combined effects of Cu+Hyp on oxidative stress. Overall, the results of our study will improve our understanding of the effects of Cu+Hyp on biochemical and physiological markers of oxidative stress, and as a euryhaline model, killifish will provide essential information for improving ambient freshwater and marine water quality criteria.

Table 2.1: Concentrations of water ions (µM), copper (µg/L), pH, dissolved oxygen (DO, mg/L) and dissolved organic carbon (DOC, mg/L). Values presented as mean ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 4$ replicates for all treatments).

Treatment	Ctrl	Cu (25 µg/L)	Hyp	Cu+Hyp (25 µg/L)
Na^+	925.7 ± 14.2	976.6 ± 21.9	958.9 ± 33.3	963.1 ± 20.6
Mg^{2+}	313.4 ± 1.9	281.2 ± 0.9	302.1 ± 6.7	307.5 ± 2.1
Ca^{2+}	736.9 ± 8.6	756.3 ± 3.8	707.3 ± 14.3	657.5 ± 5.2
K^+	66.7 ± 2.5	46.5 ± 0.8	49.1 ± 1.0	48.4 ± 1.3
Cu	0.7 ± 0.1[a]	23.4 ± 0.9[b]	1.1 ± 0.4[a]	23.6 ± 0.8[b]
pH	8.33 ± 0.08	8.21 ± 0.03	8.27 ± 0.04	8.27 ± 0.05
DO	9.10 ± 0.00[a]	9.10 ± 0.00[a]	2.33 ± 0.03[b]	2.51 ± 0.04[b]
DOC	3.09 ± 0.62	2.18 ± 0.06	2.03 ± 0.07	2.22 ± 0.13

Figure 2.1: Levels of calcium (**A**), magnesium (**B**), potassium (**C**) and sodium (**D**) ions in tissues of killifish exposed to copper (CU), hypoxia (HYP) or a combination of copper and hypoxia (CU+HYP). Values are presented as means ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 8$ for all treatments).
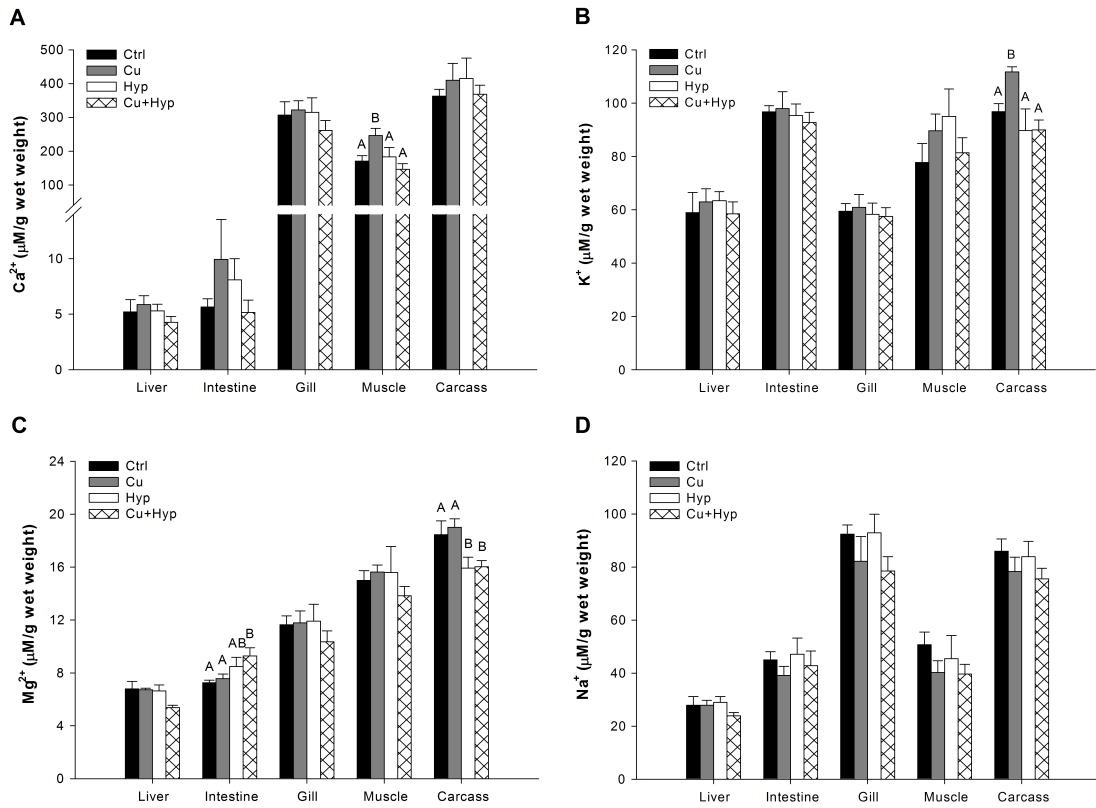
Figure 2.2: Accumulation of copper concentration (μg/g tissue) in tissues of killifish exposed to copper (Cu), hypoxia (Hyp) or a combination of copper and hypoxia (Cu+Hyp). Values are presented as means ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 10$ for all treatments).
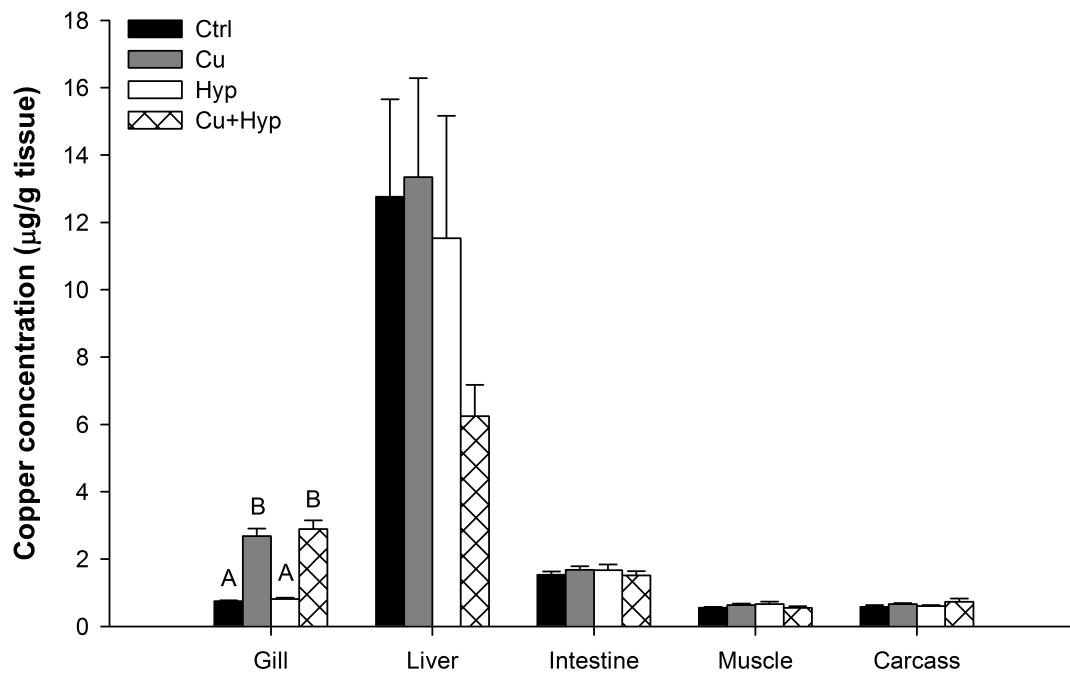
Figure 2.3: Protein carbonyl content (nmol/mg protein) in the gill (**A**) and liver (**B**) and thiobarbituric acid reactive substances (TBARS) levels (μM MDA/mg protein) in the gill (**C**) and liver (**D**) of killifish exposed to copper (Cu), hypoxia (Hyp) or a combination of both (Cu+Hyp). Gill protein carbonyl content data were analyzed with log-transformed values. Values are presented as means ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 8$ for all treatments).

A Gill Protein Carbonyls

B Liver Protein Carbonyls

C Gill TBARS

D Liver TBARS
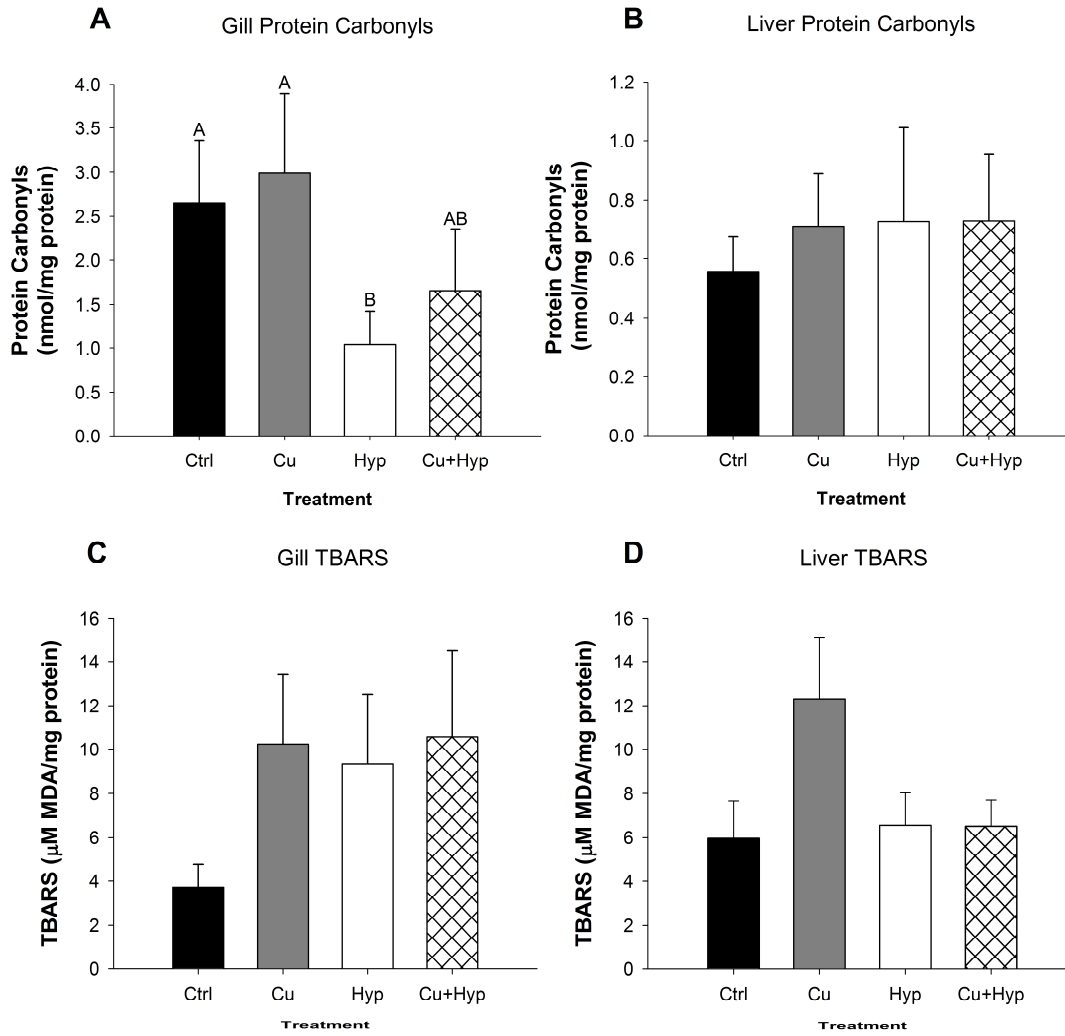
Figure 2.4: Catalase (CAT) activity in the gill (**A**) and liver (**B**) and superoxide dismutase (SOD) activity (U/mg protein) in the gill (**C**) and liver (**D**) of killifish exposed to copper (Cu), hypoxia (Hyp) or a combination of copper and hypoxia (Cu+Hyp). Values are presented as means ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 8$ for all treatments). 1U = μmol/min.
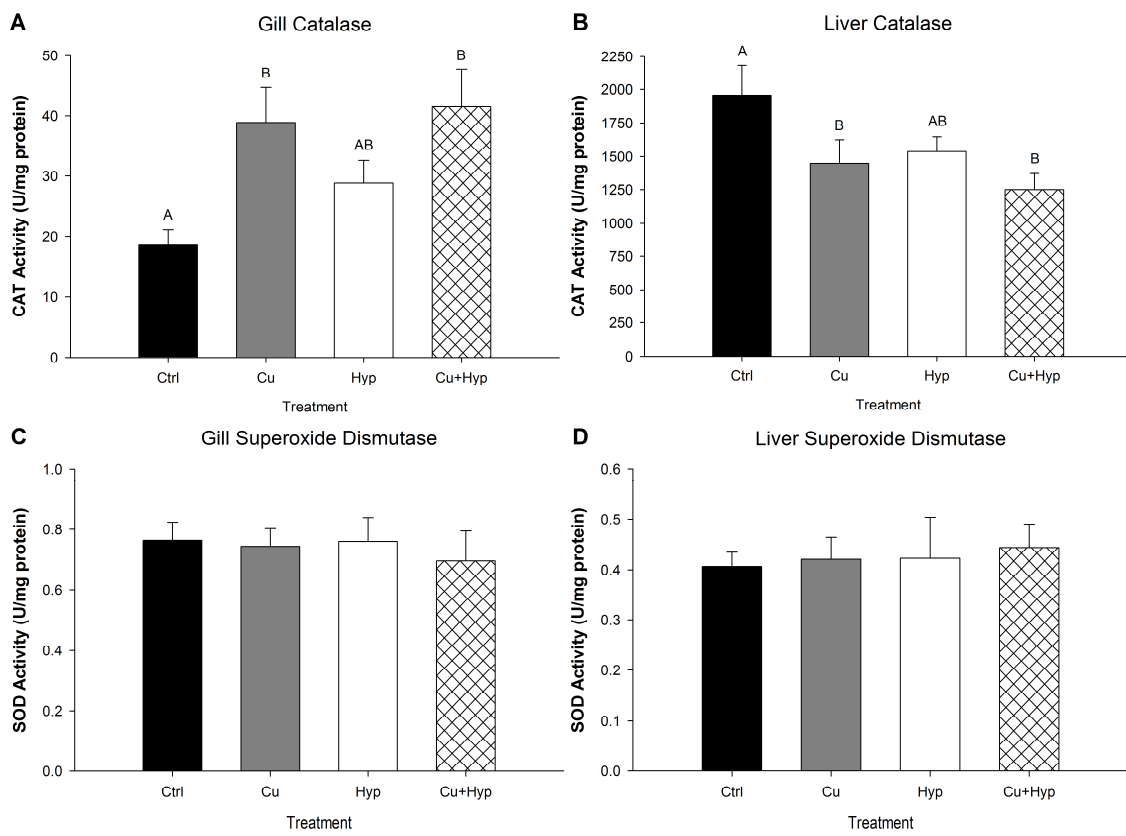
Figure 2.5: Gill (**A**) and liver (**C**) cytochrome *c* oxidase (COX) activity (U/mg protein) and gill (**B**) and liver (**D**) citrate synthase (CS) activity of killifish exposed to copper (Cu), hypoxia (Hyp) or a combination of copper and hypoxia (Cu+Hyp). Values are presented as means ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 8$ for all treatments). 1U = μmol/min.

A   Gill Cytochrome *c* oxidase

B   Liver Cytochrome *c* oxidase

C   Gill Citrate Synthase

D   Liver Citrate Synthase
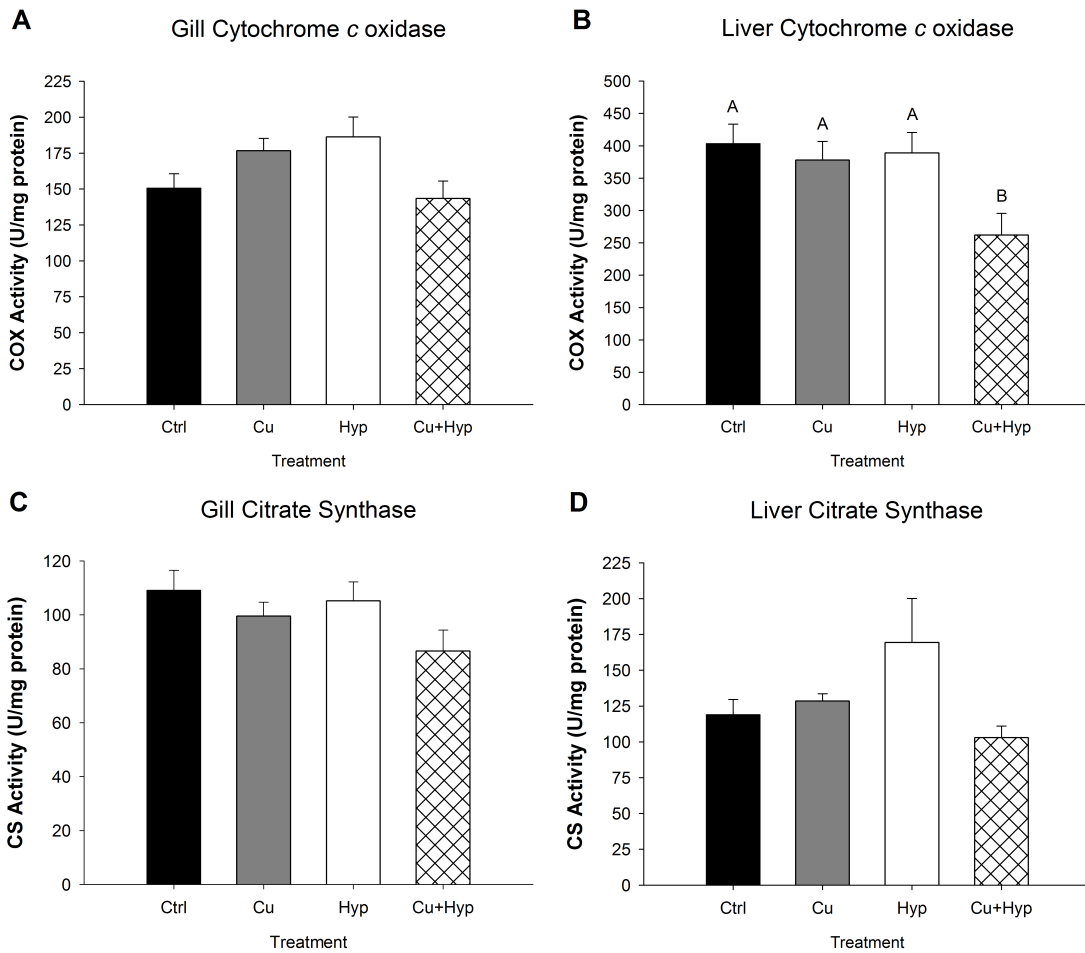
CHAPTER 3

Oxidative Stress and Metabolic Responses to Copper in Freshwater- and Seawater-Acclimated Killifish, *Fundulus heteroclitus*.

Abstract

In freshwater (FW), the main mechanism of copper (Cu) toxicity has been characterized as ionoregulatory disturbance, however, the mechanisms of Cu toxicity in seawater are less well understood. We investigated the effects of salinity on copper-induced oxidative stress and metabolic responses in adult killifish *Fundulus heteroclitus* acclimated to FW (0 ppt) and SW (35 ppt). We exposed FW and SW-acclimated killifish to either low Cu (LC, FW $51.2 \pm 1.2\mu g/L$; SW $49.0 \pm 1.4\mu g/L$) or high Cu (HC, FW $205.6 \pm 7.6\mu g/L$; SW $215.6 \pm 8.9\mu g/L$) and compared them to controls (CTRL, FW $0.65 \pm 0.11\mu g/L$; SW $0.65 \pm 0.11\mu g/L$). No changes in oxygen consumption rate (MO_2) were induced by low or high Cu exposure in FW or SW. However, exposure to LC and HC reduced opercular frequency in FW and SW, although the response was much greater in FW. Exposure to LC and HC increased hematocrit (Hct) and hemoglobin (Hb) in FW and SW, accompanied with reduced blood lactate concentration in FW-acclimated fish. Cu accumulation at the gills was significantly reduced in SW-acclimated killifish compared to FW, however no changes in gill oxidative damage were observed. In general, Cu increased catalase (CAT) maximal enzyme activity (Vmax). The Vmax of superoxide dismutase (SOD) either decreased or remained unchanged depending on tissue-type. Cu induced minimal changes in key enzymes involved in carbohydrate metabolism in all tissues. LC and HC induced oxidative stress and metabolic responses, however minimal differences existed between FW- and SW-acclimated killifish. Therefore, it is unclear whether salinity protects against Cu-induced oxidative stress and metabolic responses.

Introduction

Copper (Cu) is an essential micronutrient at trace amounts, but at elevated levels may cause toxicity to many organisms, including fish. Water chemistry has an important influence on metal speciation, bioavailability and the level of toxicity (reviewed in Campbell, 1995). While anthropogenic inputs affect the concentrations of copper (Cu), pollutants are not the sole factor contributing to Cu toxicity. In marine environments, changes in pH and natural dissolved organic matter (DOM) affect metal speciation and bioavailability but changes in salinity are a major factor affecting toxicity. The speciation of Cu differs with salinity, altering the type and prevalence of Cu complexes available.

The main form of Cu that exerts toxicity is the free ionic Cu^{2+} ion, which is considered to be more bioavailable than inorganic and organic Cu complexes (Hamelink et al. 1994). For example, in seawater (SW) Cu is mainly in the form of $CuCO_3$ and $Cu(CO_3)_2^{2-}$, which is generally thought not to exert toxicity, but may be available for accumulation (Grosell et al. 2004; Blanchard and Grosell, 2006). Only a small percentage of Cu is present in the main toxic form in SW, ionic Cu^{2+}, with $CuOH^-$ and $Cu(OH)_2$ also present and able to exert toxicity (Chakoumakos et al. 1979; Erickson et al. 1996; Paquin et al. 2002). Competition with other cations for binding impact Cu uptake and may ameliorate effects of Cu toxicity on aquatic organisms. With increasing salinity, Cu uptake and Cu accumulation in the gills in fishes has been observed to decrease (Grosell and Wood, 2002; Blanchard and Grosell, 2006). The degree of complexation of Cu with organic ligands and the concentration of dissolved cations has been found to offer a certain degree of protection against Cu toxicity with increasing salinities (Wright, 1995). Therefore, it is very important to take into account water chemistry to predict metal toxicity.

It has been generally established that the mechanisms of Cu toxicity on fish in FW and SW are different (e.g. Blanchard and Grosell, 2006). When exposed to environmentally relevant Cu concentrations in FW, fish mortality involves osmoregulatory disturbances (Grosell et al. 2002). Cu is readily taken up at the gills and accumulates. Cu entering the blood will then accumulate in other organs, such as the intestine and liver (Grosell et al. 2002). In FW, excess accumulation of Cu can affect Na^+ uptake at the gills, and possibly affect the ionoregulation abilities of the fish (Grosell and Wood, 2002; Lauren and McDonald, 1987). Other studies using Cu exposed zebrafish (*Danio rerio*) and killifish show that acute exposure to sublethal Cu concentrations can also affect gill permeability and increase levels of oxidative stress (e.g. Grosell et al. 2004; Craig et al. 2007).

In SW, waterborne Cu likely affects both the gills and intestine. In SW, fishes must drink to compensate for water lost to their hyperosmotic environment and absorption of this ingested water is driven by active transport of Na^+ and Cl^- across the gastro-intestinal tract (Loretz, 1995; Larsen et al. 2002; Marshall and Grosell, 2005; Blanchard and Grosell, 2006). Thus, the intestine is in direct contact with Cu, which can accumulate as a result of drinking. Gastro-intestinal transport processes are affected by acute 96-h and prolonged 30-day Cu exposure, as observed in the marine gulf toadfish *(Opsanus beta)* (Grosell et al. 2004). Grosell et al. (2004) found that Na^+ and Cl^- concentrations decreased in intestinal fluids of all intestinal segments in fish exposed to Cu, however, prolonged exposure increased Na^+ and Cl^- concentrations in a segment-specific manner. The mechanisms of toxicity in SW are not as well defined as in FW. Past studies have shown evidence for impaired ion regulation at Cu concentrations >400 μg Cu/L (e.g. Stagg and Shuttleworth, 1982; Wilson and Taylor, 1993; Grosell et al. 2004), increased production of

reactive oxygen species resulting in oxidative stress (Main et al. 2010) and DNA damage (Costa et al. 2002) and impaired mitochondrial ATP formation (Viant et al. 2002).

Although Cu is essential, in excess concentrations Cu exhibits the ability to produce reactive oxygen species (ROS) via the Fenton reaction, which can result in oxidative stress when levels exceed the ROS defenses (e.g. catalase, superoxide dismutase) or an inability to repair oxidative damage (Dorval and Hontela, 2003; Craig et al. 2007; Almroth et al. 2008; Bopp et al. 2008; Eyckmans et al. 2011). Various biomarkers of oxidative damage have been identified, which include, lipid peroxidation, levels of protein carbonyls and DNA damage. Oxidative damage due to Cu exposure has been observed in the gill, liver and intestine of numerous fish species. In soft-water acclimated zebrafish, exposure to copper increased protein carbonyl concentrations in the gill and liver, accompanied with increases in catalase activity (Craig et al. 2007). Copper exposure elevated lipid peroxidation levels in the liver and intestine of *Esomus danricus*, however in contrast to Craig et al. 2007, Vutukuru et al. (2006) observed declines in antioxidant enzyme activity.

At a whole animal level, oxygen consumption rates in fish exposed to Cu have been observed to decrease (Beaumont et al., 2003). Proposed explanations for the observed metabolic depression include gill damage, such as disruption of branchial structure as a result of apoptotic cell death, secretion of mucus which bind metals and impedes rates of diffusion, inhibition of enzymatic reactions related to respiration, as well as damage of oxygen receptors in the gills **(**Hughes and Shelton, 1958; Hughes, 1966; Wendelaar-Bonga and Lock, 1992; McDonald and Wood, 1993). In carp, increasing concentrations of Cu were found to disrupt the fish's ability to regulate their oxygen consumption (De Boeck et al. 1995). The effects of Cu have also been observed to change expression of genes and enzymes

involved in cellular respiration, including glycolysis, Kreb's cycle and electron transport chain (Hansen et al. 1992; Couture and Kumar, 2003; Craig et al. 2007).

The goal of this study was to investigate the effects of salinity on the copper-induced oxidative stress and metabolic responses in the killifish, *F. heteroclitus*, in attempt to better understand the mechanisms of sublethal Cu toxicity. We hypothesized that increased salinity would provide protection against Cu-induced oxidative stress and minimize changes in aerobic metabolism. We measured oxygen consumption rates, hematological parameters and key enzymes involved in aerobic metabolism to assess metabolic status between Cu exposed FW- and SW-acclimated fish. In addition, we examined biochemical and physiological endpoints associated with oxidative stress. Together, these results will help to further elicit the mechanisms responsible for Cu toxicity across salinities in a model euryhaline teleost.

Methods

Experimental Animals

Adult killifish of mixed sex (body mass: 1.7 – 8.6 g) were collected from the wild (Aquatic Research Organisms, Hampton, NH, USA) and acclimated to 35 parts per thousand (ppt) in an aerated 300-liter aquarium with carbon filtration for a minimum of two weeks. Approximately 200 killifish were then acclimated to either freshwater (FW, 0 ppt) or seawater (SW, 35 ppt). FW killifish were gradually acclimated to decreasing salinity levels from 35 parts per thousand (ppt) to 0 ppt. FW was dechlorinated Hamilton, ON tap water (moderately hard: $[Na^+]$ = 0.6 mequiv L^{-1}, $[Cl^-]$ = 0.8 mequiv L^{-1}, $[Ca^{2+}]$ = 1.8 mequiv L^{-1}, $[Mg^{2+}]$ = 0.3 mequiv L^{-1}, $[K^+]$ = 0.05 mequiv L^{-1}; titration alkalinity 2.1 mequiv L^{-1}; pH⎕8.3; hardness⎕140 mg L^{-1} as $CaCO_3$ equivalents). SW was made by the addition of Instant Ocean sea salt to FW (Big Al's Aquarium Supercenter, Woodbridge, ON, CA). FW-acclimated killifish were maintained in carbon-filtered aerated, 45-liter tanks, with 20% water-renewed daily for a minimum of 2-weeks prior to experimentation. SW-acclimated killifish were maintained in aerated, 300-liter tanks with carbon filtration for a minimum of 2-weeks prior to experimentation. During acclimation, fish were fed daily with a commercial tropical fish food (Big Al's Aquarium, Woodbridge, ON, CA) and maintained under natural photoperiod (12:12-h light:dark) at approximately 18 °C. Fish were fasted for 48 hours prior to the start of experiments and throughout the 96-hour exposure. No mortalities were observed in any of the experimental treatment or control tanks.

Copper Exposure

Killifish (*n* = 180) were removed from their respective FW or SW acclimation tanks and placed into 8-liter experimental tanks (*n* = 10 per tank, 3 treatments and 3 replicates per treatment). The three experimental conditions (*n* = 30 each) used were: Controls (Ctrl; FW, SW), Low Cu (LC;

FW, SW) and High Cu (HC; FW, SW), using a stock Cu solution made from $CuSO_4$ dissolved in 1% HNO_3, which had no significant effect on water pH. Tank water was renewed daily by 80%, with thoroughly mixed renewal water prepared 24-h in advance for FW (Na^+, 1091.2 ± 11.2 μM, Ca^{2+}, 686.4 ± 5.3 μM, Mg^{2+}, 347.0 ± 2.8 μM, K^+, 49.1 ± 1.8 μM; pH~8.3) and SW (Na^+, 469.61 ± 11.7 mM, Ca^{2+}, 9.3 ± 0.1 mM, Mg^{2+}, 54.0 ± 0.9 mM, K^+, 8.2 ± 0.1 μM; pH ~8.3) tanks.

Metabolic Rate

Oxygen consumption rate (MO_2) were determined on individual fish by using closed respirometry as previously described (Blewett et al. 2013). Fish were held in individual custom-made respirometers that had been covered with duct tape to prevent light in, filled with water from their respective treatments. Fish were allowed to acclimate to the chambers for 2-h before respirometry measurements were taken. We performed preliminary trials to determine the necessary amount of time for killifish needed to acclimate prior to respirometry to minimize the effects of stress on metabolic rate. During these preliminary trials it was determined that 2-h was sufficient for oxygen consumption measurements to not be affected by stress. The temperature was controlled by a recirculating system and was maintained at 18 °C. The respirometers were then closed to produce an air-tight seal. Measurements were taken over a 2-h period using an oxygen electrode, during which 5 ml water samples were taken at 0 min and 120 min for the measurement of partial pressure of oxygen (PO_2).

Sampling

Total and dissolved water samples were taken prior to each water replacement, with dissolved samples filtered through a 0.45 μm filtration disc (Pall Life Sciences, East Hills, NY, USA) to measure water chemistry

parameters. After 96-h exposures, killifish were quickly euthanized by cephalic concussion and gill, liver, intestine and remaining carcass were removed and weighed and quickly frozen in liquid N_2. Blood samples were collected from the dorsal aorta using heparinized capillary tubes and microhematocrit tubes blood by severing the tail behind the anal fin for measuring hematocrit and hemoglobin, respectively. Blood lactate levels were immediately measured using a hand-held lactate meter (Lactate Pro, Arkray, Kyoto, Japan). Blood samples were kept on ice until centrifuged using a microhematocrit centrifuge (International Equipment Company, Needham Heights, MA, USA) at 14,000 g for 2 min to determine hematocrit value (Hct). Hemoglobin (Hb) was determined spectrophotometrically from 5 μL blood following reaction with Drabkin's reagent (Sigma Chemical Co, USA). Using the Hct and Hb values obtained, we calculated mean cell hemoglobin concentration (MCHC).

Tissue and Water Analyses

Copper concentration in tissue and total and dissolved water samples were measured by Graphite Furnace Atomic Absorption Spectroscopy (GFAAS, Spectra AA 220Z, Varian Palo Alto, CA) as described previously (Craig et al. 2007). Tissue samples were digested in a volume of 2N nitric acid equivalent to five times their volume (Trace metal grade, Fisher Scientific, Ottawa, ON, CA) at 60 °C for 48 hours, and vortexed after 24 hours. Tissue digests were then diluted as necessary and dissolved Cu concentrations determined using a 40 μg/L Cu standard for comparison (Fisher Scientific, Ottawa, ON, CA). An additional step was performed on seawater samples using a modified method from Toyota *et al.* (1982) to eliminate Na^+ interference with GFAAS measurements. Tissue and water ion compositions were measured by Flame Atomic Absorption Spectroscopy (Spectra AA 220FS, Varian, Inc., Mulgrave,

Victoria, Australia) as previously described (Craig et al. 2007) and verified with appropriate standards (Fisher Scientific, Ottawa, ON, CA). Tissue samples were diluted as necessary with 1% HNO_3 (Na^+), 1% $LaCl_3$ in 1% HNO_3 (Ca^{2+}, Mg^+) or 0.1% $CsCl_2$ in 1% HNO_3 (K^+).

Oxidative Damage

Protein carbonyls. Protein carbonyl content was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI) as previously described (Craig et al. 2007). Tissues were homogenized in 1 ml of homogenization buffer (50 mM phosphate buffer, 1 mM EDTA, pH 6.7) and centrifuged at 10,000 g for 15 min at 4 °C and the supernatant was removed for analysis. Samples and controls were prepared with 200 μl of supernatant added plus 800 μl of DNPH to the sample tube and 800 μl of 2.5 M HCl to the control tube. Sample and control tubes were then incubated in the dark for 1 h. After incubation, 1 ml of 20% TCA was added, tubes were vortexed and then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was removed, 1 ml of 10% TCA was added, and tubes were vortexed and centrifuged again. The supernatant was removed, and the pellet was washed with three sequential washes of 1 ml 1:1 ethanol:ethyl acetate followed by centrifugation. After the third wash, the pellet was resuspended in 500 μl of 2.5 M guanidine hydrochloride. Then, 220 μl from each sample and control tube was placed in a 96-well plate, and the absorbance measured at 375 nm in duplicate. Peak absorbance from the control value was subtracted from the sample value, and this corrected absorbance was used to calculate protein carbonyl content. Protein concentration of the tissue homogenate was assayed according to Bradford (1976), using bovine serum albumin (BSA) to construct standard curve at 565 nm. Protein carbonyls are expressed in nanomoles per milligram total protein.

Enzyme Activity

Frozen gill and liver tissues were powdered in liquid nitrogen with a mortar and pestle, then diluted 1:10 (mg tissue:μL) in ice-cold homogenization buffer (20mM HEPES, 1 mM EDTA, 0.1% Triton X-100, pH 7.2) using a cooled glass on glass homogenizer. All enzyme activity levels were assayed in 96-well plates using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Menlo Park, CA, USA). Assays were performed in triplicate, with an additional negative control well lacking substrate, to correct for background activity as described previously (McClelland et al. 2005). All chemicals used were purchased from Sigma Aldrich (Oakville, ON, CA) and reaction buffers were prepared fresh daily. All enzyme activity is reported as units (U) per milligram protein.

Enzyme activity of catalase (CAT), superoxide dismutase (SOD), cytochrome *c* oxidase (COX) and hexokinase (HK) were measured on fresh tissue homogenates. Enzyme activity of pyruvate kinase (PK), lactate dehydrogenase (LDH) and citrate synthase (CS) were measured after have been frozen and thawed once, twice and three times, respectively. The activities of CAT, COX, CS, HK, LDH, PK, HK were assayed as previously described (Craig et al. 2007, McClelland et al. 2005, Schippers et al. 2006). Hexokinase activity was not measured in the liver, due to low activity and high background levels. Assay conditions were as follows, COX: 50 mmol l^{-1} Tris (pH 8.0), 50 µmol l^{-1} reduced cytochrome c; HK: 50 mmol l^{-1} Hepes (pH 7.6), 5 mmol l^{-1} d-glucose (omitted in control), 8 mmol l^{-1} ATP, 8 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} NADP, 4 U glucose-6-phosphate dehydrogenase; PK: 5 mmol l^{-1} phosphoenol pyruvate (PEP; omitted in control), 50 mmol l^{-1} imidazole (pH 7.4), 5 mmol l^{-1} ADP, 100 mmol l^{-1} KCl 10 mmol l^{-1} $MgCl_2$, 0.15 mmol l^{-1} NADH, 10 mmol l^{-1} fructose 1,6-phosphate and 5 U lactate dehydrogenase (LDH); CS: 0.5 mmol l^{-1}

oxaloacetate (omitted in control), 0.3 mmol l^{-1} acetyl-CoA, and 0.1 mmol l^{-1} dithiobisnitrobenzoic acid (DTNB) in 20 mmol l^{-1} Tris (pH 8.0); LDH: 40 mmol l^{-1} Tris (pH 7.4), 0.28 mmol l^{-1} NADH, 2.4 mmol l^{-1} Pyruvate-Na; CAT: 20 mmol l^{-1} Potassium phosphate buffer (pH 7.0), 20 mmol l^{-1} H_2O_2. Superoxide dismutase (SOD) activity (CuZn-SOD and Mn-SOD) was determined by using a commercial kit (Fluka, Sigma Aldrich, Oakville, ON). The assay measures the formation of formazan dye, utilizing the reduction of tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo, Kumamoto, Japan) with superoxide radicals at 450 nm. The reaction of xanthine oxidase generates the source of superoxide radicals. One unit of SOD is defined as the amount required to inhibit the reduction of WST-1 to WST-1 formazan by 50%.

Statistical Analysis

All data have been expressed as means ± SEM. Two-way analysis of variance (ANOVA) followed by a post hoc Tukey's test were performed to evaluate potential differences between treatment groups (Ctrl, LC and HC) and salinities (FW and SW). All data were tested for normality and log-transformation was performed when necessary. For all tests, a *p-value* ≤ 0.05 was considered statistically significant. All statistical analyses were performed using SigmaStat 3.5 (Chicago, IL, USA).

Results

Water and Tissue Chemistry

Total and dissolved Cu concentrations were close to the nominal values of 50 and 200 μg Cu/L, respectively, in FW and SW (Table 3.1). In FW-acclimated fish exposed to Cu, particularly at high Cu concentrations, we observed significant secretion of mucus over the body surface of *F. heteroclitus.* Exposure to low or high Cu did not significantly change tissue ion concentrations in either FW- or SW-acclimated killifish (Figure 3.1). Salinity significantly increased Na^+ and Mg^{2+} concentrations in a tissue-specific manner, with changes observed in the gill and intestine. Gill Na^+ levels significantly increased in SW-acclimated killifish for control, low and high Cu exposures, relative to their FW counterpart. Intestine Mg^{2+} levels followed the same trend, increasing in SW-acclimated killifish for all treatments compared to their FW counterpart, however, increased salinity only significantly increased gill Mg+ levels in killifish exposed to low Cu concentrations relative to FW-acclimated killifish. In contrast, salinity had no effect on Ca^{2+} and K^+ concentrations in any tissue (Figure 3.1).

Copper Accumulation

In general, Cu exposure resulted in increased Cu accumulation across all tissues (Figure 3.2). Surprisingly, no dose-dependent accumulation of Cu was observed in any tissues. Salinity effected Cu load, with decreased Cu accumulation observed in the gill and liver in a treatment-specific manner. This effect was absent in the intestine and carcass. A significant interaction between salinity and treatment was observed only on gill Cu load. Cu load in the gill were elevated by as much as 4.4-fold, in FW-acclimated fish exposed to either low or high Cu concentrations. In SW-acclimated fish, we observed diminished Cu concentration by as much as 5.2-fold in the gill of killifish exposed to low and high Cu, relative to their respective FW counterparts (Figure 3.2A). In

SW, only exposure to high Cu significantly elevated gill Cu concentrations (1.6-fold), relative to controls. Low and high Cu exposure in FW-acclimated fish significantly increased intestinal Cu concentrations to similar levels (2.8 and 3.3 μg Cu/g tissue) compared to controls (2.0 μg Cu/g tissue). In comparison, intestinal Cu concentrations in SW-acclimated fish were significantly elevated only at the high Cu treatment (Figure 3.2B). Exposure to high Cu significantly increased liver Cu load by 1.7-fold and 1.9-fold in FW-acclimated and SW acclimated fish, respectively (Figure 3.2C). The highest concentration of Cu in all measured tissues was observed in the liver of FW-acclimated fish (81.4 μg Cu/g tissue). Acclimation to SW significantly reduced liver Cu load by 43% and 40% relative to FW, but only in fish exposed to control and high Cu treatments respectively (Figure 3.2C). Overall, salinity and Cu exposure had no effect on carcass Cu accumulation, with the exception of exposure to low Cu in SW-acclimated fish significantly increasing accumulation relative to controls and high Cu treatment FW-acclimated fish (Figure 2D).

Oxidative Damage and Antioxidant Responses

Overall, exposure to Cu tended to increase gill, liver and intestine protein carbonyl levels in both FW- and SW-acclimated fish (Figure 3.3A, B, C). However, significant increases in protein carbonyl levels were only observed in the intestine of FW-acclimated fish exposed to low and high Cu compared to controls (Figure 3.3B). Salinity had no significant effects, with the exception of SW-acclimated fish exposed to low Cu where we found a 25% reduction in intestine protein carbonyl levels relative to FW-acclimated fish exposed to the same treatment (Figure 3.3B). We observed no significant increases in gill protein carbonyl levels in FW-acclimated or SW-acclimated fish in response to low or high Cu treatments (Figure 3.3A), despite significantly increased gill Cu concentrations being

observed in these same exposures (Figure 3.2A). However, with increased salinity gill protein carbonyl levels tended to increase across all treatments. Exposure to low and high Cu in FW-acclimated fish significantly increased intestinal protein carbonyl levels by 2.7-fold and 3.0-fold, respectively relative to controls (Figure 3.3B). In contrast, there was no increase in intestinal protein carbonyl levels observed in SW-acclimated fish. Despite significantly increased liver Cu concentrations in FW-acclimated and SW-acclimated fish exposed to high Cu levels, liver protein carbonyl levels were of similar magnitude relative to their respective controls (Figure 3.3C).

Antioxidant enzymes showed different patterns of change, with Cu exposure significantly increasing CAT activity in FW and SW across all tissues relative to their respective controls, while SOD activity significantly decreased in the gill or remained similar to controls in the liver and intestine in FW and SW-acclimated fish (Figure 3.3). Salinity effects for CAT and SOD activities were minimal, and showed no specific tissue or treatment trend. A significant interaction between salinity and treatment was observed only on gill CAT activity. In FW- and SW-acclimated fish, exposure to Cu resulted in increased CAT activity in all measured tissues, with the exception of gill CAT activity in SW-acclimated fish where Cu exposure had no significant effect (Figure 3.3D, E, F). High Cu exposure significantly increased CAT activity by 2.4-fold to 10.9 U/mg protein compared to 4.7 U/mg protein in FW-acclimated controls (Figure 3.3D). Salinity significantly increased gill CAT activity in controls, to similar activity levels induced by Cu. In the intestine, CAT activity was 4-6 times greater than that of the gill. Exposure to high Cu significantly increased intestinal CAT activity by ~1.5-fold in FW- and SW-acclimated fish, while a significant increase in CAT activity at low Cu concentrations was only observed in FW-acclimated fish (Figure 3.3E). In the liver, CAT activity

was 4-10 and 40-100 times greater than that of the intestine and gills, respectively (Figure 3.3F). High Cu exposure in FW-acclimated fish and low Cu exposure in SW-acclimated fish significantly increased liver CAT activity by ~1.8-fold relative to controls. Salinity significantly decreased liver CAT activity in fish exposed to high Cu. In general, SOD activity was much lower in all tissues measured, by as much as three orders of magnitude, compared to CAT activity. In contrast to CAT activity, SOD activity significantly decreased in the gills of FW-acclimated fish exposed to high Cu and SW-acclimated fish exposed to low Cu, by 35% and 37%, respectively (Figure 3.3G). Salinity significantly decreased gill SOD activity at low Cu exposure levels. Minimal changes were observed in intestinal SOD activity, with a significant difference in SOD activity between SW-acclimated fish exposed to low and high Cu (Figure 3.3H). No significant effects of treatment or salinity on SOD activity were observed in the liver (Figure 3.3I).

Oxygen Consumption Rate and Opercular Frequency

In FW- and SW-acclimated fish, exposure to low and high Cu levels had no effect on oxygen consumption rates, with values remaining similar to controls (Table 3.2). There were no significant differences in mean weights of killifish used for oxygen consumption rate measurements. A significant interaction between salinity and treatment was observed on opercular frequency. However, opercular frequencies of Cu-exposed FW- and SW-acclimated fish were observed to significantly decrease relative to controls, with no marked differences between low and high Cu exposure levels (Table 3.2). Opercular frequency decreased to a greater extent in FW-acclimated fish, decreasing by as much as 70% to 27 breaths/min when exposed to high Cu levels compared to 92 breaths/min in controls. In comparison, opercular frequency decreased by 14% to 81 breaths/min

when exposed to high Cu levels compared to 95 breaths/min in controls. Salinity significantly increased opercular frequency in killifish exposed to low and high Cu, relative to their respective FW counterpart. In both low and high Cu-exposed FW-acclimated fish, it was visually observed that fish altered their breathing pattern from continuous to discontinuous with long bouts of apnea, as well as increased their amplitude of breath. This pattern of breathing was not observed for SW-acclimated fish exposed to Cu.

Hematological Parameters

Hematological measures showed changes in response to Cu exposure in FW-acclimated fish, however minimal changes were observed in SW (Table 3.2). Significant salinity effects in fish exposed to Cu and a significant interaction between salinity and treatment were observed in all hematological measures, with the exception of MCHC. The concentration of blood lactate decreased significantly by 34% and 39% reaching similar levels in FW-acclimated to low and high Cu levels, respectively. Changes in blood lactate levels in FW-acclimated fish exposed to low and high Cu, were also accompanied by significant increases in Hct and Hb. Exposure to low Cu in FW increased Hct by 30% and Hb by 49%, while high Cu levels showed slightly larger increases of 36% and 52%, respectively. In contrast, no effect of either low or high Cu exposure was observed in SW-acclimated fish, with the exception of Hct levels significantly differing between low and high Cu exposed fish, however neither levels differed from controls. No significant differences in MCHC across treatment or between salinities were observed.

Carbohydrate metabolism

Impacts of Cu exposure and salinity on enzymes involved in carbohydrate metabolism showed variable responses, in a tissue-specific manner. A significant interaction between treatment and salinity were observed in intestinal PK and HK activities and liver PK activity. In the gill, PK was the only enzyme that showed a significant change due to Cu exposure, with PK activity significantly decreasing by 33% and 29%, in SW-acclimated fish exposed to low and high Cu, respectively (Figure 3.4A). Conversely, in the intestine exposure to high Cu significantly increased PK activity 1.5-fold, relative to controls in FW-acclimated fish (Figure 3.4B). In SW-acclimated fish, low and high Cu exposure tended to decrease intestinal PK activity by 11% and 13%, respectively. Low and high Cu levels significantly increased liver PK activity by 1.7-fold and 2.4-fold, respectively, in FW-acclimated fish. A similar trend was observed in SW, with PK activity increasing by 1.8-fold and 1.5-fold in fish exposed to low and high Cu, respectively, however the increase in response to high Cu was not significantly different from controls. Salinity significantly increased gill HK activity in all treatments, compared to controls (Figure 3.4G). In the intestine, significant effects of salinity were found, however, opposing trends were observed, with HK activity increasing in controls and decreasing in high Cu exposed fish in response to increase salinity (Figure 3.4H). In the intestine, exposure to high Cu significantly increased HK activity in FW-acclimated fish relative to controls and low Cu exposed fish (Figure 3.4H). No significant changes in gill, intestine or liver LDH activity were observed (Figure 3.4D, E, F).

Aerobic metabolism

Enzymes of aerobic metabolism showed minimal changes to Cu exposure and increased salinity in all tissues measured (Figure 3.5). A

significant interaction between treatment and salinity was observed in intestinal CS activity. In the gill, exposure to low Cu in FW and high Cu exposure in SW significantly decreased COX activity, relative to their respective controls (Figure 3.5A). In contrast, no significant changes were observed in gill CS activity and COX/CS ratio (Figure 3.5D, G). High Cu exposure in SW-acclimated fish significantly increased intestinal COX and CS activities by ~1.5 compared to their respective controls, while no changes were observed in COX/CS ratio (Figure 3.5B, E, H). However, in the intestine, a significant effect of salinity was observed in fish exposed to high Cu, with the COX/CS ratio decreasing with increased salinity (Figure 3.5H). No significant changes in enzyme activity in response to Cu and salinity were observed in the liver, with the exception of a significant decrease in COX/CS ratio in low Cu exposed FW-acclimated fish relative to controls (Figure 3.5C, F, I).

Discussion

Copper Accumulation

Overall, different tissue-specific patterns of Cu accumulation were observed in killifish, as a result of exposure concentration and salinity (Figure 3.2). In the presence of elevated Cu, increased salinity induced a drastic decrease in Cu accumulation in the gill, compared to FW, an effect that was independent of dose (Figure 3.2A). This effect on Cu accumulation confirms that the primary target of Cu toxicity in FW is the gill, consistent with previous studies (e.g. Blanchard and Grosell, 2006). Decreased Cu accumulation in the gills in SW is most likely due to the increased competition between Cu and cations for the same binding sites, as well as changes in physiology of the gill associated with the change in external ion content (Niyogi and Wood, 2004; Blanchard and Grosell, 2006; Scott et al. 2008). It appears that there is a finite ability to accumulate Cu in the gills of killifish, as increasing waterborne Cu did not lead to increases in gill Cu accumulation. Despite the finite ability of the gills to accumulate Cu, increased [Cu] is reflected in an increased Cu content in downstream organs, such as the liver.

In contrast to the effects in the gills, acclimation to SW had no effect on Cu accumulation in the intestine, despite SW-acclimated fish employing different osmoregulatory strategies relative to FW (Figure 3.2B). In FW, both low and high Cu exposure elevated intestinal Cu load, while in SW only high Cu exposure induced a significant increase in intestinal Cu. Similar Cu accumulation in the intestine of FW- and SW-acclimated fish may be explained by increased intestinal HCO_3^- secretion in response to elevated salinity, which can bind Cu reducing its bioavailability (Genz et al. 2008). Our results indicate that the intestine is a likely target for Cu toxicity in both FW- and SW-acclimated fish.

In the liver, Cu accumulation in SW-acclimated fish decreased relative to FW, with significant changes observed in fish exposed to high Cu levels (Figure 3.2C). The final accumulation of Cu in the liver depends on the amount of uptake that occurs at the gill and intestine (Blanchard and Grosell, 2006), therefore higher levels of Cu uptake at the gills in FW could account for differences observed in the liver. Thus, it suggests acclimation to SW had a protective effect reducing gill Cu accumulation, and presumably fish would also accumulate less Cu in the liver. It is also interesting to note that, relative to controls, only high Cu exposure increased liver Cu accumulation in FW and SW. The liver is the main organ involved in Cu homeostasis. Cu that accumulates in the liver is then excreted mainly via the bile (Grosell et al. 1998; Mazon and Fernandes, 1999). However, at high Cu concentrations it appears that killifish lose the capacity to regulate homeostatic mechanisms, or they could be accumulating Cu in the liver, perhaps bound to MT to maintain overall physiological homeostasis by making Cu biologically unavailable. It is difficult to determine if certain homeostatic mechanisms (i.e. distribution and utilization, storage and detoxification and efflux pathways; reviewed in La Fontaine and Mercer, 2007) are more affected than others.

In general, salinity and Cu exposure had no effect on carcass Cu accumulation (Figure 3.2D). This suggests the carcass is not likely to be a target of Cu toxicity, which is in agreement with previously published results (Kamunde et al. 2002a; Grosell et al. 2004; Blanchard and Grosell, 2006).

Oxidative Damage and Antioxidant Responses

Oxidative damage and antioxidant enzymes in the oxidative stress response pathway have been suggested as sensitive indicators of Cu toxicity in fish (e.g. Craig et al. 2007; Sampaio et al. 2008). Protein

carbonylation and lipid peroxidation are considered consequences of oxidation of protein side chains and lipids by ROS (Halliwell and Gutteridge, 2001). Softwater-acclimated zebrafish exposed to sublethal (15 μg/L) Cu concentrations showed elevated gill and liver protein carbonyls and SOD activity and inhibited CAT activity (Craig et al. 2007). These antioxidant responses were correlated with increased Cu tissue accumulation (Craig et al. 2007). Similar trends were observed in pacu (*Piarctus mesopotamicus*) exposed to 0.4 mg/L Cu for 48-h, with significant increases in liver lipid peroxidation and SOD activity, and reduced CAT activity (Sampaio et al. 2008). These changes were associated with increased plasma Cu concentrations (Sampaio et al. 2008). In the present study, exposure to sublethal Cu concentrations (50 and 200 μg/L) only induced changes in intestinal oxidative damage, measured as protein carbonyls, in FW-acclimated fish (Figure 3.3B). However, this response was absent in the gill, despite the fact they significantly accumulated increased Cu, relative to controls, and in the liver, which had the highest accumulation of Cu relative to other tissues. This suggests that Cu-induced oxidative damage may affect tissues to different extents at sublethal concentrations, and may not necessarily follow the same patterns throughout the entire fish (Loro et al. 2012).

Oxidative damage has been shown to be induced by Cu, but is dependent on the dose being more effective as an indicator of toxicity at high concentrations. In *Esomus danricus* exposed to sublethal (0.55 mg/L) Cu for 96-h no changes in lipid peroxidation in visceral tissue were observed (Vutukuru et al. 2006). Conversely, at lethal (5.5 mg/L) Cu levels significantly increased lipid peroxidation in the tissue was noted (Vutukuru et al. 2006). While exposure to low or high Cu concentrations had no significant effect on gill protein carbonyls in the current study, levels tended to increase in a dose-dependent manner in both FW- and SW-

acclimated fish (Figure 3.3A). Thus, the exposure concentration of Cu used in this study may not have been high enough to induce significant oxidative damage in killifish.

Another possibility is that killifish possess protective mechanisms that combat and detoxify elevated ROS, thus mitigating the level of oxidative damage. Antioxidants prevent cellular damage caused by metabolically- and environmentally-produced ROS, such as hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-) and hydroxyl radical (Halliwell and Gutteridge, 1990). Although exposure to Cu can induce antioxidant enzyme activity in fish, it can also inhibit enzyme activity depending on exposure concentration, length of exposure and species (reviewed in Grosell, 2012). A distinct salinity-independent pattern of antioxidant enzyme activities was noticed in killifish exposed to Cu. FW-acclimated killifish responded to Cu exposure with an increased activity of CAT activity in the gill, liver and intestine (Figure 3.3D-F). This was accompanied by a decrease in SOD activity in the gill, and no changes in SOD activity in the liver and intestine (Figure 3G-I). An increase in CAT activity represents a direct response to ROS to detoxify and combat Cu-induced oxidative stress, therefore alleviating the levels of damage to proteins, lipids and DNA. This response may explain the inability of low and high Cu exposure to induce protein carbonyls in the current study. In SW-acclimated fish, Cu had no effect on gill CAT activity (Figure 3.3D). This could be due to the low levels of Cu that accumulated in the gills, as well as a tendency for CAT activity to increase with increased salinity (Martinez-Alvarez et al. 2005).

However, our results were opposite to the patterns observed by Craig et al. (2007) for zebrafish and Sampaio et al. (2008) for pacu who saw SOD activity increased and either no change or decreased activity of CAT, respectively. SOD is the first enzyme involved in detoxifying ROS,

converting superoxide anion radicals produced in the ROS cascade to H_2O_2. CAT is the next enzyme in the cascade, converting H_2O_2 to water and oxygen. Sanchez et al. (2005) showed that exposure to Cu initially induced SOD activity after 4-days, followed by a transitory decrease after 21-days. This transitory decrease in SOD was also observed in blue mussels exposed to Cu (Manduzio et al. 2003). This decrease in SOD may result from Cu directly binding to the enzyme inhibiting activity as previously suggested in fish (Pedrajas et al. 1995). Thus, it is likely that the resulting hydrogen peroxide produced from the initial increased SOD activity, subsequently induces CAT activity (Sanchez et al. 2005). Similar to our results, Paris-Palacios et al. (2000) observed increased CAT activity in zebrafish chronically exposed to Cu for 2-weeks at concentrations of 40 and 140 μg Cu/L. This supports the idea that exposure length and concentration impact antioxidant enzyme activity. For these reasons, we suggest that protein carbonyls and antioxidant enzymes are not a sensitive measure to indicate sublethal Cu toxicity in killifish.

Copper Effects on Metabolism Pathways

At the cellular level, Cu can induce changes in the activity of numerous enzymes an effect that will impact metabolic pathways, which can result in changes in cellular processes, including a reduction in ATP synthesis rates, interference with oxygen transport and mitochondrial biogenesis (Depledge, 1984; Hansen et al. 1992; Craig et al. 2007). Metabolic pathways are continuously maintaining and regulating homeostasis, with key enzymes controlling the metabolic flux (Brooks and Storey, 1995). In the first stage of carbohydrate metabolism, key enzymes include HK, PK and LDH. HK, is involved in the initial step phosphorylating glucose to glucose-6-phosphate. PK and LDH are involved in the last steps of glycolysis converting phosphoenolpyruvate (PEP) to pyruvate and

reducing pyruvate to lactate (under anaerobic conditions), respectively. In general, a reduction in energy availability is observed in Cu-exposed fish, an effect that has been suggested to occur as a result of decreased glycolytic enzyme activities (Hansen et al. 1992; Gul et al. 2004, Carvalho and Fernandes, 2008). Inhibition of PK and HK activities was reported in the liver of *Dicentrarchus labrax* (Isani et al. 1994) and *Prochilodus lineatus* (Carvalho and Fernandes, 2008) exposed to acute sublethal Cu concentrations. Reduction in glycolytic capacity may be dependent on direct competition of Cu with essential divalent cations, such as Mg^{2+}, for protein-binding sites. These essential cations are required for catalytic activity of all kinases, thus the exclusion of them from their binding sites by the presence of Cu will induce enzyme conformational changes, altering activity (Streyer, 1981; Isani et al. 1994; Carvalho and Fernandes, 2008).

The activity of LDH indicates the capacity for lactate and pyruvate exchange and can be used as an index of anaerobic capacity. Anaerobic metabolism appears to be less sensitive to Cu inhibition than aerobic metabolism (Cheny and Criddle, 1996). Overall, inhibition of LDH in tissues is absent in Cu-exposed fish and invertebrates, and conversely, an induction of LDH is observed (e.g. Hansen et al. 1992; Cheny and Criddle, 1996; Tóth et al. 1996; Couture and Kumar, 2003). However, exposure to Cu in *Sparus auratus* led to a decrease in LDH activity in the liver (Antognelli et al., 2003). Couture and Kumar (2003) suggested that decreases in aerobic capacity due to Cu exposure might be compensated by increased anaerobic capacities, as observed in yellow perch.

In contrast, we observed a general increase in HK and PK in the intestine and liver, accompanied by no changes in LDH in killifish exposed to Cu (Figure 3.4B,C,H). These changes are suggestive of increased carbohydrate metabolism. Although Cu may directly inhibit glycolytic enzymes altering energy metabolism, it has also been observed to

interfere with mitochondrial function. This is possibly as a result of Cu-induced oxidative stress, reducing the amount of energy from oxidative phosphorylation (Pourahmad and O'Brien, 2000; Krumschnabel et al. 2005). Craig et al. (2007) observed a substantial decrease in the COX-to-CS ratio in the liver of soft-water acclimated zebrafish exposed to acute sublethal Cu. The enzyme activities of CS, a key enzyme in tricarboxylic acid cycle (TCA) cycle, and COX, a key enzyme in aerobic metabolism, can be used as indicators of mitochondrial density and mitochondrial inner membrane, respectively (Capkova et al. 2002). In addition, changes in the ratio of COX-to-CS can indicate mitochondrial dysfunction (Capkova et al. 2002). Cu may inhibit proper COX assembly or function, as excess Cu can disturb the interaction of COX with oxygen, and the latter as a result of alterations in the lipid membrane of the mitochondria due to increased ROS levels (Radi and Matkovics, 1988; Capkova et al. 2002; Bury et al. 2003; Bagnyukova et al. 2006). Similarly, we observed a significant decrease in the COX-to-CS ratio in the liver of Cu-exposed killifish in FW (Figure 3.5I). In addition, gill COX activity decreased in FW- and SW-acclimated killifish exposed to Cu, suggesting an inhibitory effect on the electron transport chain (Figure 3.5A). However, the opposite trend was observed in the intestine, with COX activity increasing (Figure 3.5B). This suggests that the effect of Cu on mitochondrial function may be tissue-dependent. Cu induced minimal changes on CS activity, with an increase in intestinal CS observed only in SW-acclimated fish exposed to high Cu (Figure 3.5E). Therefore, excess Cu could lead to a reduction in oxidative metabolism and an increased switch to carbohydrate metabolism to maintain energy needs as suggested in the earthworm, *Lumbricus rubellus* (Bundy et al. 2008). Overall, the results of this study show that acute sublethal Cu concentrations alter energy metabolism in FW- and SW-acclimated killifish, with a varying degree of effects on enzymes involved

in carbohydrate metabolism and mitochondrial oxidative metabolism in a tissue-specific manner.

Oxygen Consumption and Opercular Frequency

One of the common physiological responses to metal toxicity is a change in oxygen consumption rate (Connell et al. 1999). In general, oxygen consumption rate has been observed to decrease in fish exposed to sublethal Cu concentrations (Beaumont et al. 2003). Exposure to Cu appears to disrupt a fish's ability to regulate their oxygen consumption, with possible causes including gill damage, epithelium swelling, increased secretion of mucus and disruption of sensory mechanisms (McDonald and Wood, 1993; De Boeck et al. 2004). De Boeck et al. (1995) found that oxygen consumption dropped significantly immediately after Cu exposure in the common carp, (*Cyprinus carpio*). In Cu-exposed *Tilapia mossambica,* Balavenkatasubbaiah (1984) observed a reduction in oxygen consumption rate, accompanied by a decrease in enzyme activities of succinate and LDH, which they suggested indicated a switch from aerobic to anaerobic metabolism. Couture and Kumar (2003) observed reduced oxygen consumption in yellow perch exposed to Cu, accompanied by decreased CS activity in the muscle, which could indicate reduced aerobic capacities. Suppression of aerobic metabolism is primarily thought to be controlled at a cellular and biochemical level, and this ultimately reduces ATP-consuming processes that contribute to standard metabolic rate, improving survival in response to environmental stress (Richards, 2010). As shown above, there is clear evidence that Cu induces changes at a cellular and biochemical level in FW- and SW-acclimated killifish. However, our results suggest FW- and SW-acclimated killifish have the ability to regulate their respiration rate when exposed to acute sublethal Cu at environmentally relevant concentrations (Table 3.2).

This difference could be explained by the ability of fish to recover after a 96-h exposure. Gill damage induced after 96-h exposure to sublethal Cu in *Prochilodus scrofa* was observed to gradually recover after 7-days in clean water, with complete recovery noted on day 45 (Cerqueira and Fernandes, 2002). Similarly, De Boeck et al. (1995) observed an apparent recovery in oxygen consumption after 1-week of continuous exposure to Cu, suggesting that repair of gill tissue may have occurred during this period. Therefore, exposure length and time of measurement appear to be important factors to consider when measure oxygen consumption rates to evaluate Cu toxicity.

Cu altered opercular frequency of killifish, with a significant decrease observed in SW that was even more extensive in FW (Table 3.2). Decreased opercular frequency may help reduce absorption of Cu through the gills, similar to the results observed by Bhat et al. (2012) in Indian major carp (*Labeo rohita*) exposed to pesticides. Conversely, opercular frequency has also been observed to increase when fish are exposed to Cu. In largemouth bass *(Micropteris salmoides)* exposure to Cu significantly increased opercular frequency, however, after 60 minutes opercular frequency returned to control values (van Aardt and Hough, 2007). Cu also altered the breathing pattern of FW-killifish from a continuous to a discontinuous pattern characterized by short breathing episodes alternating with longer periods of apnea. It is difficult to determine whether this change in breathing pattern is a compensatory mechanism, or whether oxygen sensors were impaired, however, it is interesting to note that despite this marked change it did not alter oxygen consumption rates.

Hematological Parameters

The absence of changes observed in oxygen consumption rate in Cu exposed killifish can be related to the hematological changes observed after exposure to Cu. A common observation in fish exposed to metals is a stress response. An increase in Hct and Hb after acute exposure to Cu was found by Svobodova (1982) in common carp, Mazon et al. (2002) in *P. scrofa* and Cyriac et al. (1989) in Mozambique tilapia (*Oreochromis mossambicus*). In the present study, Cu exposure increased Hct and Hb levels in FW- and SW-acclimated killifish, which indicates a Cu-induced stress response (Table 3.2). An increase in Hct and Hb may have compensated for the oxygen debt (i.e. metabolizing lactate; Hochachka et al. 1978) induced by Cu, therefore, enabling Cu-exposed fish to maintain oxygen consumption rates similar to their relative controls. Increases in Hct and Hb could occur to elevate the blood oxygen carrying capacity, a mechanism that enables an organism to transport more oxygen and increase carrying capacity to meet increased oxygen demands (Larsson et al. 1985; Cyriac et al. 1989). Cell swelling has also been observed to occur in Cu-exposed carp (*Cyprinus carpi*o) as indicated by an increase in mean cell volume (MCV) (Witeska et al. 2010). This was suggested to be an adaptive response increasing surface area over which oxygen diffusion can occur (Witeska et al. 2010). Carvalho and Fernandes (2006) found Cu induced a significant increase in mean cell hemoglobin (MCH) and MCHC in *Prochilodus scrofa,* also suggesting a stimulation of hemoglobin synthesis. In contrast, we observed no changes in MCHC in the present study. However, Cu may play a role in stimulation of hemoglobin, a compensatory hemopoietic response to Cu-induced damage, as the liver accumulates the highest levels of Cu in fish and is also one of the heme synthesis sites (Kraemer et al. 2005; Witeska et al. 2010). Exposure to 26.9 μg Cu/L for 3 days elevated Hb and Hct in rainbow trout, but levels

returned to control values by 14-days of exposure, which Dethloff et al. (1999) suggested was due to acclimation to Cu.

Another possible explanation for increased Hct and Hb in Cu-exposed fish may be as a result of increased oxygen demand caused by anaerobic metabolism due to converting lactate by-products and increased energy expenditure for detoxification (Saravanan and Natarajan, 1991; Witeska et al. 2010). However, we found no changes in LDH activity in FW- and SW-acclimated fish and observed blood lactate concentrations decreased in Cu-exposed FW-acclimated fish, suggesting a reduction in anaerobic metabolism (Table 3.2). Consequently, it is likely that Cu induced a stress response in killifish, which was compensated for by increased Hct and Hb levels. The blood lactate values observed in Cu-exposed killifish are similar to those measured in *F. heteroclitus* exposed to hypoxia (14.4 mmol/L; Virani and Rees, 2000). Due to the method in which we measured lactate, the concentrations obtained most likely represent stress-induced lactate levels, not resting values.

Conclusions

In this research, I have shown that oxidative stress and metabolic responses induced by Cu in killifish acclimated to SW differed only slightly from that in FW, with the biggest differences observed in Cu accumulation at the gills and opercular frequency. It is clear from this study that Cu induces changes at many levels of biological organization, from the cellular level to whole body changes. Our results suggest that measurements of oxidative damage and aerobic metabolism can be used to assess the toxicity of Cu on fish, however, the levels of damage and changes in metabolism may vary depending on exposure concentration and duration. To further elicit a better understanding of the mechanisms involved in Cu toxicity at SW, we suggest chronic exposure periods and

Cu concentrations greater than environmentally-relevant levels be investigated. While the killifish, is an ideal model due to its euryhaline capabilities, it is also quite tolerant of environmental perturbations, therefore, we suggest a more sensitive model may be better suited to elicit Cu toxicity mechanisms.

Table 3.1. Measured total and dissolved Cu concentrations (μg Cu/L) in freshwater (FW, 0 ppt) and seawater (SW, 35 ppt). Total and dissolved Cu concentrations in control water showed no significant difference and are presented as one. Values are presented as means ± SEM.

Salinity	CTRL Total Cu (μg/L)	LC (50 μg Cu/L) Total Cu (μg/L)	Dissolved Cu (μg/L)	HC (200 μg Cu/L) Total Cu (μg/L)	Dissolved Cu (μg/L)
FW	1.62 ± 0.24	53.06 ± 1.35	51.22 ± 1.16	204.60 ± 3.71	205.56 ± 7.57
SW	5.00 ± 0.14	46.24 ± 1.45	49.03 ± 1.41	237.61 ± 4.13	215.60 ± 8.85

Table 3.2. Oxygen consumption rate, opercular frequency and hematological measures (lactate, hematocrit (Hct), hemoglobin (Hb) and mean cell hemoglobin concentration (MCHC)) in killifish acclimated to freshwater (FW) or seawater (SW) and exposed to low Cu (LC) or high Cu (HC). Values are presented as means ± SEM. Different letters indicate significant differences among treatments within the same salinity. (*) denotes significant differences between FW and SW-acclimated fish within the same treatment (two way anova, $p<0.05$).

	FW			SW		
	CTRL	LC	HC	CTRL	LC	HC
Oxygen consumption						
Mean weight of fish (g)	2.41 ± 0.14	2.52 ± 0.12	2.90 ± 0.12	2.12 ± 0.08	2.19 ± 0.12	2.40 ± 0.29
O_2 consumption rate (μM O_2/g wet weight/h)	9.62 ± 0.68	9.19 ± 0.79	9.12 ± 0.44	9.32 ± 0.88	7.82 ± 0.82	9.55 ± 0.80
Opercular frequency (breaths/min)	92.54 ± 3.21^A	29.40 ± 2.38^B	27.49 ± 2.03^B	95.22 ± 3.03^A	84.23 ± 3.51^{B*}	81.75 ± 3.18^{B*}
Hematological measures						
Lactate (mmol/L)	12.20 ± 0.43^A	8.08 ± 0.56^B	7.47 ± 0.53^B	13.81 ± 1.10	13.87 ± 0.68*	14.34 ± 0.73*
Hct (%)	34.44 ± 1.47^A	44.72 ± 1.50^B	46.75 ± 1.13^B	32.27 ± 1.57^{AB}	31.37 ± 1.48^{A*}	37.77 ± 2.90^{B*}
Hb (g/dL)	7.12 ± 0.58^A	10.64 ± 0.44^B	10.84 ± 0.50^B	6.49 ± 0.44	7.21 ± 0.45*	7.77 ± 0.50*
MCHC (g/L)	195.2 ± 13.8	233.5 ± 10.5	231.7 ± 11.0	206.2 ± 15.4	241.6 ± 23.9	205.7 ± 10.9

Figure 3.1. Concentrations of sodium (Na$^+$; **A**), calcium (Ca^{2+}; **B**), potassium (K$^+$; **C**) and magnesium (Mg^{2+}; **D**) in gill, liver, intestine (INT) and carcass (CARC) of killifish exposed to control (CTRL), low (LC) or high copper (HC) in freshwater (FW) or seawater (SW). Values are presented as means ± SEM of n = 6 per treatment. Values that do not share the same letter indicate significant differences among treatments within the same salinity. (*) indicates significant differences between FW- and SW-acclimated fish within the same treatment (two-way ANOVA, $p \leq$ 0.05).
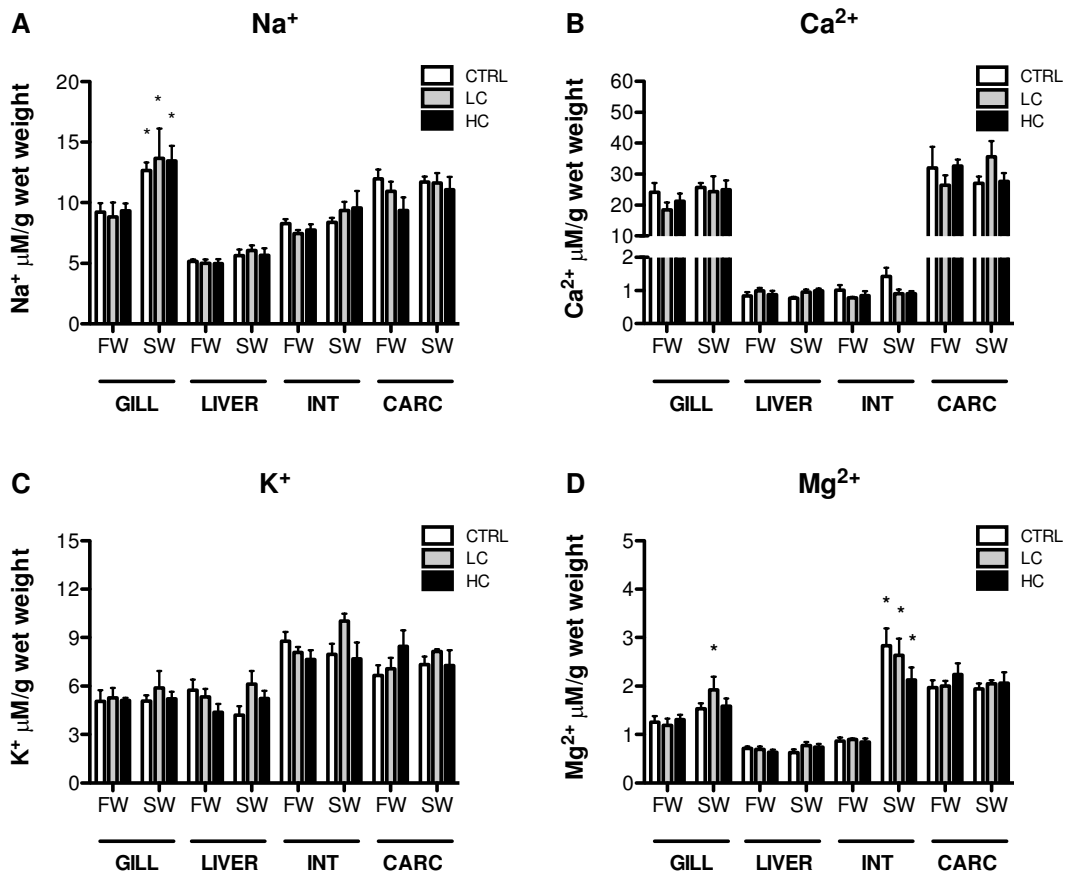
Figure 3.2. Gill (**A**), intestine (**B**), liver (**C**) and carcass (**D**) Cu load (μg/g tissue) in killifish acclimated to freshwater (FW) or seawater (SW) and exposed to low Cu (LC) or high Cu (HC). Values are presented as means ± SEM of n = 6 per treatment. Different letters indicate significant differences among treatments within the same salinity. (*) denotes significant differences between FW- and SW-acclimated fish within the same treatment (two-way ANOVA, $p < 0.05$).

A   GILL CU LOAD

B   INTESTINE CU LOAD

C   LIVER CU LOAD

D   CARCASS CU LOAD

Figure 3.3. Gill, intestine and liver protein carbonyl content (nmol/mg protein; **A**, **B**, **C**, respectively), catalase activity (U/mg protein; **D**, **E**, **F**, respectively) and superoxide dismutase (U/mg protein; **G**, **H**, **I**) in killifish acclimated to freshwater (FW) or seawater (SW) and exposed to low Cu (LC) or high Cu (HC). Values are presented as means ± SEM of n = 8 per treatment. Different letters indicate significant differences among treatments within the same salinity. (*) denotes significant differences between FW- and SW-acclimated fish within the same treatment (two-way ANOVA, p<0.05).

A GILL PROTEIN CARBONYL

B INTESTINE PROTEIN CARBONYL

C LIVER PROTEIN CARBONYL

D GILL CAT

E INTESTINE CAT

F LIVER CAT

G GILL SOD

H INTESTINE SOD

I LIVER SOD

83
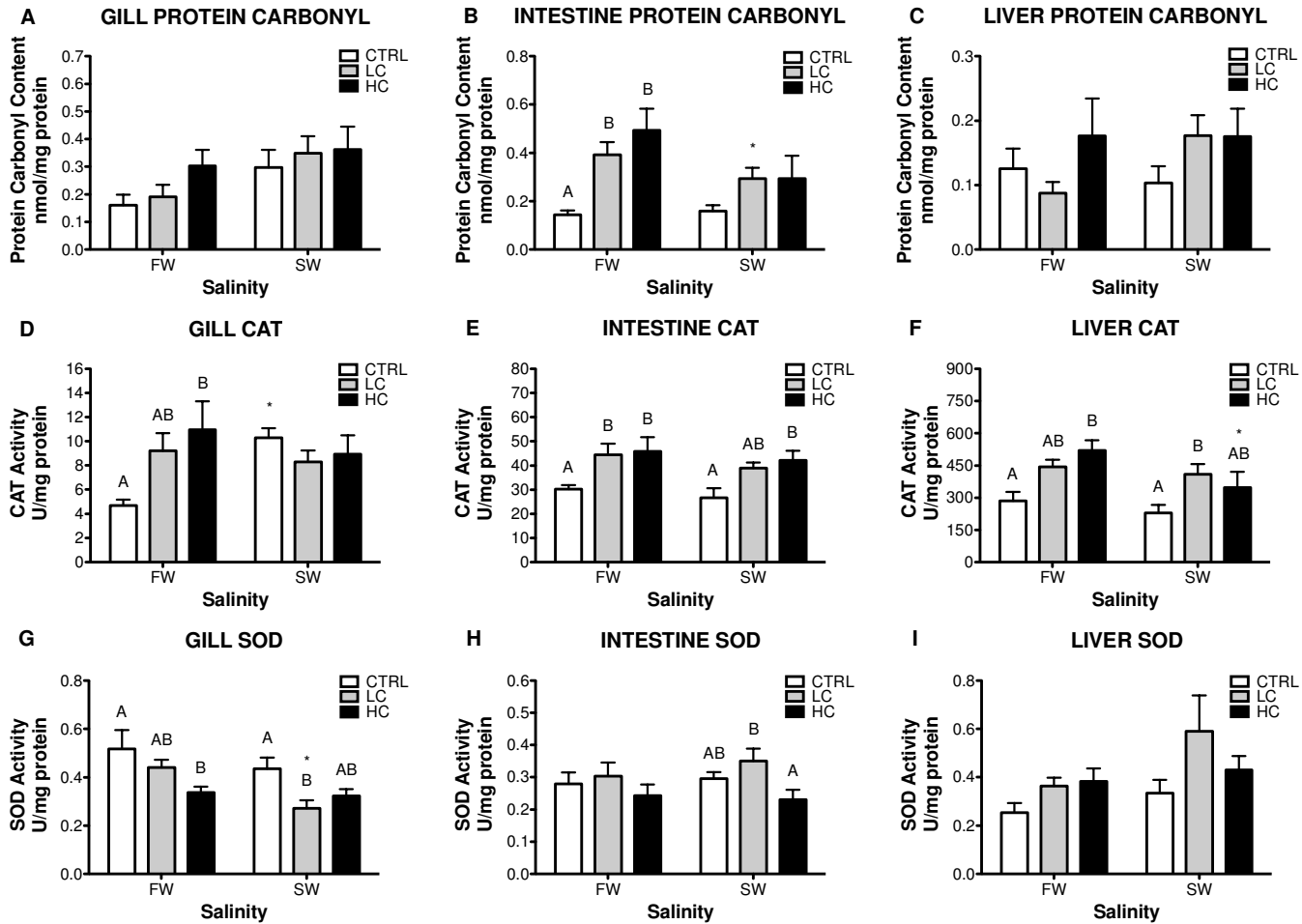
Figure 3.4. Gill, intestine and liver enzyme activities of carbohydrate metabolism (pyruvate kinase (PK), **A**, **B**, **C**, respectively; lactate dehydrogenase (LDH), **D**, **E**, **F**, respectively; and hexokinase (HK), **G**, **H**, respectively) in killifish acclimated to freshwater (FW) or seawater (SW) and exposed to low Cu (LC) or high Cu (HC). Values are presented as means ± SEM of n = 6 per treatment. Different letters indicate significant differences among treatments within the same salinity. (*) denotes significant differences between FW- and SW-acclimated fish within the same treatment (two-way ANOVA, $p<0.05$).
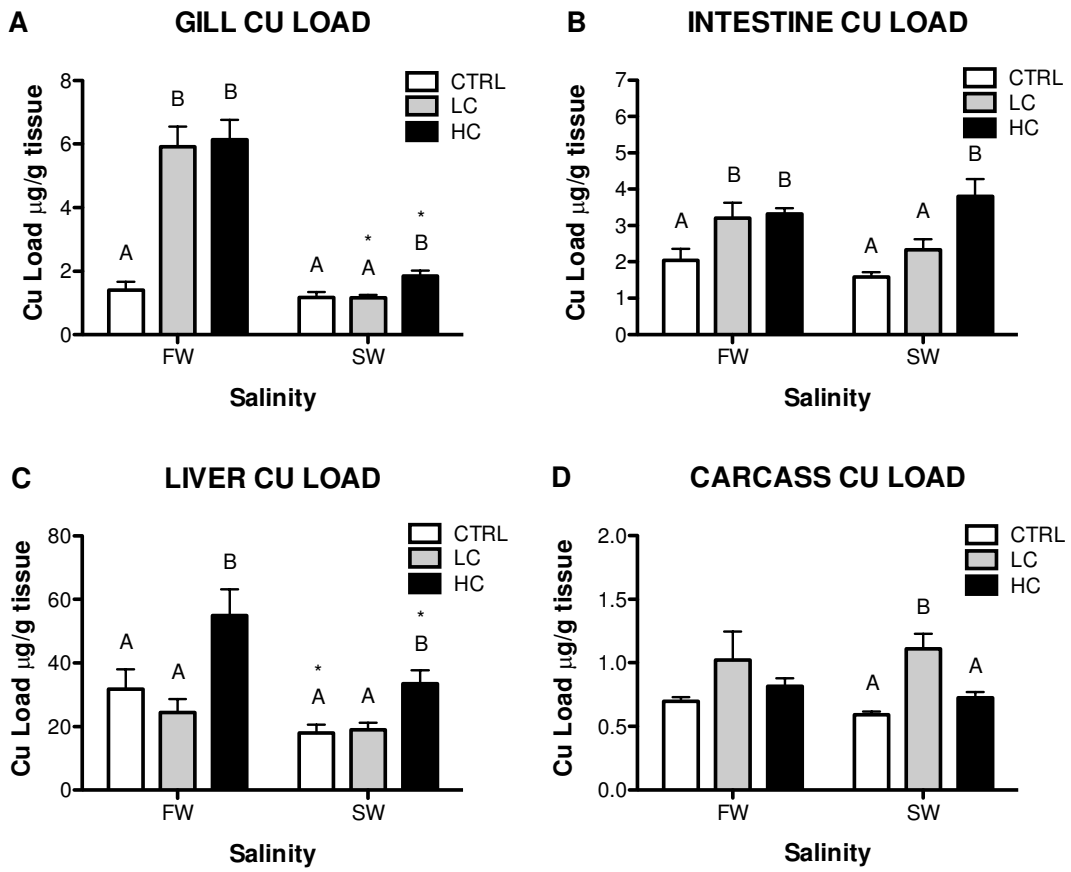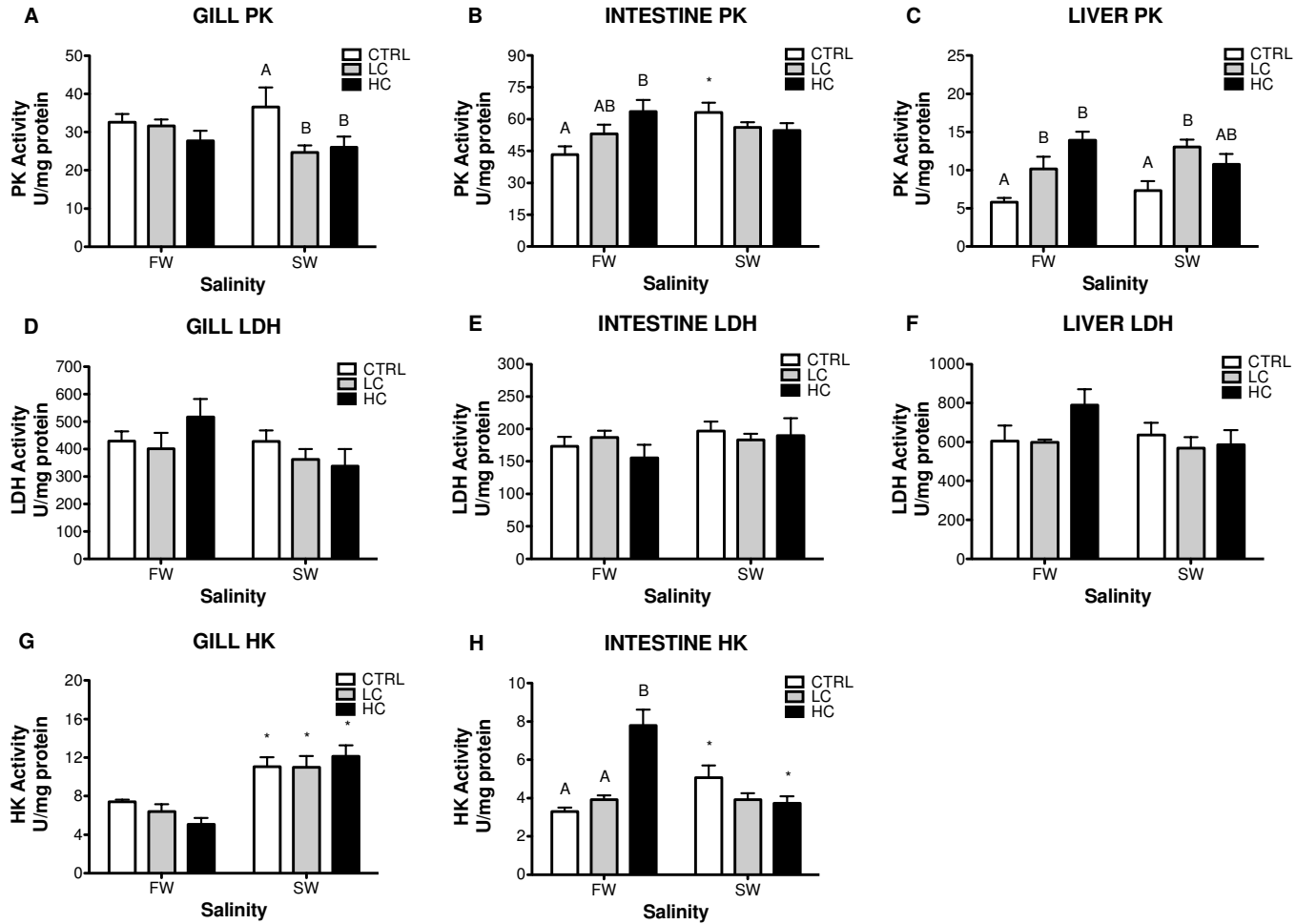
Figure 3.5. Gill, intestine and liver enzyme activities of aerobic metabolism (cytochrome *c* oxidase (COX), **A**, **B**, **C**, respectively; citrate synthase (CS), **D**, **E**, **F**, respectively; and COX/CS ratio, **G**, **H**, **I**, respectively) in killifish acclimated to freshwater (FW) or seawater (SW) and exposed to low Cu (LC) or high Cu (HC). Values are presented as means ± SEM of n = 6 per treatment. Different letters indicate significant differences among treatments within the same salinity. (*) denotes significant differences between FW- and SW-acclimated fish within the same treatment (two-way ANOVA, p<0.05).
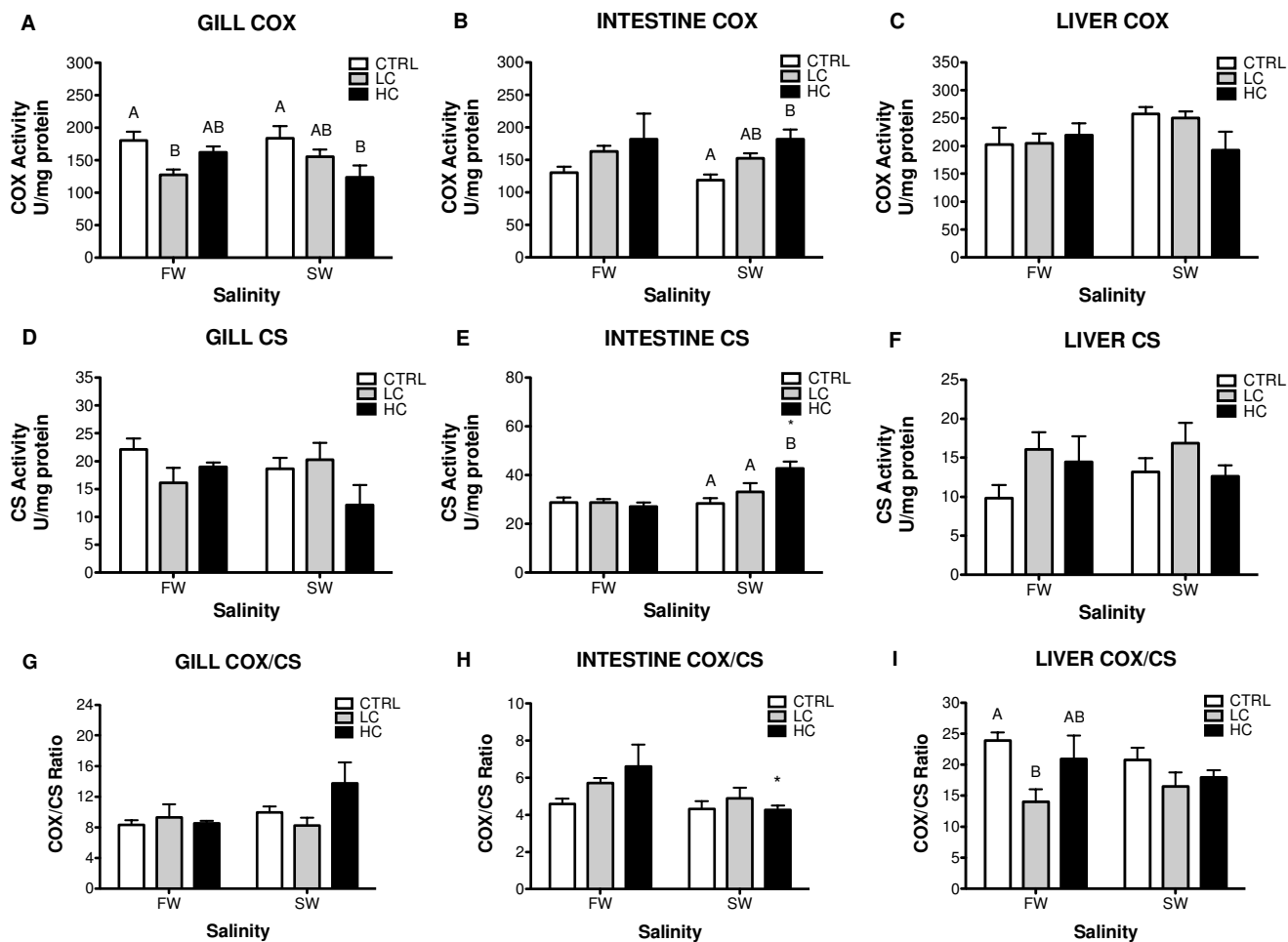
CHAPTER 4

GENERAL SUMMARY AND CONCLUSIONS

As outlined in the general introduction (Chapter 1), few studies have investigated the combined action of environmental stressors, such as acute waterborne Cu exposure and hypoxia (reviewed in Heugens et al. 2001). In addition, the mechanism of Cu toxicity in marine environments has not yet clearly been described (reviewed in Grosell, 2012). This thesis has employed physiological, hematological and metabolic endpoints to study the effects of acute waterborne Cu exposure in a euryhaline model species. This novel data will aid in the ultimate goal of creating accurate predictive models to better protect and regulate natural aquatic environments, including FW and saline conditions, without underestimating toxicological effects.

The combined effect of environmental stressors on aquatic organisms is a relevant area of research as these conditions are common aspects of natural environments. In this study, we demonstrated that the combination of sublethal environmentally relevant Cu concentrations and hypoxia induced antioxidant protection pathways and reduced oxidative capacity of target tissues in FW-acclimated killifish (Chapter 2). My findings suggest that the combined effects of waterborne Cu+Hyp do not work synergistically in terms of oxidative stress, which may suggest different mechanisms exist. Conversely, evidence to suggest hypoxia acts in an antagonistic manner reducing Cu-induced oxidative stress in killifish was observed, which would ultimately affect the toxicity of Cu.

The pollution of marine environments occurs mainly in coastal areas (Forbes, 1991), where organisms may simultaneously be exposed to metal contaminants and fluctuating dissolved oxygen concentrations and salinities, which is why most research focuses on estuarine organisms. In an attempt to understand Cu-hypoxia interactions, we utilized the

capabilities of killifish to acclimate to FW (reviewed in Burnett et al. 2007), therefore eliminating potential salinity interactions. However, no clear relationship or trend between Cu+Hyp was evident in terms of oxidative stress across tissues (gill and liver). While the exposure concentration of Cu used in this study was of environmental relevance, the concentration is well below the median lethal concentration (LC50) of adult killifish. At 14 ppt, Lin and Dunson (1993) found the LC50 of Cu to be 1.7 mg/L in adult killifish, however, no specific 96-h LC50 data exists in FW and SW (35 ppt). Data for juveniles (2-weeks of age) killifish does exist, with 96-h LC50 values for Cu found to be 18 and 294 μg/L in FW and SW, respectively (Grosell et al. 2007). As we observed no mortalities in adult killifish exposed to ~25 μg Cu/L, it is clear the 96-h LC50 is much higher for adults in both FW and SW. Therefore, exposure to higher concentrations of Cu may help to better elucidate mechanisms of Cu toxicity in killifish, in terms of oxidative stress responses.

Furthermore, we found that the oxidative stress and metabolic responses induced by Cu in SW-acclimated killifish, differed only slightly from FW (Chapter 3). My findings confirmed salinity affected Cu accumulation, reducing Cu accumulation in SW at the gills relative to FW, while on a whole-animal scale Cu reduced opercular frequency, with a more pronounced decrease observed in FW compared to SW, despite no change in oxygen consumption rates. In addition, Cu increased red blood cell parameters in FW and SW conditions. In general, metal toxicity is known to decrease with increased salinity, with salinity protecting against Cu uptake in fish (Blanchard and Grosell, 2005, 2006; Grosell, 2012). However, it is unclear whether salinity protects against Cu-induced oxidative stress and metabolic responses. There are several possible reasons for this. Firstly, the concentrations of Cu to which killifish were exposed did not induce high levels of protein carbonyls or changes in

oxygen consumption rates in FW or SW, making it difficult to determine any patterns. Secondly, measurements of oxidative damage and antioxidant enzyme responses may not be sensitive enough to indicate changes in oxidative stress in a tolerant fish species. Lastly, it is possible that oxidative stress and metabolic responses were missed, and may have occurred prior to 96-h. Therefore, we suggest that exposure to increased concentrations of Cu, surpassing environmentally relevant levels be used and that time course experiments on both acute and chronic scales be performed to better assess the oxidative stress and metabolic responses of killifish. We also want to emphasize the importance of combining various physiological endpoints, such as oxidative stress, metabolic responses and hematological parameters, as it has already been well established that physiology rather than chemistry seems to be governing the effects of Cu in saline environments (Grosell et al. 2007). Studies that utilize a variety of physiological endpoints will provide a more detailed and accurate model of an aquatic organism's physiology.

Overall, the results of this thesis clearly demonstrate the importance of investigating combined environmental stressors and examining physiological endpoints at varying salinities (Chapter 2 and 3). It is also hoped that the necessity to further examine oxidative stress responses in combination with other physiological endpoints, including metabolic responses is evident (Chapter 3). The results of this thesis will further increase our breadth of knowledge and understanding of how changing environmental conditions, toxicity and physiology of an organism interact with one another to build more accurate predictive and regulatory models reflecting that of freshwater and saline environments.

CHAPTER 5

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