

## Oxidative stress and metabolic responses to copper in freshwater- and seawater-acclimated killifish, *Fundulus heteroclitus*



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### ARTICLE INFO

#### Article history:

Received 22 August 2014

Received in revised form 18 February 2015

Accepted 20 February 2015

Available online 21 February 2015

#### Keywords:

Copper

Protein carbonyls

Oxygen consumption rate

Freshwater

Salinity

Catalase

Killifish

Metabolism

### ABSTRACT

In freshwater (FW), many of the main mechanisms of copper (Cu) toxicity have been characterized; however, toxicity mechanisms in seawater (SW) are less well understood. We investigated the effects of salinity on Cu-induced oxidative stress and metabolic responses in adult killifish, *Fundulus heteroclitus*. We exposed FW and SW-acclimated killifish to either low Cu (LC, 50 g/L) or high Cu (HC, 200 g/L) for 96 h and compared them to controls (CTRL) under the same salinities without added Cu. Cu exerted minimal influence on tissue ion levels in either FW or SW. Salinity generally protected against Cu bioaccumulation in the gills and liver, but not in the carcass. Hematocrit (Hct) and hemoglobin (Hb) levels were increased by LC and HC in both FW and SW, and blood lactate was reduced in FW-killifish exposed to LC and HC. Rates of oxygen consumption were similar across treatments. Salinity reduced Cu load in gill, liver and intestine at LC but only in the gills at HC. In general, Cu increased gill, liver, and intestine catalase (CAT) activity, while superoxide dismutase (SOD) either decreased or remained unchanged depending on tissue-type. These changes did not directly correlate with levels of protein carbonyls, used as an index of oxidative stress. Cu-induced changes in carbohydrate metabolic enzymes were low across tissues and the effect of salinity was variable. Thus, while salinity clearly protects against Cu bioaccumulation in some tissues, it is unclear whether salinity protects against Cu-induced oxidative stress and metabolic responses.

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### 1. Introduction

Copper (Cu) is an essential micronutrient required at trace amounts, but at elevated levels may cause toxicity to many organisms, including fishes. While anthropogenic inputs affect the concentrations of Cu, other factors may strongly modify Cu toxicity. Water chemistry has an important influence on toxicity and in marine environments, pH, salinity, and natural dissolved organic matter (DOM) will affect metal speciation and bioavailability (reviewed in Campbell, 1995). However, salinity is regarded

as a major regulator of metal ion toxicity, by increasing protective cations and altering physiology (Wright, 1995; Wood, 2012). Indeed gill uptake and accumulation in fishes has been shown to decrease with increasing salinity (Grosell and Wood, 2002; Blanchard and Grosell, 2006). However, Cu toxicity to fish appears to follow a biphasic relationship with greatest toxicity in FW, lowest toxicity at isosmotic salinities, and intermediate toxicity in full strength SW (Grosell et al., 2007). Therefore, it is very important to take water chemistry into account when predicting metal ion toxicity.

The mechanisms by which Cu exerts its toxic effects on fish appear to be different in freshwater (FW) and seawater (SW) (e.g., see Blanchard and Grosell, 2006; Grosell et al., 2007; Grosell, 2012). In FW, waterborne Cu toxicity at environmentally relevant Cu exposure levels seems to primarily involve ionoregulatory disturbances that affect osmoregulatory homeostasis (Lauren and McDonald, 1987; Grosell and Wood, 2002; Grosell, 2012). Cu is readily taken up by, and accumulates at the gills, but also enters the blood to accumulate in other organs, such as the intestine and liver

**Abbreviations:** Cu, copper; DOM, dissolved organic matter; FW, freshwater; SW, seawater; ROS, reactive oxygen species; CAT, catalase; COX, cytochrome c oxidase; CS, citrate synthase; HK, hexokinase; LDH, lactate dehydrogenase; SOD, superoxide dismutase; PO<sub>2</sub>, partial pressure of oxygen; Hct, hematocrit; Hb, hemoglobin.

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(Grosell, 2012). Accumulation of Cu can affect Na<sup>+</sup> uptake at the gills, and likely cause ionoregulatory disturbances in downstream organs as a result. Other studies using Cu-exposed zebrafish (*Danio rerio*) and killifish (*Fundulus heteroclitus*) show that acute exposure to sublethal Cu concentrations can also affect gill permeability and induce oxidative stress (e.g., Grosell et al., 2002; Craig et al., 2007).

In SW, waterborne Cu affects gills but also the intestine, since SW fishes must drink to compensate for water lost to the environment. Drinking SW drives active transport of Na<sup>+</sup> and Cl<sup>-</sup> across the gastro-intestinal tract and water follows (Loretz, 1995; Larsen et al., 2002; Marshall and Grosell, 2005; Blanchard and Grosell, 2006). 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). Cu exposure in SW also increases tissue reactive oxygen species (ROS) levels, resulting in oxidative stress (Main et al., 2010), DNA damage (Costa et al., 2002), and impaired mitochondrial ATP formation (Viant et al., 2002). In addition to inducing reactive oxygen species (ROS), likely via its participation in the Fenton and Haber-Weiss reactions, excess Cu may interfere with ROS defenses (e.g., activities of catalase (CAT), superoxide dismutase (SOD)) and/or the ability to repair oxidative damage (Dorval and Hontela, 2003; Craig et al., 2007; Almroth et al., 2008; Bopp et al., 2008; Eyckmans et al., 2011). Oxidative damage with Cu exposure has been observed as changes in biomarkers (e.g., protein carbonyls, lipid peroxidation, and DNA damage products) in the gill, liver and intestine of numerous fish species. For example in soft-water acclimated zebrafish, exposure to Cu increased protein carbonyl content in the gill and liver, accompanied by increases in activity of the ROS defense enzyme catalase (Craig et al., 2007). Cu exposure elevated lipid peroxidation levels in the liver and intestine of Indian flying barb (*Esomus danricus*) but led to a decline in antioxidant enzyme activity (Vutukuru et al., 2006).

Cu exposure can also affect whole-organism performance by decreasing metabolic rate (Beaumont et al., 2003). The putative cause of reduced oxidative metabolism appears to be gill damage, manifested as either a disruption of branchial structure, secretion of mucus that binds metals and impedes rates of diffusion, inhibition of respiratory enzymes, and/or damage to the gill's oxygen-sensing receptors (Hughes and Shelton, 1958; Hughes, 1966; Wendelaar-Bonga and Lock, 1992; McDonald and Wood, 1993). In carp (*Cyprinus carpio*), increasing concentrations of Cu were found to disrupt the ability to regulate their oxygen consumption (De Boeck et al., 1995). The effects of Cu have also been observed to change expression of genes encoding enzymes and other proteins involved in cellular respiration, including glycolysis, Krebs' cycle and the 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 acclimation on the Cu-induced oxidative stress and metabolic responses in the adult killifish, *F. heteroclitus*. In recent studies salinity was found to be protective against waterborne Zn toxicity and markedly protected *F. heteroclitus* against oxidative stress and ionoregulatory disturbances (Loro et al., 2012, 2014). We hypothesized that increased salinity would similarly provide protection against Cu-induced oxidative stress and would also minimize disruption of aerobic metabolism seen in freshwater. Thus, we determined rates of oxygen consumption, 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, as well as ionoregulatory status. Together, these results will help to further understand the mechanisms responsible for Cu toxicity across salinities in a model euryhaline teleost.

## 2. Materials and methods

### 2.1. Experimental animals

All procedures were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

Adult killifish of mixed sexes (body mass: 1.7–8.6 g) were collected from the wild SW environment (Aquatic Research Organisms, Hampton, NH, USA) and acclimated to 35 ppt in an aerated 300-L aquarium with carbon filtration for a minimum of two weeks. Approximately, 200 killifish were then acclimated to either FW (0 ppt) or SW (35 ppt). Killifish for the FW group were first transferred from 35 ppt to 0 ppt by gradually diluting the sea water over a two week period with dechlorinated Hamilton, ON tap water (moderately hard: [Na<sup>+</sup>]=0.6 mequiv L<sup>-1</sup>, [Cl<sup>-</sup>]=0.8 mequiv L<sup>-1</sup>, [Ca<sup>2+</sup>]=1.8 mequiv L<sup>-1</sup>, [Mg<sup>2+</sup>]=0.3 mequiv L<sup>-1</sup>, [K<sup>+</sup>]=0.05 mequiv L<sup>-1</sup>; titration alkalinity 2.1 mequiv L<sup>-1</sup>; pH ~8.3; hardness ~140 mg L<sup>-1</sup> as CaCO<sub>3</sub> equivalents). FW-acclimated killifish then were maintained in carbon-filtered aerated 45-liter tanks with 20% water-renewal daily for a minimum of 2-weeks prior to experimentation. SW was made by the addition of Instant Ocean sea salt to de-chlorinated tap water (Big Al's Aquarium Supercenter, Woodbridge, ON, CA). SW-acclimated killifish were maintained in aerated, 300-L tanks with carbon filtration for a minimum of 2-weeks prior to experimentation with fish densities equivalent to FW-acclimated fish. 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 h prior to the start of experiments and throughout the 96-h exposure. No mortalities were observed in any of the experimental treatment or control tanks.

### 2.2. Copper exposure

Killifish ( $n = 180$  total) were removed from their respective FW or SW acclimation tanks (90 each) and placed into 8-L experimental tanks at 2 salinities (0 ppt or 35 ppt), each undergoing 3 treatments (targets of 0, 50 or 200 µg/L Cu) with a total of 3 replicates per treatment,  $n = 10$  fish per tank). The experimental conditions in FW and SW used a stock Cu solution made from CuSO<sub>4</sub> dissolved in 1% HNO<sub>3</sub>, which had no significant effect on water pH. Tank water underwent 80% renewal daily with thoroughly mixed renewal water prepared 24-h in advance for FW (Na<sup>+</sup>, 1.09 ± 0.01 mM, Ca<sup>2+</sup>, 0.69 ± 0.01 mM, Mg<sup>2+</sup>, 0.35 ± 0.00 mM, K<sup>+</sup>, 0.05 ± 0.00 mM; pH ~8.3) and SW (Na<sup>+</sup>, 469.6 ± 11.7 mM, Ca<sup>2+</sup>, 9.3 ± 0.1 mM, Mg<sup>2+</sup>, 54.0 ± 0.9 mM, K<sup>+</sup>, 8.2 ± 0.1 mM; pH ~8.3) tanks.

### 2.3. Metabolic rate

Oxygen consumption rates (MO<sub>2</sub>) were determined for individual fish using closed chamber respirometry as previously described for killifish (Blewett et al., 2013). Fish were held in individual custom-made respirometers filled with the same 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 to acclimate prior to respirometry to minimize the effects of transfer stress on metabolic rate. Temperature was controlled by a recirculating system and was maintained at 18 °C. The respirometers were then closed, and sealed with plastic paraffin film (Parafilm™). Water samples (5 mL) were taken at 0 min and 120 min for the measurement of partial pressure of oxygen (PO<sub>2</sub>) using a Clarke-Type O<sub>2</sub> electrode (Cameron Instruments, Port Aransas, TX, USA) set to the experimental temperature and

connected to an AM Systems Polarographic Amplifier (Model 1900, Carlsberg, WA, USA) digital dissolved oxygen meter.

#### 2.4. Tissue and water sampling

Water samples were taken prior to each water replacement, and analyzed for total and dissolved ion concentrations. Samples for dissolved ions were filtered through a 0.45 m filtration disc (Pall Life Sciences, East Hills, NY, USA) before determination of water chemistry parameters. After 96-h exposures, killifish were quickly euthanized by cephalic concussion and gills, liver, intestine and remaining carcass were weighed and quickly frozen in liquid N<sub>2</sub>. Blood samples were collected from the dorsal aorta using heparinized capillary tubes and microhematocrit tubes by severing the tail behind the anal fin for measuring hematocrit and hemoglobin, respectively. Blood lactate levels were measured immediately after blood sampling using a hand-held lactate meter (Lactate Pro Arkray, Kyoto, Japan). The remaining 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 (Hct) value. 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).

#### 2.5. 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 2 N nitric acid equivalent to five times their volume (Trace metal grade, Fisher Scientific, Ottawa, ON, CA) at 60 °C for 48 h, and vortexed after 24 h. An additional step was performed on SW samples using a method (Nadella et al., 2009) modified from Toyota et al. (1982) to eliminate Na<sup>+</sup> interference with GFAAS measurements. Briefly, Cu was precipitated from 1 mL of sample by adding 1 μL of lanthanum oxide (10 mg La mL<sup>-1</sup>) and 75 μL of 1 M Na<sub>2</sub>CO<sub>3</sub>, which brought the pH of the sample to approximately 9.8. The solution was gently stirred in a hot water bath maintained at 80 °C for 30 min to allow flocculation of precipitate. The solution was centrifuged at 5000 rpm for 15 min and the supernatant discarded. The remaining precipitate was dissolved in 1 mL of 1N HNO<sub>3</sub>. 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 digests were diluted as necessary with 1% HNO<sub>3</sub> (Na<sup>+</sup>), 1% LaCl<sub>3</sub> in 1% HNO<sub>3</sub> (Ca<sup>2+</sup>, Mg<sup>2+</sup>) or 0.1% CsCl<sub>2</sub> in 1% HNO<sub>3</sub> (K<sup>+</sup>). Water dissolved organic matter was not measured in these experiments but routinely it has been found to be ~3 mg/L in Hamilton tap water.

#### 2.6. Protein carbonyls

Protein carbonyl content was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI) as previously described (Craig et al., 2007). Protein concentration of the tissue homogenate was assayed at 595 nm according to Bradford (1976), using bovine serum albumin (BSA) as a standard. Protein carbonyls are expressed in nanomoles per milligram total protein.

#### 2.7. Enzyme activity

Frozen gill and liver tissues were powdered using a liquid N<sub>2</sub>-cooled mortar and pestle, then diluted 1:10 (wt:vol) in ice-cold

homogenization buffer (20 mM 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 Spectra-Max Plus 384 spectrophotometer (Molecular Devices, Menlo Park, CA, USA). Assays were performed in triplicate, plus measurements lacking substrate to correct for background activity as described previously (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 activities are reported as units (U) per milligram protein, where 1 U = μmol/min.

The apparent V<sub>max</sub> of catalase (CAT), superoxide dismutase (SOD), cytochrome c oxidase (COX) and hexokinase (HK) were measured on fresh tissue homogenates. Enzyme activities of pyruvate kinase (PK), lactate dehydrogenase (LDH) and citrate synthase (CS) were measured after homogenates were frozen and thawed once, twice and three times, respectively. The activities of CAT, COX, CS, HK, LDH, PK and HK were assayed as previously described (McClelland et al., 2006; Schippers et al., 2006; Craig et al., 2007). Hexokinase activity was not measured in the liver, due to low activity and high non-specific background levels (see above). Assay conditions were as follows (in mM unless otherwise indicated), COX: 50 Tris (pH 8.0), 50 reduced cytochrome c; HK: 50HEPES (pH 7.6), 5 D-glucose (omitted in control), 8 ATP, 8 MgCl<sub>2</sub>, 0.5 NADP, 4 U glucose-6-phosphate dehydrogenase; PK: 5 phosphoenol pyruvate (PEP; omitted in control), 50 imidazole (pH 7.4), 5 ADP, 100 KCl, 10 MgCl<sub>2</sub>, 0.15 NADH, 10 fructose 1,6-phosphate and 5 U lactate dehydrogenase (LDH); CS: 0.5 oxaloacetate (omitted in control), 0.3 acetyl-CoA, and dithiobisnitrobenzoic acid (DTNB) in 20 Tris (pH 8.0); LDH: 40 Tris (pH 7.4), 0.28 NADH, 2.4 Pyruvate-Na; CAT: 20 potassium phosphate buffer (pH 7.0), 20H<sub>2</sub>O<sub>2</sub>. Superoxide dismutase (SOD) activity (Cu, Zn-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. One unit of SOD is defined as the amount required to inhibit the reduction of WST-1 to WST-1 formazan by 50%.

#### 2.8. 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).

### 3. Results

#### 3.1. Water and tissue ions

Total and dissolved Cu concentrations approximated the nominal values of 50 and 200 μg Cu/L, respectively, in FW and SW (Table 1). Exposure to LC or HC did not significantly change ion concentrations for most tissues in either FW- or SW-acclimated killifish (Fig. 1). However, Na<sup>+</sup> and K<sup>+</sup> decreased with HC treatment in liver of SW acclimated fish and Mg<sup>2+</sup> was reduced in liver of FW acclimated fish in LC. Increasing the water salinity significantly increased ion concentrations in a tissue-specific manner, with significant increases in Na<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> observed in the gill. SW acclimation significantly increased gill, liver and carcass Na<sup>+</sup> levels for control, LC and HC exposures, relative to their FW counterparts.

**Table 1**Measured total and dissolved Cu concentrations ( $\mu\text{g Cu/L}$ ) in freshwater (FW, 0 ppt) and seawater (SW, 35 ppt).

Salinity	CTRL	LC (target 50 $\mu\text{g Cu/L}$ )		HC (target 200 $\mu\text{g Cu/L}$ )	
	Total Cu ( $\mu\text{g/L}$ )	Total Cu ( $\mu\text{g/L}$ )	Dissolved Cu ( $\mu\text{g/L}$ )	Total Cu ( $\mu\text{g/L}$ )	Dissolved Cu ( $\mu\text{g/L}$ )
FW	1.6 $\pm$ 0.2(15)	53.1 $\pm$ 1.4(20)	51.2 $\pm$ 1.2(18)	205 $\pm$ 4(20)	206 $\pm$ 8(17)
SW	5.0 $\pm$ 0.1(10)	46.2 $\pm$ 1.5(24)	49.0 $\pm$ 1.4(18)	238 $\pm$ 4(24)	216 $\pm$ 9(17)

Total and dissolved Cu concentrations in control water showed no significant differences and so data were combined. Values are presented as means  $\pm$  SEM (sample sizes appear in parentheses).

$\text{Mg}^{2+}$  levels in all tissues followed the same trend, increasing in SW-acclimated killifish for all treatments compared to their FW counterparts. In contrast, salinity only increased  $\text{Ca}^{2+}$  in gills and  $\text{K}^+$  in whole carcass (Fig. 1).

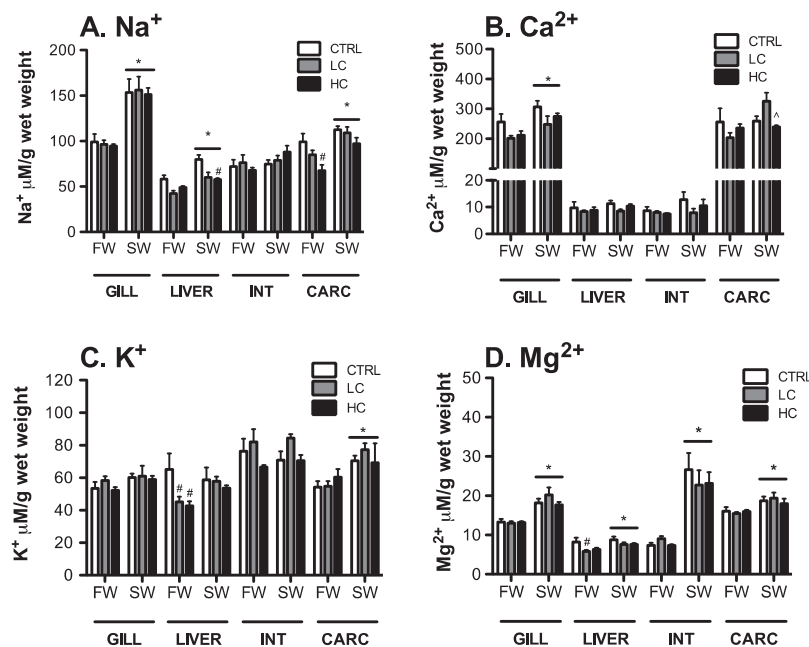
### 3.2. Copper accumulation

Cu exposure generally resulted in increased Cu accumulation across all tissues (Fig. 2). Surprisingly, no dose-dependent accumulation of Cu was observed in any tissue, except perhaps the liver which showed no accumulation at LC but did at HC. Salinity affected Cu load, which decreased with increasing salinity in both the gill and liver. However, there was a significant interaction effect between salinity and Cu treatment only for gill Cu load which was elevated by as much as 4.4-fold in FW-acclimated fish and only 1.6-fold in SW-acclimated killifish exposed to HC (Fig. 2A). In the intestine, both LC and HC exposures significantly increased Cu concentrations and to similar levels (2.8 and 3.3  $\mu\text{g Cu/g}$  tissue, respectively) compared to controls (2.0  $\mu\text{g Cu/g}$  tissue) in FW-acclimated fish. In comparison, intestinal Cu concentrations in SW-acclimated fish were significantly elevated only at the high Cu treatment (Fig. 2B). Cu burdens were much higher in the liver relative to other tissues under all conditions. Exposure to HC significantly increased liver Cu load by 1.7-fold and 1.9-fold in FW-acclimated and SW-acclimated fish, respectively (Fig. 2C). The highest tissue concentration of Cu was observed in the liver of FW-

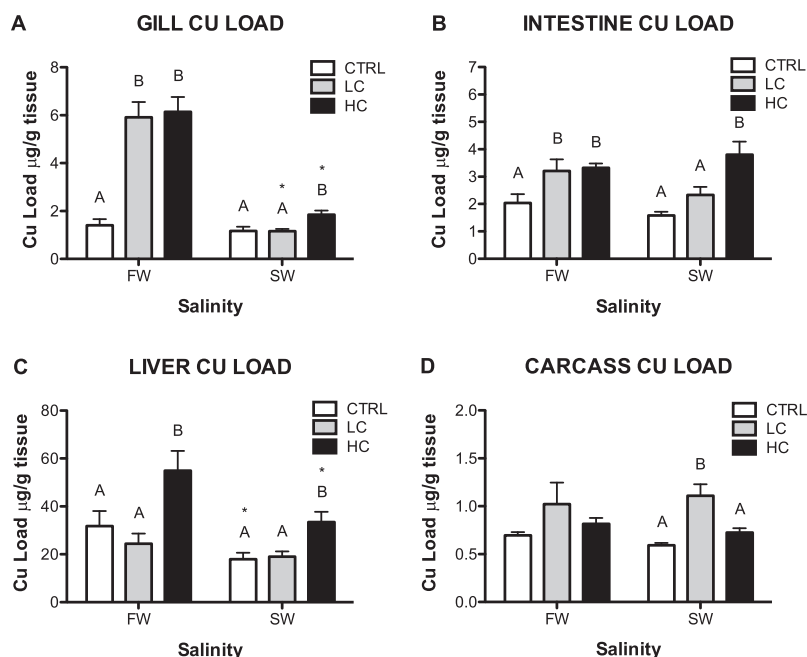
acclimated fish (81.4  $\mu\text{g Cu/g}$  tissue) exposed to HC. Acclimation to SW significantly reduced liver Cu load by 43% and 40% relative to FW, but only in fish exposed to control and HC treatments, respectively (Fig. 2C). Cu burdens were lowest in the carcass relative to other tissues under all conditions. Overall, salinity had no effect on carcass Cu accumulation, with the exception of exposure to LC in SW-acclimated fish, with significantly higher accumulation relative to controls (Fig. 2D).

### 3.3. Oxidative damage and antioxidant responses

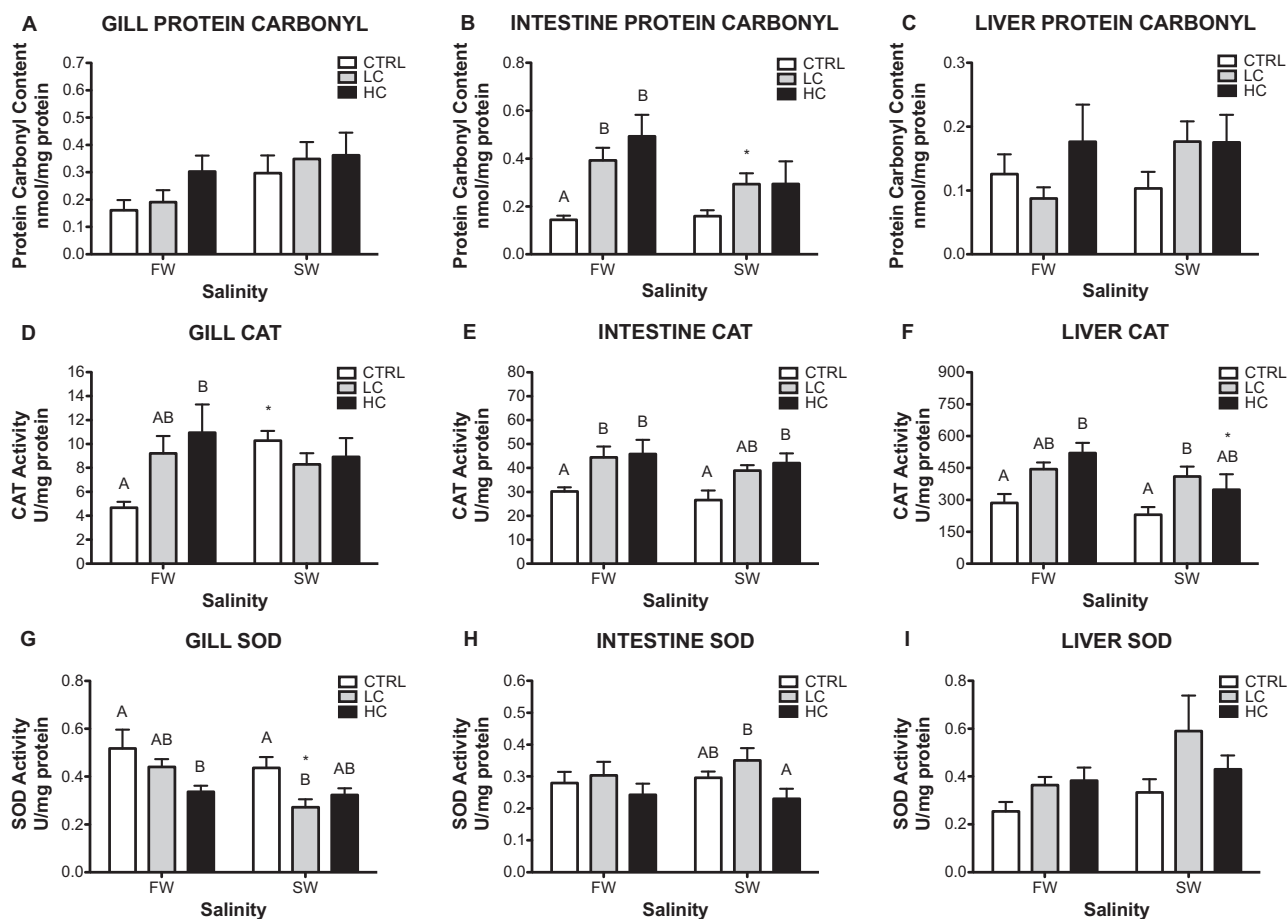
Overall, exposure to Cu tended to increase gill, liver and intestine protein carbonyl levels in both FW- and SW-acclimated fish (Fig. 3A–C). However, these changes were statistically significant only in the intestine of FW-acclimated fish exposed to LC and HC compared to controls (Fig. 3B). SW acclimation caused a 25% reduction in intestine protein carbonyl levels in LC relative to FW-acclimated fish exposed to the same treatment (Fig. 3B). Cu exposure significantly increased CAT activity in FW- and SW-acclimated fish across all tissues relative to their respective controls with the exception of gills in SW fish, while SOD activity significantly decreased in the gill or remained similar to control activities in the liver and intestine (Fig. 3). There was a significant interaction effect between salinity and treatment for gill CAT activity. Salinity on its own significantly increased gill CAT activity in controls. In the intestine, control CAT activity was 4–6 times greater than



**Fig. 1.** Tissue concentrations of sodium ( $\text{Na}^+$ ; A), calcium ( $\text{Ca}^{2+}$ ; B), potassium ( $\text{K}^+$ ; C) and magnesium ( $\text{Mg}^{2+}$ ; D) in gill, liver, intestine (INT) and carcass (CARC) of killifish exposed to control (CTRL), low (50  $\mu\text{g/L}$ , LC) or high copper (200  $\mu\text{g/L}$ , HC) for 96 h after freshwater (FW) or seawater (SW) acclimation. Values are presented as means  $\pm$  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, (#) indicates significant difference from controls within a salinity treatment and (̂) indicates significant difference from LC (two-way ANOVA,  $p \leq 0.05$ ).



**Fig. 2.** Gill (A), intestine (B), liver (C) and carcass (D) Cu load ( $\mu\text{g/g}$  tissue) in killifish acclimated to freshwater (FW) or seawater (SW) and exposed to low Cu ( $50 \mu\text{g/L}$ , LC) or high Cu ( $200 \mu\text{g/L}$ , HC) for 96 h. Values are presented as means  $\pm$  SEM of  $n=6$  per treatment. Values that do not share the same letter 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,  $p < 0.05$ ).



**Fig. 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 activity (U/mg protein; G, H, I) in killifish acclimated to freshwater (FW) or seawater (SW) and exposed to low Cu ( $50 \mu\text{g/L}$ , LC) or high Cu ( $200 \mu\text{g/L}$ , HC) for 96 h. Values are presented as means  $\pm$  SEM of  $n=8$  per treatment. Values that do not share the same letter 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,  $p < 0.05$ ).

that of the gill. Exposure to HC significantly increased intestinal CAT activity by ~1.5-fold in FW- and SW-acclimated fish, whereas exposure to LC only increased CAT activity in the FW-acclimated fish (Fig. 3E). The highest CAT activity was observed in liver and was 4–10 and 40–100 times greater than that of the intestine and gills, respectively (Fig. 3F). Liver CAT activity was increased by HC exposure in FW-acclimated fish and LC exposure in SW-acclimated fish. Salinity significantly decreased liver CAT activity but only in HC exposed fish. In contrast to CAT activity, SOD activity was significantly decreased in the gills of FW-acclimated fish exposed to HC and SW-acclimated fish exposed to LC, by 35% and 37%, respectively (Fig. 3G). No changes were observed in intestinal SOD activity with Cu exposure in FW, but there was a significant decline in SOD activity between LC and HC in SW-acclimated fish (Fig. 3H). No significant effects of treatment or salinity on SOD activity were observed in the liver (Fig. 3I).

#### 3.4. Hematological parameters

Significant salinity effects were seen in FW fish exposed to Cu and a significant interaction between salinity and treatment were observed in all hematological measures, with the exception of MCHC (Table 2). The concentration of blood lactate decreased significantly in FW fish exposed to LC and HC, by 34% and 39%, respectively. FW-acclimated fish exposed to LC and HC also showed significant increases in Hct and Hb. Exposure to LC in FW increased Hct by 30% and Hb by 49%, while HC showed slightly larger increases of 36% and 52%, respectively. In contrast, no effect of either LC or HC exposure was observed in SW-acclimated fish, with the exception of Hct levels significantly differing between LC and HC exposed fish. However, neither level differed from controls. No significant differences in MCHC across treatments or between salinities were observed.

#### 3.5. Carbohydrate metabolism

Impacts of Cu exposure and salinity on enzymes involved in carbohydrate metabolism varied in a tissue-specific manner. Significant interactions between treatment and salinity were observed in intestinal and liver PK activity (Fig. 4B and C). In the intestine, exposure to HC significantly increased PK activity by 1.5-fold, relative to controls in FW-acclimated fish (Fig. 4B). Acclimation to SW alone also increased intestinal PK activity. LC and HC 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 LC and HC, respectively, however the increase in response to HC was not significantly different from controls. In the gill, PK activity significantly decreased by 33% and 29%, in SW-acclimated fish exposed

to LC and HC, respectively (Fig. 4A). No significant changes in gill, intestine or liver LDH activity were observed (Fig. 4D–F). Salinity significantly increased gill HK activity in all treatments, compared to controls (Fig. 4G). In the intestine, HK activity increased in controls and decreased in HC exposed fish in response to increased salinity (Fig. 4H). In the intestine, exposure to high Cu significantly increased HK activity in FW-acclimated fish relative to both controls and LC treatments (Fig. 4H). HK could not be measured in the liver (see Section 2).

#### 3.6. Aerobic metabolism

In FW- and SW-acclimated fish, exposure to LC and HC had no effect on routine oxygen consumption rates (Table 2). There were no significant differences in mean weights of killifish used for oxygen consumption rate measurements. In the gill, exposure to LC in FW and HC in SW significantly decreased COX activity, relative to their respective controls (Fig. 5A). In contrast, no significant changes were observed in gill CS activity and COX/CS ratio (Fig. 5D and G). HC exposure in SW-acclimated fish significantly increased intestinal COX and CS activities by ~1.5-fold compared to their respective controls, while no changes were observed in COX/CS ratio (Fig. 5B, E and H). However, in the intestine, a significant effect of salinity was observed in fish exposed to HC, with the COX/CS ratio decreasing with increased salinity (Fig. 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 LC exposed FW-acclimated fish relative to controls (Fig. 5C, F and I).

## 4. Discussion

Our findings suggest that while increased water salinity reduced Cu accumulation in most tissues, the overall protective effect on oxidative stress and disruption of oxidation metabolism was variable. While salinity clearly affected tissue ion levels, Cu exposure had minimal impact on this trait in either FW or SW. We did observe with Cu exposure an increase in protein carbonyls in intestines of FW-acclimated fish, which declined with SW acclimation. However, neither gills nor liver showed this association, and in fact SW acclimation alone resulted in increased protein carbonyl levels in gills. In general, Cu exposure increased the activity of one oxidative stress defense enzyme, CAT (which was partially reversed by salinity in the liver), but Cu reduced the activity of another, SOD. Both Cu and salinity affected the maximal activities of glycolytic and oxidative enzymes, principally by inducing the capacity for glycolysis and decreasing COX activity, however, in a tissue-specific manner. Salinity seemed to have a greater protective effect on

**Table 2**

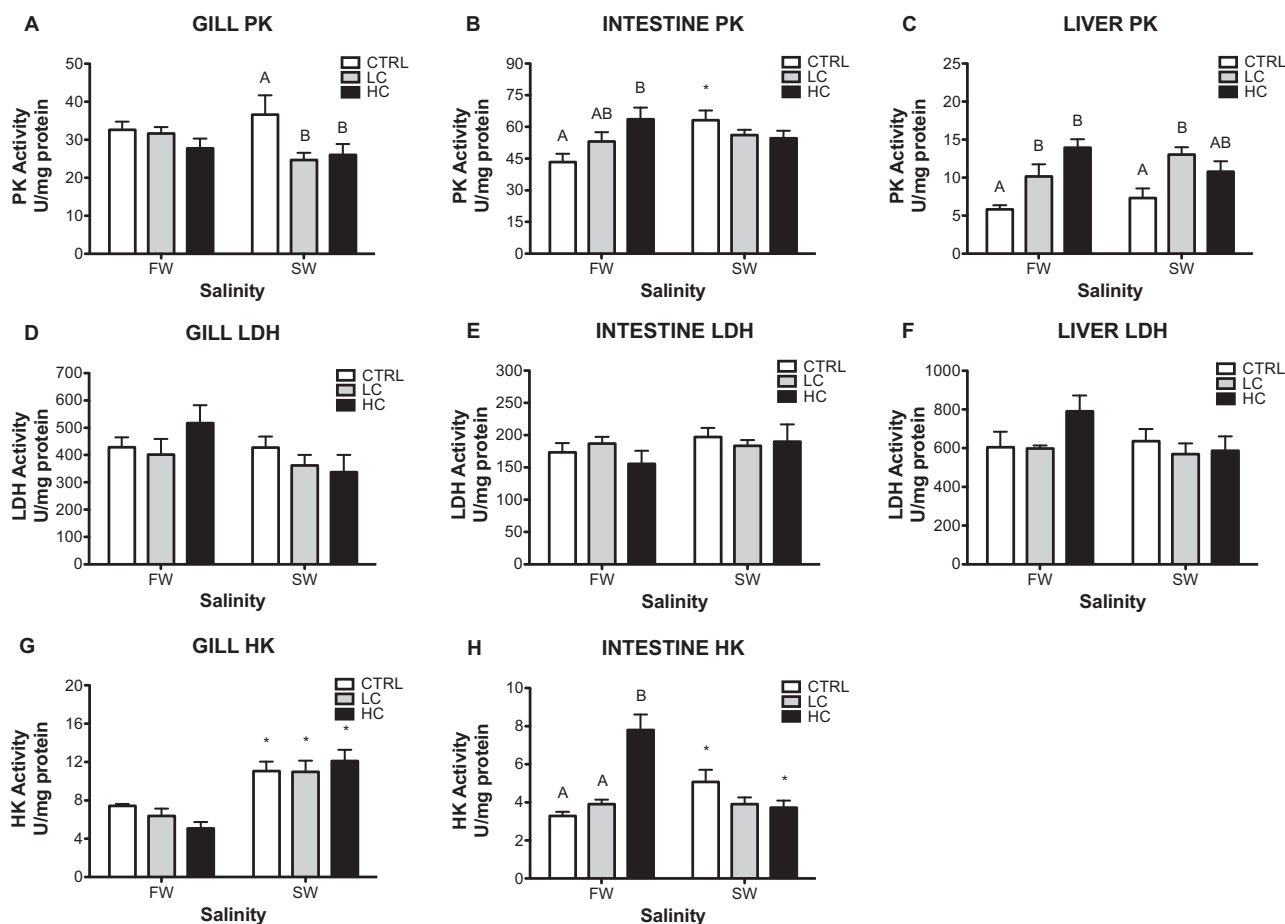
Oxygen consumption rate, and hematological measures (lactate, hematocrit (Hct), hemoglobin (Hb) and mean cell hemoglobin concentration (MCHC)) in killifish acclimated to freshwater (FW) or seawater (SW) and exposed low Cu (50 µg/L, LC) or high Cu (200 µg/L, HC) for 96 h.

	FW				SW	
	CTRL	LC	HC	CTRL	LC	HC
Body weight (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 <sub>2</sub> consumption rate (µM O <sub>2</sub> /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
<i>Hematological measures</i>						
Lactate (mmol/L)	12.2 ± 0.4 <sup>A</sup>	8.1 ± 0.6 <sup>B</sup>	7.5 ± 0.5 <sup>B</sup>	13.8 ± 1.1	13.9 ± 0.7 <sup>†</sup>	14.3 ± 0.7 <sup>†</sup>
Hct (%)	34.4 ± 1.5 <sup>A</sup>	44.7 ± 1.5 <sup>B</sup>	46.6 ± 1.1 <sup>B</sup>	32.3 ± 1.6 <sup>AB</sup>	31.4 ± 1.5 <sup>A</sup>	37.8 ± 2.9 <sup>B*</sup>
Hb (g/dL)	7.1 ± 0.6 <sup>A</sup>	10.6 ± 0.4 <sup>B</sup>	10.8 ± 0.5 <sup>B</sup>	6.5 ± 0.4	7.2 ± 0.5 <sup>†</sup>	7.8 ± 0.5 <sup>†</sup>
MCHC (g/L)	195 ± 14	234 ± 11	232 ± 11	206 ± 15	242 ± 24	206 ± 11

Values are presented as means ± SEM (N = 16–25).

Values that do not share the same letter 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,  $p < 0.05$ ).



**Fig. 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 (50  $\mu\text{g/L}$ , LC) or high Cu (200  $\mu\text{g/L}$ , HC) for 96 h. Values are presented as means  $\pm$  SEM of  $n = 6$  per treatment. Values that do not share the same letter 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,  $p < 0.05$ ).

partially reversing the Cu-induced reduction in blood oxygen carrying capacity.

#### 4.1. Copper accumulation

Overall, Cu accumulation followed tissue-specific patterns depending on exposure concentration and salinity (Fig. 2). There was a significant accumulation of Cu in gills of FW-acclimated fish consistent with this tissue as the primary target of metal ion toxicity (e.g., Blanchard and Grosell, 2006). SW acclimation reduced the Cu accumulation in gills compared to FW fish, but independent of Cu exposure level (Fig. 2A). Given the similar pH and bicarbonate alkalinity of the fresh water and sea water used in the present study, Cu speciation was likely similar in the two media, being dominated by carbonate and DOC complexes, with small amounts of hydroxides and free  $\text{Cu}^{2+}$  (Grosell, 2012). However, the concentrations of competitive cations ( $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$  and  $\text{Mg}^{2+}$ ) were many-fold higher in sea water. Therefore the reduction in Cu accumulation in the seawater exposures was likely due to the increased competition between Cu and cations for the same binding sites, as well as to a modified gill phenotype associated with water salinity (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 levels did not increase gill Cu accumulation. Despite the finite ability of the gills to accumulate Cu, increased Cu exposure is reflected in an increased Cu content in downstream organs, such as the liver.

In this study, our analyses recorded total Cu concentrations in tissues, and therefore we could not distinguish between the accumulation of  $\text{Cu}^{2+}$  (the ionic form which occurs in aerobic waters) and the reduced ionic form  $\text{Cu}^+$ . Indeed we are aware of no study in fish which has done this. Nevertheless, many Cu transport proteins are known to preferentially bind  $\text{Cu}^+$  over  $\text{Cu}^{2+}$ , and there has been considerable speculation that  $\text{Cu}^{2+}$  may be reduced to  $\text{Cu}^+$  by a Cu reductase on the gill surface prior to uptake (reviewed by Grosell, 2012). The two forms have different redox properties and would be expected to contribute differentially to ROS generation in the tissues, so internal interconversion might be responsible for obscuring Cu concentration versus ROS response relationships; this is an important area for future investigation.

Compared to gills, in the intestine SW acclimation affected Cu accumulation to a lesser extent (Fig. 2B), despite likely increased drinking rates compared to FW (Loro et al., 2014). In FW, both low and high Cu exposure elevated intestinal Cu load. After SW acclimation only the high Cu exposure led to a significant increase in intestinal Cu. The equivalent intestinal Cu accumulation regardless of salinity may be the result of increased intestinal  $\text{HCO}_3^-$  secretion in response to elevated salinity, which can bind Cu reducing its bioavailability in the SW acclimated fish (Genz et al., 2008). Our results indicate that the intestine is a likely target for Cu toxicity in both FW- and SW-acclimated fish.

Salinity decreased Cu accumulation in the liver, and SW-acclimated fish had significant lower Cu levels when both groups were exposed to HC (Fig. 2C). Since the liver serves as a Cu

scavenger, it has much higher levels than other tissues (Grosell, 2012) and Cu accumulation depends on uptake 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 between salinities in liver accumulation. These results suggest that the protective effect SW acclimation has on gill accumulation also reduces accumulation in the liver. This protection may involve either the complexation of Cu with ions in SW or perhaps competition of the ions for transport sites shared with Cu. Interestingly, relative to controls; only HC exposure increased liver Cu accumulation in both FW and SW-acclimated fish. The liver accumulates Cu and then excretes it mainly via the bile (Grosell et al., 1998; Mazon and Fernandes, 1999). However, at high Cu concentrations killifish may lose the capacity to regulate Cu homeostatically, or they could be sequestering Cu in the liver, perhaps bound to MT so as to make it biologically unavailable. It is difficult to determine which homeostatic mechanism is most effective in dealing with excess Cu (reviewed in La Fontaine and Mercer, 2007).

In the carcass (mainly muscle, skin, and bone), with the exception of LC in SW acclimated fish, neither salinity nor Cu exposure had a significant effect on Cu accumulation (Fig. 2D). This suggests that the carcass is not a major target of Cu toxicity, in agreement with previously published results (Kamunde et al., 2002; Grosell et al., 2004; Blanchard and Grosell, 2006).

#### 4.2. Oxidative damage and antioxidant responses

Oxidative damage and changes in expression and/or activity of antioxidant enzymes may be 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 ROS-induced oxidation of protein side chains and lipids, respectively (Halliwell and Gutteridge, 2001). Softwater-acclimated zebrafish exposed to sublethal (15 µg/L) Cu concentrations showed elevated gill and liver protein carbonyls but also increased SOD activity and a reduction in CAT activity that were correlated to tissue Cu levels (Craig et al., 2007). Similar trends were observed in pacu (*Piarctus mesopotamicus*) exposed to 400 µg/L Cu for 48-h, with significant increases in liver lipid peroxidation and SOD activity, and reduced CAT activity (Sampaio et al., 2008). In the present study, Cu exposures only induced changes in intestinal oxidative damage, measured as protein carbonyls, in FW-acclimated fish (Fig. 3B). This response was absent in the gill and liver, despite significant accumulation of Cu, suggesting tissue-specific responses and the absence of dose-related effects. Loro et al. (2012) reported qualitatively similar responses in various indices of oxidative stress across different tissues of killifish exposed to sublethal Zn in FW, and complete absence of these effects in SW exposed animals. As with effects on ionoregulation (Loro et al., 2014), Cu and Zn clearly differ greatly in their oxidative stress effects on killifish, and in their interactions with salinity.

Other studies have reported more consistent indications of oxidative damage at high concentrations of Cu, rather than low to moderate concentrations. In Indian flying barb (*E. danricu*) exposed to sublethal (0.55 mg/L) Cu for 96-h, no changes in lipid peroxidation were observed in visceral tissue (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 LC and HC 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 (Fig. 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 potent antioxidant mechanisms that combat and detoxify elevated ROS. Although

exposure to Cu can induce antioxidant enzyme activity in fishes, it can also inhibit enzyme activity depending on exposure concentration, length of exposure and species (reviewed in Grosell, 2012; Lushchak, 2011). FW-acclimated killifish responded to Cu exposure with increased CAT activity in the gill, liver and intestine (Fig. 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 (Fig. 3G–I). An increase in CAT activity represents a direct response to ROS and may explain why LC and HC exposure did not induce protein carbonyls in the current study. In SW-acclimated fish, Cu had no effect on gill CAT activity (Fig. 3D). This could be due to the lower levels of Cu that accumulated in the gills, as well as an induction of CAT activity with increased salinity (reviewed in Lushchak, 2011; Martínez-Álvarez et al., 2005).

Interestingly, our results show patterns distinct from those observed by Craig et al. (2007) for zebrafish and Sampaio et al. (2008) for pacu, where SOD activity increased with either no change or decreased activity of CAT, respectively. Sanchez et al. (2005) showed that exposure to Cu initially induced SOD activity after 4-days, followed by a decrease after 21-days. In killifish exposed to sublethal Zn for 96 h in FW, there was a marked decrease in SOD activity in all tissues (Loro et al., 2012), similar to the gill SOD response to Cu in the present study. This transitory decrease in SOD activity 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 and thereby inhibiting activity as previously suggested in fish (Pedrajas et al., 1995). 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.

#### 4.3. Copper effects on metabolic pathways

At the cellular level Cu can induce changes in the activity of a number of enzymes, impacting metabolic pathways and ATP synthesis rates, plus disrupt oxygen transport and mitochondrial biogenesis (Depledge, 1984; Hansen et al., 1992; Craig et al., 2007).

A general increase in PK in the intestine and liver, and HK in intestine, accompanied by no changes in LDH in either tissue with Cu exposure (Fig. 4B, C and H) suggest an increased capacity for carbohydrate metabolism. In contrast, previous studies have shown a decrease in glycolytic enzyme activities with Cu exposure (Hansen et al., 1992; Gul et al., 2004; Carvalho and Fernandes, 2008). Reduction in PK and HK activities has also been reported in the liver of European seabass (*Dicentrarchus labrax*; Isani et al., 1994) and curimba (*Prochilodus lineatus*; Carvalho and Fernandes, 2008) exposed to acute sublethal levels of Cu. Reduction in glycolytic capacity may be dependent on direct competition of Cu with essential divalent cations, such as Mg<sup>2+</sup>, 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).

Overall, LDH activity is generally unaffected or even increased in Cu-exposed fishes and invertebrates (e.g., Hansen et al., 1992; Cheney and Criddle, 1996; Tóth et al., 1996; Couture and Kumar, 2003). However, exposure to Cu in seabream (*Sparus auratus*) led to a decrease in LDH activity in the liver (Antognelli et al., 2003). Couture and Kumar (2003) suggested that Cu-induced decreases in aerobic capacity might lead to a compensatory induction of anaerobic capacity, as observed in yellow perch (*Perca flavescens*). While it is thought that anaerobic metabolism appears to be less sensitive to Cu inhibition than aerobic metabolism (Cheney and Criddle,



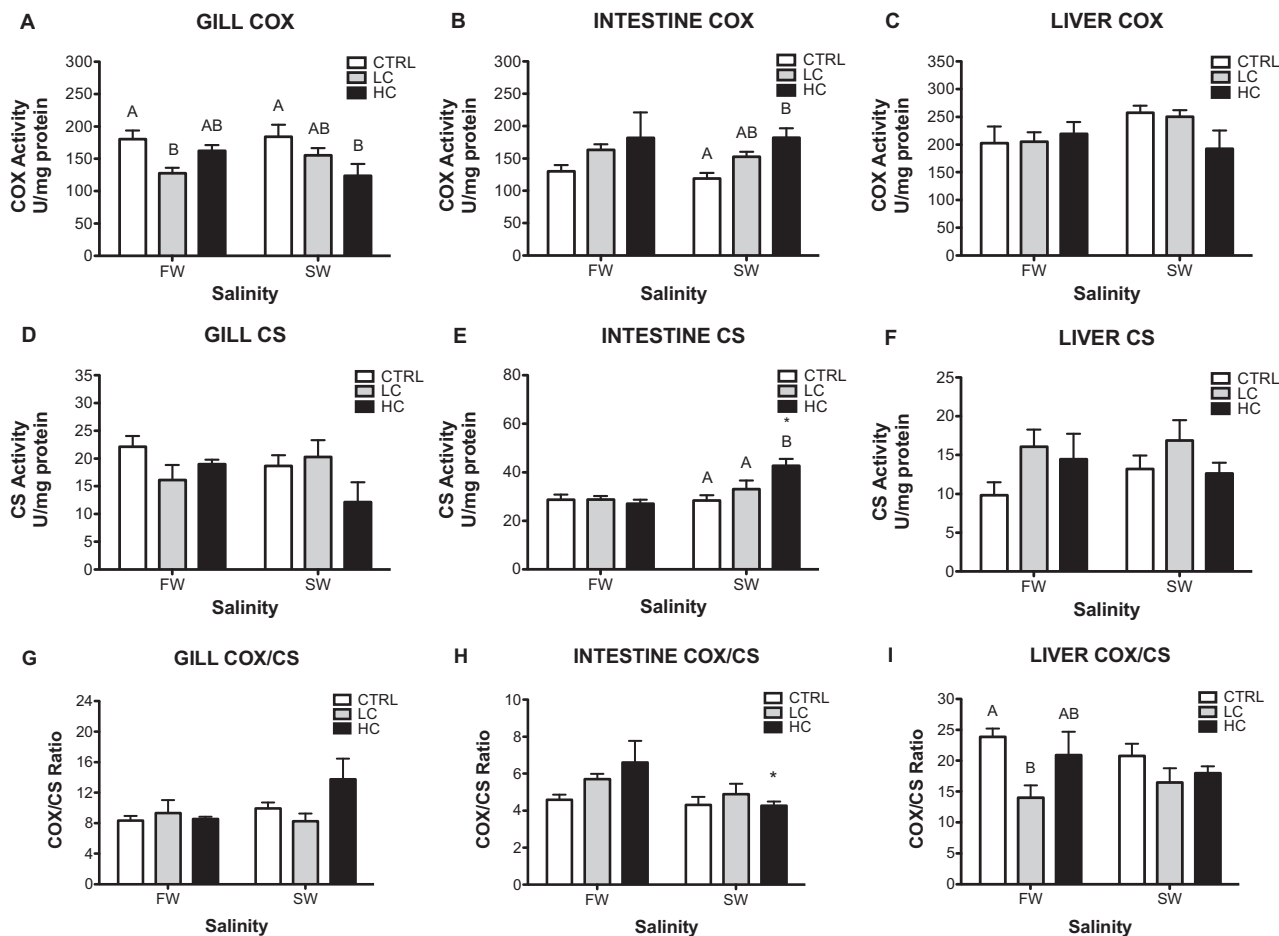
1996), the reduction in blood lactate observed (Table 2) suggests this might not be the case in killifish.

Cu has also been observed to interfere with mitochondrial function, possibly as a result of 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 livers of zebrafish exposed to acute sublethal Cu. The enzyme activities of CS, a key enzyme in the tricarboxylic acid cycle (TCA) cycle, and COX, a key enzyme in aerobic metabolism, can be used as indicators of mitochondrial quantity and quality, respectively (Capkova et al., 2002). In addition, changes in this ratio can indicate mitochondrial dysfunction (Craig et al., 2007; Capkova et al., 2002). Cu may inhibit proper COX assembly or function, as excess Cu can disturb the interaction of COX with oxygen, as a result of alterations in the surrounding membrane lipids due to increased ROS levels (Radi and Matkovic, 1988; Capkova et al., 2002; Bury et al., 2003; Bagnyukova et al., 2006). Similar to the findings of Craig et al. (2007) on Cu-exposed zebrafish, we observed a significant decrease in the COX-to-CS ratio in the liver of Cu-exposed killifish in FW (Fig. 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 components (Fig. 5A). However, the opposite trend was observed in the intestine, with Cu exposure leading to an increase in COX activity (Fig. 5B). Cu exposure had minimal effects in CS activity, which increased in the intestine but only in SW-acclimated fish exposed to HC (Fig. 5E). Overall, the

results of this study show that acute sublethal Cu concentrations alter components of energy metabolism in FW- and SW-acclimated killifish, but to varying degrees on enzymes involved in carbohydrate metabolism and mitochondrial oxidative metabolism and in a tissue-specific manner.

#### 4.4. Oxygen consumption

One common physiological response to metal ion toxicity is a change in organismal oxygen consumption rate (Connell et al., 1999), which may decrease in fishes exposed to sublethal Cu concentrations (Beaumont et al., 2003). De Boeck et al. (1995) found that oxygen consumption dropped immediately after Cu exposure in the common carp. Our results suggest FW- and SW-acclimated killifish retain the ability to regulate metabolic rate when exposed to acute sublethal Cu at environmentally relevant concentrations (Table 2). Gill damage may be a major cause of metal-induced reductions in oxygen consumption but that induced after 96-h exposure to sublethal Cu in curimba 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 in carp, 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 measuring oxygen consumption rates to evaluate Cu toxicity. Whether Cu exposures causes significant gill



**Fig. 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 (50 µg/L, LC) or high Cu (200 µg/L, HC) for 96 h. 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. (\*) denotes significant differences between FW- and SW-acclimated fish within the same treatment (two-way ANOVA,  $p < 0.05$ ).

damage in killifish, or if gill repair occurs over the time frame of our metabolic measurement (cf. McDonald and Wood, 1993) are important factors to consider in future investigations.

#### 4.5. Hematological parameters

In agreement with studies on carp, curimba (*Prochilodus scrofa*) and Mozambique tilapia (*Oreochromis mossambicus*) in acute Cu challenges (Svobodova, 1982; Mazon et al., 2002; Cyriac et al., 1989), killifish exposed to Cu for 96 h showed increased Hct and Hb (Table 2). These changes may be the result of a common generalized stress response in fishes to metal ion toxicity (Craig et al., 2009) or in response to systemic hypoxia as a result of impaired gas transport at the gills. Increases in Hct and Hb could occur to elevate the blood oxygen carrying capacity (Larsson et al., 1985; Cyriac et al., 1989) and to increase extraction at the gills. Notably, we found plasma lactate levels were significantly reduced in these LC and HC treatments in FW (Table 2). Carvalho and Fernandes (2006) found Cu induced a significant increase in mean cell hemoglobin (MCH) and MCHC in curimba, suggesting a stimulation of hemoglobin synthesis. In contrast, we observed no changes in MCHC. Exposure of rainbow trout to 27 µg Cu/L for 3 days elevated Hb and Hct but levels returned to control values by 14-days of exposure, which Dethloff et al. (1999) suggested was due to acclimation to Cu. So, at high concentrations Cu may play a role in the stimulation of a compensatory hemopoietic response to Cu-induced damage, involving the liver as it accumulates the highest levels of Cu and is also one of the heme synthesis sites (Kraemer et al., 2005; Witeska et al., 2010).

## 5. Conclusions

In this study, we have shown that although salinity protects against Cu accumulation in gills and liver, oxidative stress and metabolic responses induced by Cu in killifish acclimated to SW differed only slightly from those in FW. It is clear from this study that Cu induces changes at many levels of biological organization. Our results suggest that measurements of oxidative damage and aerobic metabolism can be used to assess the sublethal toxicity of Cu to fish, however, the levels of damage and changes in metabolism may vary depending on exposure concentration and tissue examined. To further elicit a better understanding of the mechanisms involved in Cu toxicity in SW, we suggest that chronic exposure periods and Cu concentrations greater than environmentally relevant levels (e.g., >200 µg/L) be investigated.

## Acknowledgements

This research was supported by an NSERC CRD grant (Scott Smith, P.I.) with co-funding and/or in-kind contributions from the International Copper Association (ICA), the Copper Development Association (CDA), the International Zinc Association (IZA), the International Lead Zinc Research Organization (ILZRO), the Nickel Producers Environmental Research Association (NiPERA), Teck Resources, and Vale Inco. CMW was supported by the Canada Research Chairs Program. The authors also thank the industrial partners for helpful comments on the manuscript.

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