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The oxidative stress response in freshwater-acclimated killifish (*Fundulus heteroclitus*) to acute copper and hypoxia exposure

Victoria E. Ransberry, Tamzin A. Blewett, Grant B. McClelland *

Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada

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ABSTRACT

Aquatic organisms face multiple stressors in natural ecosystems. Here we examine the effects of moderate hypoxia and low-level copper (Cu) on freshwater (FW)-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 killifish for 96 h to Cu in normoxia (total 23.4 ± 0.9 μg Cu L^{-1}), or either no Cu (2.33 ± 0.01 mg O_2 L^{-1}) or with Cu in hypoxia (23.6 ± 0.8 μg Cu L^{-1}; 2.51 ± 0.04 mg O_2 L^{-1}), and compared them to normoxic controls with no added Cu (0.7 ± 0.1 μg Cu L^{-1}; 9.10 ± 0.00 mg O_2 L^{-1}) at a hardness of 140 mg L^{-1} as $CaCO_3$ equivalents. Gills showed significant Cu accumulation with both excess waterborne Cu in normoxia and in hypoxia. This was accompanied by increases in gill catalase (CAT) activity but with no significant changes in either protein carbonyls or lipid peroxidation (TBARS). Hypoxia alone decreased gill protein carbonyls. Liver showed no change in Cu load, but a significant decline in CAT activity occurred with Cu in normoxia. Liver showed an increase in TBARS with Cu in normoxia. Cu when combined with hypoxia caused a significant decline in cytochrome *c* oxidase (COX) and citrate synthase (CS) activity in gill and liver. Thus, low waterborne levels of Cu and moderate hypoxia both affected gill and liver phenotypes. However, killifish are tolerant of Cu and hypoxia, and there was no evidence of a synergistic response to exposure to the two stressors combined compared to each stressor alone.

1. Introduction

Many coastal ecosystems currently suffer from eutrophication, resulting in hypoxic conditions (Diaz and Rosenberg, 1995). Organic enrichment can trigger eutrophication, which can be further facilitated by anthropogenic inputs to the system. Anthropogenic influences can also result in increased levels of metals in marine environments, such as copper (Cu) (Flemming and Trevors, 1989). Cu, 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 Cu concentrations range from 0.2 to 300 μg L^{-1} in FW systems (USEPA, 2007), and anthropogenic input can result in Cu concentrations ranging from 100 μg L^{-1} to 200 mg L^{-1} in mining areas (USEPA, 2007). 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, 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 marine environments with both organic matter and metals.

Excess waterborne Cu may exert many negative effects on fish, such as disrupting ion homeostasis and growth (Laurén and McDonald, 1987ab). Cu is also highly reactive with hydrogen peroxide (H_2O_2) that is constitutively produced or stress induced, leading to the generation of reactive oxygen species (ROS) (Harris and Gitlin, 1996). Cu may also enhance ROS generation as it can interfere with the protective antioxidant systems by inhibiting the activities of ROS-scavenging enzymes, superoxide dismutase (SOD) and catalase (CAT), and depleting cellular glutathione concentrations (Pruell and Engelhardt, 1980; Freedman et al., 1989; Harris, 1992). There is also a close link between metal-induced oxidative stress and mitochondrial function (Stohs and Bagchi, 1995) because of Cu's role in assembly and function of cytochrome *c* oxidase (Uauy et al., 1998) and Cu, Zn-SOD (Harris, 1992).

Similarly, changes in environmental O_2 availability can alter ROS production, and both hyperoxia and hypoxia are thought to lead to increased oxidative stress (reviewed in Lushchak, 2011). Hypoxia has been observed to increase SOD and CAT maximal enzyme activities in numerous fish, including goldfish, common carp, and goby (Lushchak et al., 2001, 2005; Lushchak and Bagnyukova, 2007). These data suggest

Abbreviations: Cu, copper; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; COX, cytochrome *c* oxidase; HIF-1, hypoxia inducible factor 1; HRE, hypoxia responsive elements; TBARS, thiobarbituric acid reactive substances; CS, citrate synthase; ppt, parts per thousand; FW, freshwater; DO, dissolved oxygen; DOC, dissolved organic carbon.

* Corresponding author at: Department of Biology, McMaster University, 1280 Main St. W, Hamilton, ON L8S 4K1 Canada. Tel.: +1 905 525 9140x24266; fax: +1 905 522 6066.
E-mail addresses: vransberry@gmail.com (V.E. Ransberry), blewetta@mcmaster.ca (T.A. Blewett), grantm@mcmaster.ca (G.B. McClelland).

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that exposure to excess dissolved and bioavailable waterborne Cu and reduced dissolved O_2 may have an additive or synergistic effect on inducing oxidative stress. Moreover, Cu on its own has been shown to induce a hypoxia-like response in different model organisms (e.g., Sampaio et al., 2008), suggesting multiple interacting effects of combined exposure.

The killifish, *Fundulus heteroclitus*, is a non-migratory euryhaline teleost, inhabiting intertidal marshes along the east coast of North America. Killifish are often found in tidal shallows that experience large fluctuations in daily dissolved oxygen levels, temperature, and salinity. Thus, this species is an excellent model for physiological responses to natural and anthropomorphic environment changes (reviewed in Burnett et al., 2007). As coastal areas are highly populated resulting in increased influence of anthropogenic sources to water quality, killifish will likely face a wide range of environmental stressors, including elevated Cu and hypoxia.

The goal of this study was to assess the potential interaction effects of two stressors currently faced by aquatic organisms, Cu and hypoxia. We used killifish acclimated to freshwater to minimize the ameliorating effect of salinity on Cu toxicity (e.g., Bianchini et al., 2004) and exposed them for 96 h to an environmentally relevant Cu concentration (25 μg L^{-1}) and hypoxia, each alone and in combination. We hypothesized that the combination of sub-lethal Cu and hypoxia would synergistically induce oxidative stress as well as decrease the quantity and quality of tissue mitochondria. Alternatively, the two stressors could act additively or antagonistically. After 96-h exposure, we measured indicators of oxidative damage (protein carbonyl content and lipid peroxidation, TBARS). We examined the oxidative stress defenses in killifish by measuring the maximal enzyme activities of ROS detoxifying enzymes (CAT and SOD) and indices of mitochondrial quantity and quality (citrate synthase (CS) and COX, respectively). These results will uncover some mechanisms of the potential interaction effects of multiple stressors on fish.

2. Materials and methods

2.1. Experimental animals

Adult killifish of mixed sex (average body weight: 3.21 ± 0.09 g) were collected from the wild by Aquatic Research Organisms (Hampton, NH, USA). Once received at McMaster University, fish were kept in artificial SW for 2 weeks before being gradually acclimated to decreasing salinity levels to 10 ppt in an aerated 300-liter aquarium with carbon filtration and kept at this salinity for a minimum of 2 weeks. Approximately 192 killifish were then acclimated to FW conditions in carbon-filtered, aerated, 45-liter tanks for a minimum of 2 weeks prior to experimentation. FW was dechlorinated City of Hamilton tap water (moderately hard (all in mEq L^{-1}): $[Na^+]$ = 0.6, $[Cl^-]$ = 0.8, $[Ca^{2+}]$ = 1.8, $[Mg^{2+}]$ = 0.3, $[K^+]$ = 0.05; titration alkalinity = 2.1; pH ~8.0; hardness ~140 mg L^{-1} as $CaCO_3$ equivalents (see Table 1 for full water chemistry). During the acclimation period, fish were fed daily with a commercial tropical fish flake to satiation (Big Al's Aquarium Supercenter, Woodbridge, ON) and maintained under a constant photoperiod (12:12-h light:dark) and at approximately 18 °C. Fish were fasted for 48 h prior to the start of experiments and throughout the 96-h exposure period. All procedures were approved by the McMaster University Animal Research Ethics Board and in accordance with the Guidelines of the Canadian Council on Animal Care.

2.2. Copper and Hypoxia Exposure

Killifish (n = 192) were removed from their FW acclimation tanks and placed into 8-liter darkened experimental tanks (n = 12 fish per tank, 4 treatments, and 4 replicates per treatment). The four experimental conditions (n = 48, per treatment) used for 96-h exposures were as follows: (1) no copper controls (no Cu, 0.7 ± 0.1 μg Cu L^{-1}) or (2) with excess copper (+Cu, 23.4 ± 0.09 μg Cu L^{-1}) in normoxia (both at 9.10 ± 0.00 mg O_2 L^{-1}) and (3) no Cu (1.1 ± 0.4 μg Cu L^{-1}) or (4) +Cu (23.6 ± 0.1 μg Cu L^{-1}) in hypoxia (2.33 ± 0.03 and 2.51 ± 0.04 mg O_2 L^{-1}, respectively). Each Cu replicate exposure tank was prepared using a stock Cu solution made from $CuSO_4$ dissolved in 1% HNO_3 (Sigma Aldrich, Oakville, ON), which had no significant effect on water pH (Table 1). Tank water was renewed daily by 80%, with thoroughly mixed renewal water prepared 24 h in advance. In the no Cu and +Cu in normoxia, treatment water was fully oxygen-saturated by means of an airstone. For no Cu and +Cu treatments in hypoxia, tanks were covered with plastic wrap, and dissolved oxygen (DO) concentration of the water was lowered by bubbling a mixture of compressed N_2 gas and air in the appropriate proportions using mass flow controllers (Sierra Instruments, Monterey, CA, USA) and 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). At the end of 96 h, fish in all experimental treatments were treated with 1 mM EDTA (Sigma Aldrich Oakville, ON) followed by a 1-min rinse with deionized water to remove loosely bound Cu on gill tissue or the exterior of the body.

Table 1
Body mass of fish used (in g, n = 39–40 per treatment), and exposure water composition as ions (μM), copper (Cu, μg L^{-1}), pH, dissolved oxygen (DO, mg L^{-1}), and dissolved organic carbon (DOC, mg L^{-1}). Values for water chemistry presented as mean ± SEM of 4 replicate per treatment). Values that do not share the same letter indicate a significant difference ($p \leq 0.05$).

	Normoxia		Hypoxia	
Treatment	No Cu	Cu	No Cu	Cu
Body mass (g)	3.34 ± 0.16	3.22 ± 0.20	3.26 ± 0.15	2.99 ± 0.16
Na^+ (μM)	925.7 ± 14.2	976.6 ± 21.9	958.9 ± 33.3	963.1 ± 20.6
Mg^{2+} (μM)	313.4 ± 1.9[a]	281.2 ± 0.9[b]	302.1 ± 6.7[a]	307.5 ± 2.1[a]
Ca^{2+} (μM)	736.9 ± 8.6[a]	756.3 ± 3.8[a]	707.3 ± 14.3[b]	657.5 ± 5.2[b]
K^+ (μM)	66.7 ± 2.5[a]	46.5 ± 0.8[b]	49.1 ± 1.0[b]	48.4 ± 1.3[b]
Cu (μg L^{-1})	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 (mg L^{-1})	9.10 ± 0.00[a]	9.10 ± 0.00[a]	2.33 ± 0.03[b]	2.51 ± 0.04[b]
DOC (mg L^{-1})	3.09 ± 0.62	2.18 ± 0.06	2.03 ± 0.07	2.22 ± 0.13

2.3. Tissue and water analyses

Water samples were taken prior to each water renewal, filtered through a 0.45-μm filtration disc (Pall Life Sciences, East Hills, NY, USA) to measure water chemistry parameters from each replicate. Data from replicates for each treatment were combined and reported in Table 1. After 96-h exposures, killifish were quickly euthanized by cephalic concussion and gills, liver, muscle, intestine, and remaining carcass were sampled, weighed, and quickly frozen in liquid N_2. 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). Briefly, 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 then vortexed after 24 h. Tissue digests were then diluted as necessary and dissolved Cu concentrations determined using a 40 μg Cu L^{-1} standard for comparison (Fisher Scientific, Ottawa, ON). 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 Environment Canada 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^{2+}), or 0.1% $CsCl_2$ in 1% HNO_3 (K^+). Total dissolved organic carbon (DOC) concentration of water samples was measured directly using a Shimadzu TOC-

$V_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan) as described previously (Al-Reasi et al., 2012).

2.4. Oxidative damage

2.4.1. Protein carbonyls

Tissue protein carbonyl content was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI) as previously described (Craig et al., 2007). All protein concentrations of the tissue homogenates were assayed according to Bradford (1976), and bovine serum albumin (BSA) was used to construct a standard curve at 565 nm. Protein carbonyls are expressed in nanomoles per milligram total protein.

2.4.2. 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 s at 40 V on ice in homogenization buffer (100 mM Tris–HCl, 2 mM EDTA, 5 mM $MgCl_2$•$6H_2O$, pH 7.75) containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor, then centrifuged at 1,600x*g* for 10 min 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 freshly prepared color reagent (thiobarbituric acid (TBA), acetic acid and sodium hydroxide). Sample and standards were placed into boiling water for 1 h, immediately removed and incubated on ice for 10 min, then centrifuged at 1,600x*g* for 10 min 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 described earlier and TBARS levels are presented as micromolar MDA per milligram total protein.

2.5. Enzyme activity

Frozen gill and liver tissues were powdered using a liquid nitrogen-cooled mortar and pestle, then diluted 1:20 (mg tissue:μL) in ice-cold buffer (20 mM HEPES, 1 mM EDTA, 0.1% Triton X-100, pH 7.2) and homogenized for 1 min 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 (U) per milligram protein, where 1 U = μmol min^{-1}.

Maximal enzyme activity of CAT was measured according to Claiborne (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).

Maximal enzyme activity of SOD (combined Cu, Zn-SOD and Mn-SOD) was determined by using a commercial kit (Fluka, Sigma Aldrich, Oakville, ON, CA). 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%.

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).

CS activity was measured after tissue homogenates were frozen and re-thawed 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).

2.6. Statistical analysis

All data have been expressed as means ± SEM. For all tests, a p-value ≤ 0.05 was considered statistically significant. A two-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test was performed using copper and hypoxia as independent variables, to evaluate potential differences between treatment groups. All statistical analysis was performed using SigmaStat 3.5 (Chicago, IL, USA).

3. Results

3.1. 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 no Cu in normoxia (control) tanks. Single and combined exposure to Cu and hypoxia had no effect on mean weight of adult killifish compared to no Cu normoxic controls. Mean weights of fish for no Cu and +Cu in normoxia were 3.34 ± 0.16 g and 3.22 ± 0.20 g, and for no Cu and +Cu in hypoxia were 3.26 ± 0.15 g and 2.99 ± 0.16 g, respectively (Table 1).

3.2. Tissue and water chemistry

Water chemistry was determined for each treatment and levels of major cations Na^+, Mg^{2+}, Ca^{2+}, K^+, and DOC, which were relatively consistent across treatments. However, Mg^{2+} and K^+ were lower in the +Cu in normoxia treatment (11–43%, $F_{1,12} = 47.1$, $p < 0.001$ and $F_{1,12} = 85.1$, $p < 0.001$, respectively), K^+ was lower in the hypoxia with no Cu treatment (36%, $F_{1,12} = 26.8$, $p < 0.001$) and Mg^{2+} lower in the +Cu in hypoxia treatment ($F_{1,12} = 52.7$, $p < 0.001$) compared to no Cu in normoxia (Table 1). DOC did not vary across treatments ($p > 0.05$). Cu concentrations were low in the no Cu treatments in both normoxia and hypoxia (0.7 ± 0.1 and 1.1 ± 0.4 μg L^{-1}, respectively) and approached the target exposure level of 25 μg Cu L^{-1} in the +Cu treatments (in normoxia, 23.4 ± 0.9 and in hypoxia, 23.6 ± 0.8 μg L^{-1}). The hypoxia treatments (no Cu and +Cu) had similar dissolved O_2 of 2.33 ± 0.03 and 2.51 ± 0.04 mg O_2 L^{-1}, respectively, and were significantly lower than both normoxia treatments (9.10 ± 0.00 mg O_2 L^{-1}; Table 1). Water temperature in all tanks averaged 19.9 ± 0.12 °C and did not vary over the course of the experiments.

When killifish were exposed to excess waterborne Cu in hypoxia, there was a significant decrease in Ca^{2+} in skeletal muscle (+44%, $F_{1,16} = 7.26$, $p = 0.016$, Fig. 1A). Although Cu did not have an overall significant effect on muscle, Ca^{2+} *post hoc* analysis showed a significant increase with Cu exposure in normoxia ($p = 0.021$). Hypoxia in the absence of Cu increased in K^+ in the carcass (+15%, $F_{1,16} = 9.04$, $p = 0.008$, Fig. 1B) compared to no Cu controls. *Post hoc* analysis revealed a significant effect of Cu in normoxia on carcass K^+ ($p = 0.04$). However, this effect was absent when killifish were exposed to Cu in combination with hypoxia. Whole carcass samples showed a significant decrease in Mg^{2+} when exposed to hypoxia alone (13%, $F_{1,16} = 12.6$, $p = 0.003$) and when combined with Cu (14%, *post hoc analysis*, $p = 0.015$, Fig. 1D) relative to no Cu in normoxia. In comparison, Cu in combination with hypoxia caused a 27% increase of Mg^{2+} in the intestine ($p = 0.029$, Fig. 1D). There were no significant changes in ion

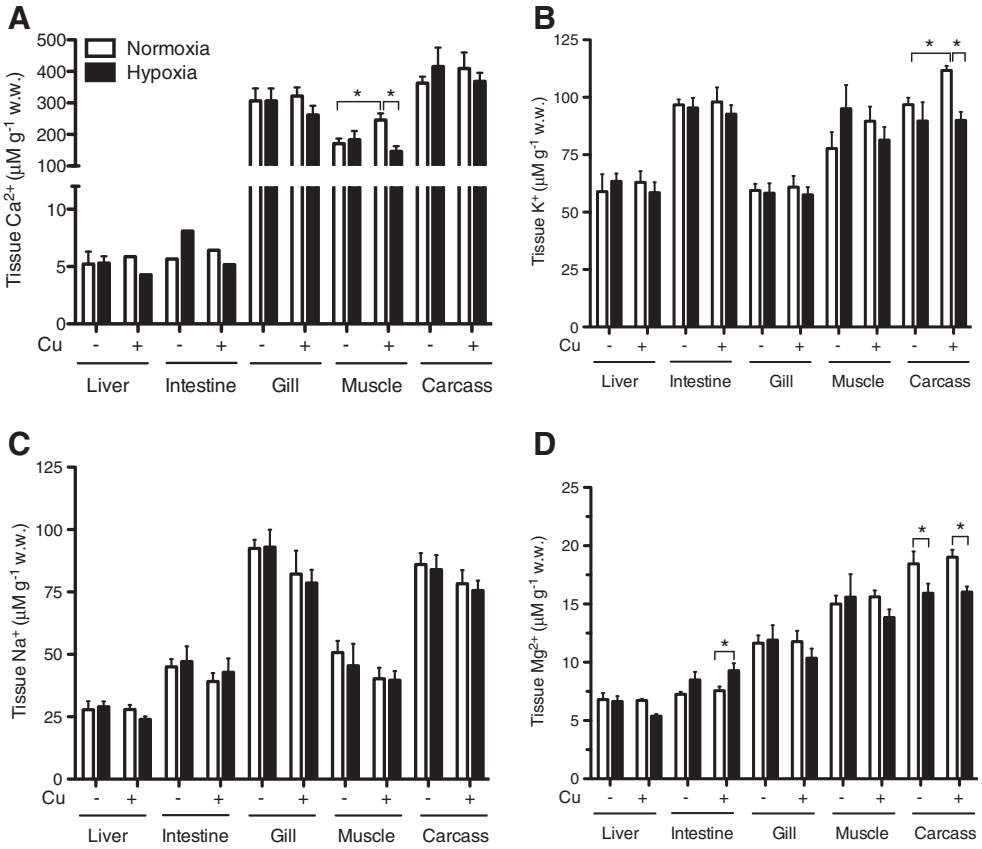

Fig. 1. Tissue levels of calcium (A), potassium (B), sodium (C), and magnesium (D), ions of killifish exposed to no copper or 25 μg L^{-1} (−Cu, +Cu, respectively), in normoxia (white bars) or hypoxia (black bars). Values are presented as means ± SEM. Values that are connected by a bracket are significantly different from each other (*$p \leq 0.05$, $n = 8$ for all treatments).

levels observed in either the gill or liver with the experimental treatments.

3.3. Copper accumulation

In the gill, Cu concentrations were significantly elevated ($F_{1,36} = 140.9$, $p < 0.001$) when fish were exposed to Cu in normoxia and with Cu in hypoxia (Fig. 2), compared to no Cu in normoxia. While the concentration of Cu was higher in the liver compared to the gill, intestine, and muscle (as much as 23-fold), it was not affected by any of the treatments. In the intestine, muscle, and carcass, no changes in Cu concentrations were observed between controls and experimental treatment groups (Fig. 2).

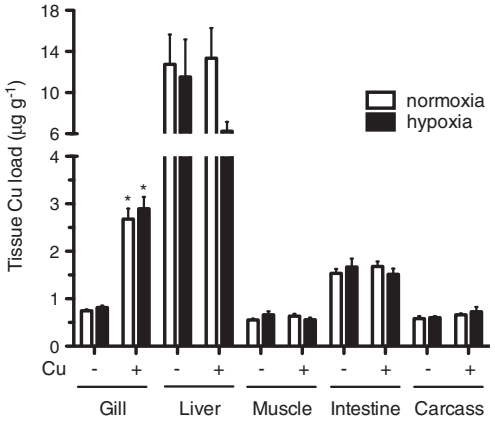

Fig. 2. Accumulation of copper (μg g^{-1} tissue) in tissues of killifish exposed to no copper or 25ug l^{-1} copper (−Cu, +Cu, respectively), in normoxia (white bars) or hypoxia (black bars). Values are presented as means ± SEM (*$p \leq 0.05$, $n = 10$ for all treatments).

3.4. Indices of oxidative damage

Levels of protein carbonyls were higher in gills than in the liver. However, despite increased Cu accumulation in the gill with Cu exposure, gill protein carbonyl content significantly decreased in hypoxia ($F_{1,28} = 4.47$, $p = 0.044$, Fig. 3A). Neither Cu ($F_{1,27} = 0.13$, $p = 0.72$) nor hypoxia exposure ($F_{1,27} = 0.19$, $p = 0.66$) or combined ($F_{1,27} = 0.12$, $p = 0.73$) had any effect on protein carbonyl content in the liver (Fig. 3B). Another index of oxidative damage, that of lipid peroxidation (TBARS), increased 2.8-fold in response to Cu in normoxia in the gill but this did not reach statistically significance ($F_{1,26} = 1.61$, $p = 0.22$). TBARS were found to be significantly higher after normoxic +Cu exposure in liver of killifish (*post hoc* test, $p = 0.04$, Fig. 3D).

3.5. Enzyme activities

3.5.1. Catalase

In the gill, Cu in normoxia and in combination with hypoxia induced a significant 2-fold ($p = 0.013$) and 2.2-fold ($p = 0.023$) increase in catalase activity, respectively (two-way ANOVA, $F_{1,27} = 12.9$, $p < 0.05$, Fig. 4A). In contrast, there was a significant decrease in catalase activity in the liver with Cu in normoxia ($F_{1,28} = 5.32$, $p < 0.05$, Fig. 4B). Catalase activity showed a decline in liver with hypoxia, but it was not significantly different from no Cu controls in normoxia ($F_{1,28} = 3.58$, $p = 0.069$).

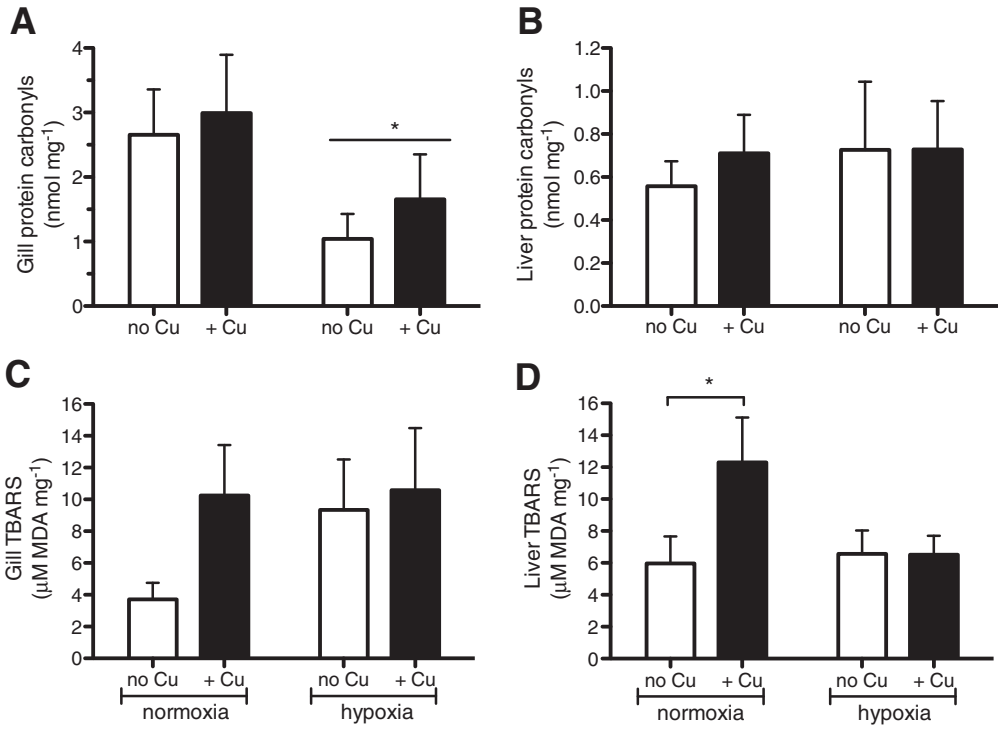

Fig. 3. Protein carbonyl content (nmol mg^{-1} protein) in the gill (A) and liver (B) and thiobarbituric acid reactive substances (TBARS) levels (μM MDA mg^{-1} protein) in the gill (C) and liver (D) of killifish exposed to no copper or 25ug l^{-1} copper (no Cu, + Cu, white and black bars, respectively), in normoxia or hypoxia. Values that are connected by a bracket are significantly different from each other (*$p \leq 0.05$, $n = 8$ for all treatments). The line in panel A denotes an overall significant effect of hypoxia on protein carbonyl content.

3.5.2. Superoxide dismutase

The apparent Vmax of SOD was similar between gill and liver, but neither Cu ($F_{1,27} = 0.31$, $p = 0.58$), hypoxia alone ($F_{1,27} = 0.12$, $p = 0.73$), nor hypoxia in combination with Cu ($F_{1,27} = 0.09$, $p = 0.77$) had any effect on SOD activity in either tissue of killifish (Fig. 4C, D).

3.5.3. Cytochrome c oxidase and citrate synthase

The activities of COX and CS were assayed to determine the effect of Cu and hypoxia treatment on indexes of mitochondrial quality and quantity, respectively. CS activity in the gill was affected by Cu ($F_{1,28} = 4.20$, $p = 0.05$) and tended to decreased with Cu exposure in

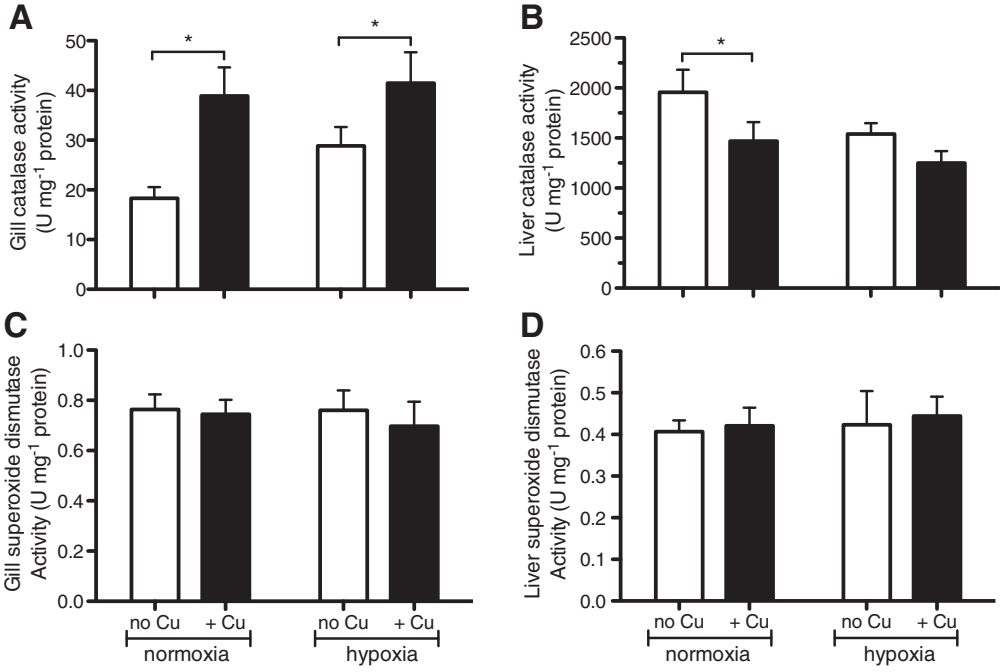

Fig. 4. Catalase (CAT) activity in the gill (A) and liver (B) and superoxide dismutase (SOD) activity (U mg^{-1} protein) in the gill (C) and liver (D) of killifish exposed to no copper or 25 μg l^{-1} copper (no Cu, + Cu, white and black bars, respectively), in normoxia or hypoxia. Values are presented as means ± SEM. Values that are connected by a bracket are significantly different from each other (*$p \leq 0.05$, $n = 8$ for all treatments). 1U = μmol min^{-1}.

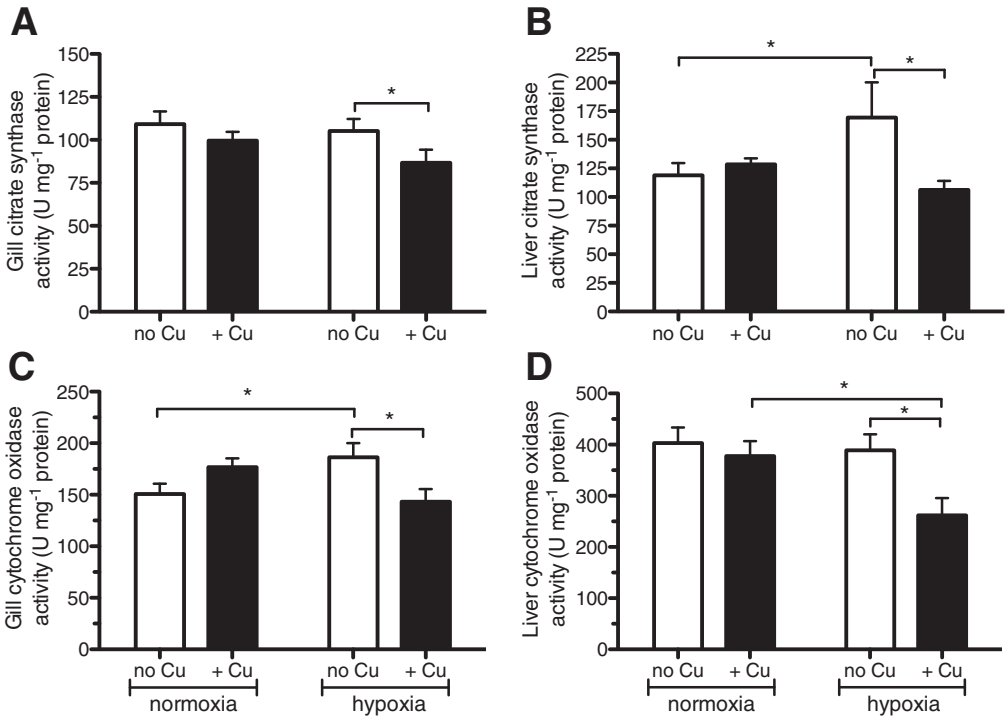

Fig. 5. Gill (A) and liver (B) citrate synthase (CS) activity and gill (C) and liver (D) cytochrome *c* oxidase (COX) activity (U mg^{-1} protein) of killifish exposed to no copper or 25 $\mu g\ l^{-1}$ copper (no Cu, +Cu, white and black bars, respectively), in normoxia or hypoxia. Values are presented as means ± SEM. Values that are connected by a bracket are significantly different from each other ($^*p \leq 0.05$, $n = 8$ for all treatments). 1U = $\mu mol\ min^{-1}$.

hypoxia ($p = 0.066$). In liver, CS showed an interaction effect between Cu and hypoxia ($F_{1,29} = 4.85$, $p = 0.036$) with *post hoc* analysis showing an increase due to hypoxia alone ($p = 0.042$) and a decrease when hypoxia and Cu were experienced together (Fig. 5B, $p = 0.01$). COX activity also showed an interaction effect in gills ($F_{1,27} = 9.32$, $p = 0.005$) with an increase in gill with hypoxia in the absence of Cu ($p = 0.031$) and a decline with Cu in hypoxia (Fig. 5C, $p = 0.013$). Under normoxic conditions, Cu did not affect liver COX activity, but activity was reduced when Cu was combined with hypoxia (Fig. 5D, $p = 0.07$).

4. Discussion

4.1. General findings

Our findings suggest that the combined effects of waterborne Cu and hypoxia do not act synergistically to affect oxidative stress. Indices of oxidative damage, protein carbonyls and TBARS, only significantly increased with exposure to excess Cu in normoxia in liver and not when excess Cu was combined with hypoxia. The combination of Cu and hypoxia did, however, reduce the maximal activity of COX in gill and liver, suggesting that when experienced together Cu and hypoxia reduced the oxidative capacity of these tissues. A 96-h 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 and increased liver CS and gill COX activities. The combined effects of Cu and hypoxia also caused some minor disturbances in tissue ions. Thus, our data suggest that in killifish the effects of combined Cu and hypoxia are neither additive nor are they synergistic, at least at the levels of Cu and hypoxia tested here. These results provide important insight into the combined effects of two stressors that commonly co-occur in freshwater and marine environments (Cu and hypoxia) and the biochemical and physiological responses they elicit in this euryhaline species.

4.2. Antioxidant responses

Copper-induced ROS formation is well documented among fish species, with the most notable response in the gill (Bopp et al., 2008) and liver (Manzl et al., 2004). While others show ROS generation increases as O_2 levels increase, hypoxia has also been observed to either increase or decrease ROS production (Chandel et al., 2000; Lushchak et al., 2005). The activities of SOD and CAT are part of the same metabolic pathway conversing various reactive molecules to H_2O_2 and then H_2O (Cooper et al., 2002); however, in the present study, we did not observe parallel changes in the activity of these two enzymes in response to Cu and/or hypoxia (Fig. 4). Sanchez et al. (2005) showed that after 4 days of exposure to Cu, SOD activity was initially induced but was followed by a transitory decrease after 21 days. Therefore, it is possible that in our exposures, there was an initial increase in activity occurred prior to 96 h and that the subsequent H_2O_2 produced induced CAT activity.

We found that gill CAT activity increased in response to Cu alone and in combination with hypoxia (Fig. 4), indicating an activation of defensive mechanisms occurred, presumably to prevent excessive oxidative damage. Since hypoxia alone had little effect on CAT activity, the induction in the Cu and hypoxia exposure likely occurs in response to Cu as it does in Cu in normoxia (Fig. 4). In contrast to the gills, Cu in normoxia resulted in a decrease in CAT activity in the liver, which was slightly attenuated in Cu in hypoxia. This reduced CAT activity may be due to excessive levels of ROS, as it has been shown that ROS can inactivate CAT (Halliwell and Gutteridge, 1989). However, this is not supported by data showing an absence of oxidative damage by Cu in the liver (Fig. 3), suggesting reduced CAT activity occurs by some other mechanism.

There were no significant changes in SOD activity in either the gill or liver. While studies on other fish species (e.g., *Piaractus mesopotamicus*, Sampaio et al., 2008) have shown an induction of SOD with Cu exposure, our data suggest killifish may maintain constitutively high SOD activities capable of catabolizing any ROS induced by accumulating Cu. Alternatively, it is also possible that in killifish SOD activity is induced

only at higher levels of waterborne Cu (Ransberry et al., 2015). The lack of response in SOD activity while CAT activity was induced or reduced by the same treatments demonstrates a mismatch between two reactions that are functionally linked. As well as suggesting a more complex response to stress than earlier presumed, it also emphasizes the need to use multiple biomarkers to assess oxidative stress.

4.3. Oxidative damage

Gills are a primary target of Cu toxicity due to their direct interaction with the external environment (Brungs et al., 1973; Buckley et al., 1982; Stagg and Shuttleworth, 1982). Indeed, we found significant Cu accumulation in the gill of killifish exposed to Cu alone and when combined with hypoxia (Fig. 2). However, hypoxia had no effect on Cu accumulation, compared to Cu alone, in agreement with previous studies on other species (Pilgaard et al., 1994). This Cu accumulation was not associated with an increase in oxidative damage to proteins or lipids (Fig. 3). This suggests that the increase in CAT activity (Fig. 4) in the gills may have been sufficient to reduce any Cu-induced ROS generation. The accumulation of protein carbonyls in response to Cu accumulation appears to be both time- and dose-dependent. For example, in soft-water acclimated zebrafish (*Danio rerio*) Craig et al. (2007, 2010) found increased protein carbonyls with both acute and chronic exposures but to relatively 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 or to a degree detectable using indexes we selected. However, under the condition tested, lipid peroxidation may be 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 response to zinc-induced oxidative stress compared to protein carbonyl levels and any other antioxidant measurement.

It has been speculated that reduced dissolved O_2 also affects oxidative stress in fishes, but via mechanism that are still unclear (Chandel and Shumacker, 2000). In killifish, hypoxia significantly decreased gill protein carbonyls, while gill lipid peroxidation tended to increase (Fig. 3). Similarly, Lushchak et al. (2005) observed that lipid peroxidation increased, while protein carbonyl content decreased or remained unchanged during hypoxia, in the liver of the common carp, *Cyrinus carpio*. In killifish, when Cu exposure was combined with hypoxia, gill protein carbonyls also tended to decrease compared to controls. However, it is possible that hypoxia induced a response in killifish to mitigate oxidative stress upon reoxygenation, a response known as "preparation to oxidative stress" (Hermes-Lima et al., 1998). It would be interesting to examine the combined effects of Cu and hypoxia on the recovery response of killifish, to determine whether their ability to effectively cope with reintroduction to normoxia is impaired.

The liver is the main detoxifying organ in the fish, and as such is equipped with a high antioxidant capacity (Das et al., 1998). However, the liver also possesses a high metabolic rate, which also makes it a target for oxidative damage (Ji et al., 1988). The Cu exposure level used in this study did not lead to a significant accumulation of Cu in the liver (Fig. 2). In fact, it may only be at higher Cu exposures, when the capacity for accumulation at gills may be reached, that excess Cu "spills over" to other organs such as liver (see Ransberry et al., 2015). The absence of Cu accumulation is reflected in a lack of induction of protein carbonyls or TBARS in this tissue. However, TBARS did show a slight but nonsignificant increase with the Cu only treatment (Fig. 3). Moreover, reduction in liver CAT activity suggests a Cu-independent effect on levels of this enzyme. It is also possible that other antioxidant defenses were induced to combat oxidative damage induced by Cu and/or hypoxia, such as glutathione and glutathione peroxidases (Lushchak et al., 2001; Hermes-Lima and Zenteno-Savín, 2002). While we measured a number of indicators of oxidative stress, future studies should aim to examine additional variables to gain a comprehensive picture of combined Cu and hypoxia stress.

4.4. Mitochondrial quantity and quality

Cu is essential for mitochondrial function, but when in excess, the mitochondrion is a major target for Cu toxicity (Sheline and Choi, 2004; Arciello et al., 2005). The enzyme activities of CS, a key enzyme in TCA cycle, and COX, a inner membrane enzyme key to aerobic metabolism, can be used as indicators of mitochondrial volume density and mitochondrial inner membrane, respectively (Capkova et al., 2002). In our study, hypoxia stimulated the activity of CS in liver when in the absence of Cu. This was reversed when experienced with Cu, suggesting the induction mitochondrial biogenesis by hypoxia is impaired by excess Cu. Similarly, stimulation of COX activity by hypoxia in gills was reversed when Cu and hypoxia were experience together. In contrast, Cooper et al. (2002) observed no change in gill CS activity by hypoxia in spot fish (*Leiostomus xanthurus*), and Craig et al. (2007) found Cu exposure did not induce gill COX activity in soft-water acclimated zebrafish when water Na^+ and Ca^+ were reintroduced. Similarly, in gilthead sea bream (Perez-Jimenez et al., 2012) and carp (Zhou et al. 2000), hypoxia had little effect on CS activity. Thus, it appears the effect of hypoxia on the CS activity may be quite variable and perhaps dependent on the degree and length of hypoxia exposure.

We observed a significant decrease in liver COX activity in killifish exposed to Cu in combination with hypoxia (Fig. 5). A reduction in COX activity after exposure to Cu and hypoxia may suggest a decrease in COX protein content. 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). Since an initial phase of metabolic depression is facilitated by decreasing oxidative enzyme activities in hypoxia-tolerant animals (Lutz et al., 1984; LaManna et al., 1996), this may be a strategy used by killifish to cope with Cu and hypoxia together. Neither Cu or hypoxia alone reduced COX activity; thus, both stressors act together to depress COX activity.

A decrease in the ratio of COX/CS can also be used as a marker of mitochondrial dysfunction (Capkova et al., 2002). We did not see any significant reductions in this enzyme ratio across treatments. Therefore, it suggests that neither Cu singly nor combined with hypoxia exposure induced mitochondrial dysfunction in adult killifish.

4.5. Limitation of the study

Since killifish showed tolerance to the environmentally relevant Cu levels, even in hypoxia, some possible interactive effects of the two stressors may not have been detected. Future studies may choose to use a less tolerant species or more extreme Cu exposure level in killifish to uncover mechanisms of multiple stressors. Moreover, although 96 h is a standard exposure for toxicology studies, it may not be sufficient to initiate all of the physiological responses.

4.6. Conclusions

We show here that killifish are tolerant of the combined exposure to environmentally relevant levels of Cu and hypoxia. These data suggest induced antioxidant protection pathways but a reduced oxidative capacity in a tissue-specific manner with the gills being the most sensitive. Moreover, we find that hypoxia may have an antagonistic effect on Cu-induced lipid peroxidation in freshwater-acclimated killifish. Overall, the results of our study will improve our understanding of the effects of Cu and hypoxia on biochemical and physiological markers of oxidative stress, and as a vertebrate euryhaline model, killifish will provide essential mechanistic information for improving water quality criteria.

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