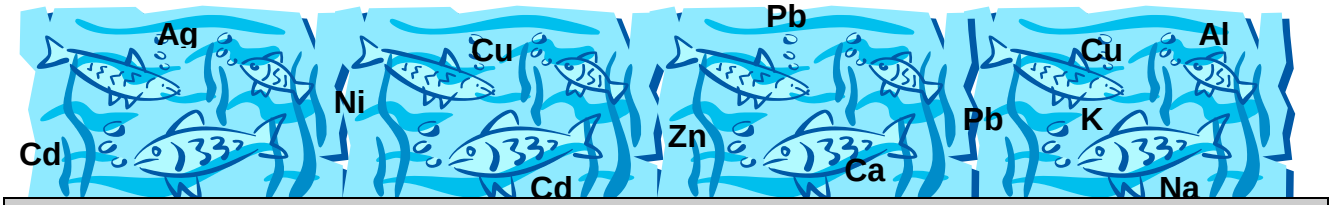
NSERC – Industry Project on Metal Bioavailability
Research Newsletter



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NEWS

The aim of this newsletter is to report on people and the projects they are working on. The star of this newsletter is **Victoria Ransberry**, who will be sharing some of her M.Sc. work, entitled "Effects of acute waterborne copper and hypoxia on the oxidative stress response in freshwater-adapted killifish, *Fundulus heteroclitus*", thanks! Just so the other labs don't feel left out, I've also thrown in some scientific snippets of interest. This small selection of studies highlights what the current 'CRD marine biotic ligand model (BLM) project' is all about: the integration of biology and chemistry to help produce a BLM for metal toxicity in marine and estuarine environments. Huzzah.

Comings and goings (May 2012 – Nov. 2012):

The Smith lab says hello (again) to **Holly Gray**, who is now a Research Assistant. Holly contributes to TOC, ASV metals as well as fluorescence measurements and rotifer toxity experiments for Zn. **Jessie Cunningham**, also a Research Assistant to both Smith and McGeer, now contributes to Pb measurements by voltammetry, ICPMS and GFAAS, mostly in support of a recent Pb chronic study with mysids. A new Ph.D. student has also joined the Smith lab albeit from a distance (co-supervised with Céline Guéguen at Trent University in Peterborough Ontario). **Weibin (Ben) Chen** will be working on DOC quality and impacts on metal speciation and toxicity with a particular emphasis on organic sulphides. There are also a couple of 4th year undergraduates in the Smith lab: **Scott Holmes** is performing Ni toxicity tests with rotifers in saltwater grab samples from various locations, as well as measuring bioaccumulation. **Giselle Webster** is continuing work on validating the use of Pb ISE for salt water applications and, similar to Scott H, also performing Pb toxicity tests with rotifers in saltwater grab samples.

There are two new M.Sc. students in the McGeer lab. **Oliver Vukov** is working on the toxicity of rare earth elements (currently cerium) to *Hyalella azteca* and *Daphnia pulex*. His work is supported by Environment Canada and Avalon Rare Metals Inc. **Tyler Weinhardt** is studying the impacts of suspended sediments on fish. His work is part of a larger project assessing the massive slumps that are occurring on the Peel River plateau of the Northwest Territories due to climate change.

Over in the Wood lab, in September, (**Dr**.) **Hassan Al-Reasi** successfully defended his Ph.D. thesis at McMaster University entitled: "Quantifying the direct and indirect effects of dissolved organic matter (DOM) on aquatic organisms: Interactions with pH and quality measures." Hassan was jointly supervised by Chris Wood (McMaster U.) and Scott Smith (Wilfrid Laurier U.). Hassan is going back to Oman to take up an Assistant Professor Position at Sultan Qaboos University. Congratulations Hassan! **Dr. Kevin Brix**, fresh from his Ph.D under the direction of Dr. Martin Grosell at the University of Miami, will be joining the Wood lab as a Postdoctoral Fellow in November. Kevin has an extensive background in metals research, and will be doing studies on both basic and applied problems at McMaster. Congratulations on your Ph.D. Kevin, and welcome to Canada!

Finally, Grant McClelland's undergrad. **Daniel Li**, left the lab in May to start a M.Sc. for John Peever at University of Toronto on the neurobiology of sleep.

New Funding:

- **K. Wilkinson,** along with **C. Fortin, J. McGeer, S. Smith** and **P. Campbell** (supporting organizations and collaborators include Avalon Rare Metals Inc., Environment Canada, Natural Resources Canada and PerkinElmer Canada Inc.) have just received a NSERC strategic grant to study the aquatic bio-geochemistry and impacts of rare-earth and platinum group elements.

- **G. Scott, G. McClelland** and **C. Wood** have received a NSERC RTI for swim tunnel respirometers for studying the effects of environmental stress on fish, which will allow them to study swimming energetics in fish of a variety of sizes and under a variety of conditions.

Upcoming Conferences and Meetings:

The 33rd Annual SETAC North America Meeting takes place at Long Beach, California, from 11th – 15th November, 2012.

*There will be a special marine metals session this year at SETAC entitled the "**Fate and Effects of Metals: Marine Concerns**", which will be chaired by Scott Smith, myself and Jim McGeer. The session runs from 8:00am to 11:50am in room 201A on Tuesday the 13th. The respective posters are also on the same day in the Exhibit Hall.*

This session will focus on basic saltwater-specific bioavailability research, physiological mechanisms, chemistry (speciation) and the role of organic matter and salinity on metal toxicity to a wide range of organisms found in estuarine and marine environments. Accepted papers include (presenter in bold):

- **Cooper, C.**, Tait, T., McGeer, J., Santore, R., Smith, S. Influence of salinity and DOC on acute Cu toxicity to the rotifer *Brachionus plicatilis*. (Platform 249. 8:50am-9:10am)

- **Tellis, M.**, Lauer, M., Nadella, S., Bianchini, A., Wood, C. Toxic physiological effects of Pb, Zn, Cu and Ni on early life stages of the sea urchin (*Strongylocentrotus purpuratus*). (Platform 251. 10:15am-10:35am)

- Lauer, M., Cavicchioli Azevedo, V., **Bianchini, A.** Is energy metabolism impairment the unifying response to copper exposure in marine invertebrates? (Platform 253. 11:05am-11:25am)

There are also a number of other metal bioavailability orientated sessions. The following platforms and posters will be presented by people associated with Laurier and McMaster. Presenters are in bold, also included are session names, dates, times and locations (in chronological order):

- **Santore, R.**, Ryan, A. C., Arnold, R., Bianchini, A., Rosen, G., Cooper, C., Delos, C. A Biotic Ligand Model based revision to the U.S. water quality criteria for copper in saltwater for marine and estuarine organisms. (In *"Fate and effects of metals: Regulatory and risk assessment perspective"*. Tues. 13th. Grand Ballroom. Platform 285. 5:00pm)

- Diamond, R., **Smith, S.** Nadella, S., Bianchini, A., Wood, C. Saltwater Pb speciation and toxicity for Mytilus embryo tests in the presence of various sources and concentrations of organic matter. (In *"Marine sediment and water toxicity evaluations"*. Tues. 13th. Exhibit Hall. Poster TP126)

- **Tait, T.**, Smith, S. Flow-through ISE Determination of free Cu in sea water and influence of DOC source on free Cu and toxicity to *Brachionus plicatilis*. (In *"Marine sediment and water toxicity evaluations"*. Tues. 13th. Exhibit Hall. Poster TP131)

- Avila, T., Furci, B., Abel Machado, A., **Bianchini, A.** Influence of seawater-derived organic matter on acute Zn toxicity in the copepod *Acartia tonsa* in a wide range of salinities. (In *"Marine sediment and water toxicity evaluations"*. Tues. 13th. Exhibit Hall. Poster TP132)

- Nasir, R., Cunningham, J., Smith, S., **McGeer, J.** Effect of salinity and natural organic matter on the toxicity of Cu to *Americamysis bahia*. (In *"Marine sediment and water toxicity evaluations"*. Tues. 13th. Exhibit Hall. Poster TP133)

- Al-Reasi, H., **Smith, S.**, Wood. C. Characterization of freshwater natural dissolved organic matter: quality perspectives for direct and indirect interactions with organisms. (In *"Fate and effects of metals: aquatic biological perspectives"*. Wed. 14th. Room 102A/B. Platform 397. 8:00am)

- **Livingstone, K.**, McGeer, J. Does ecosystem disturbance influence the toxicity mitigation quality of natural organic matter? (In *"Fate and effects of metals: aquatic biological perspectives"*. Wed. 14th. Room 102A/B. Platform 398. 8:25am)

- **Costa, E. J.**, McGeer, J. Mechanism of nanoparticle silver accumulation in *Daphnia pulex*. (In *"Assessing the risks of nanosilver in the environment"*. Wed. 14th. Room 101B. Platform 394. 10:40am)

- Chan, K., **McGeer, J.** Mitigation of acute and chronic Ni toxicity to *Hyalella azteca* by Ca and natural organic matter in very soft waters. (In *"Fate and effects of metals: aquatic biological perspective - BLM"*. Wed. 14th. Room 102A/B. Platform 495. 3:10pm)

- **Machado, A.**, Wood, C., Bianchini, A., Gillis, P. Using a suite of biomarkers and bioindicators to assess the effects of complex contaminant mixtures on chronically exposed wild freshwater mussels. (In *"Linking mechanism of action to physiological and ecological effects in aquatic species"*. Wed. 14th. Exhibit Hall. Poster WP017)

- **Vukov, O.**, McGeer, J. Developing a site specific understanding of the toxicity of rare earth elements to *Daphnia pulex* and *Hyalella azteca*. (In *"Fate and effects of metals: aquatic biological perspectives"*. Wed. 14th. Exhibit Hall. Poster WP052)

Finally, there's a Metals Advisory Group Reception scheduled for 6:00pm-9:00pm (Mon. 12th. Room 202A)

The following peer reviewed papers and book chapters were published by the Metals Bioavailability Group (May 2012 – Nov. 2012):

- Al-Reasi, H. A., Smith, D. S., Wood, C. M. (2012). Characterization of freshwater natural dissolved organic matter (DOM): mechanistic explanations for protective effects against metal toxicity and direct effects on organisms. *Environ. Internat.* (In revision).

- Cooper, C. A., Tait, T., Gray, H., Webster, G., Santore, R. C., McGeer, J.C., Smith, D. S. (2012). Influence of salinity and dissolved organic carbon on acute Cu toxicity to the rotifer *Brachionus plicatilis. Environ. Sci. Technol.* (Completed draft under internal review).

- McClelland, G.B., Scott, G.R. (2013) Muscle Plasticity. In: Evans, D.H., and Currie, S. (ed.) *The Physiology of Fishes*, 4th Ed.

- Nadella, S. R., Tellis, M., Diamond, R. L., Smith, D. S., Bianchini, A., Wood, C. M. (2012) Toxicity of Pb and Zn to developing mussel and sea urchin embryos: dissolved organic matter and salinity effects, and critical tissue residues (completed draft under external review).

- Ransberry, V. E., McClelland, G. B. (2012) Effects of Acute Waterborne Copper Exposure and Hypoxia on the oxidative stress response in freshwater-adapted killifish, *Fundulus heteroclitus. Aquat. Tox.* (In Preparation).

- Tellis, M. S., Lauer, M. M., Nadella, S., Bianchini, A., Wood, C. M. (2012). Ionic status, calcium uptake, and Ca^{2+}-ATPase activity during early development in the purple sea urchin (*Strongylocentrotus purpuratus*). *J. Exp. Mar. Biol. Ecol.* (submitted).

- Zimmer, A. M., Barcarolli, I. F., Wood, C. M., Bianchini, A. (2012). Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both freshwater and seawater. *Aquat. Tox.* 122-123: 172-180.

Scientific tit-bits:

- **Tamzin Blewett, Som Niyogi and Chris Wood:**

Salinity-dependence of waterborne Ni and Zn uptake and toxicity *Carcinus maenas* was investigated. Crabs were exposed to a range of environmentally-relevant levels of Ni and Zn at five different salinities (100%, 80%, 60%, 40%, 20% SW). Although preliminary, results indicated that the primary site of uptake for both Ni and Zn was the gill, and anterior and posterior gills demonstrated different accumulation patterns. Gill perfusion studies with isolated gills confirmed this finding. Other tissues displayed only low levels of accumulation. Salinity appeared to have only a moderate influence on metal accumulation. Ni and Zn accumulation were measured in various tissues, and tissue and haemolymph samples were preserved for subsequent ionoregulatory, acid-base, enzymatic, and oxidative stress measurements. These assays will be conducted in upcoming months.

- **Jessie Cunningham, Oliver Vukov, Rabia Nasir and Jim McGeer:**

In July and August this crack team of scientists conducted a chronic flow through test for the sublethal effects of Pb on mysids held at 25 ppt salinity. Endpoints included growth and reproduction as well as survival. At high exposure concentrations the survival of adults was reduced. The accumulation of Pb in surviving individuals was assessed after 28 d of exposure and therefore it may be possible to examine links between accumulation and effects.

- **Tara Tait and Scott Smith:**

Tara has tested cupric ion-selective electrode (ISE) protocols based on published methods. The published ISE method involves external calibration against strong metal buffer solutions and we were able to measure free copper in the 10^{-10} M and lower range. Challenges in the development of this method included reproducibility (up to 4 orders of magnitude variation in some free Cu measurements) and this is consistent with previously observed reproducibility of ISE in the literature. Tara has developed a novel internal calibration method that dramatically reduces variability to within approximately half an order of magnitude or better. She is now able to recover known speciations using titrations of 10 μM tryptophan as a model ligand with total copper in the range ≥5 μg/L. Her cupric ISE measurement methods are being used to support exposure characterization for the invertebrate LC50 tests.

Research Highlight:

Effects of acute waterborne copper and hypoxia on the oxidative stress response in freshwater-adapted killifish, *Fundulus heteroclitus*

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Abstract

Although oxidative stress can be induced in fish by copper (Cu) and hypoxia exposure alone, few studies have investigated the combined physiological and toxicological effects of these environmentally relevant stressors. In this study, we examined whether Cu exposure reduced the ability of *Fundulus heteroclitus* to cope with hypoxia, and whether the combined effects of Cu and hypoxia act synergistically or antagonistically to induce changes in oxidative stress and mitochondrial oxidative capacity in adult *F. heteroclitus*. Freshwater-acclimated *F. heteroclitus* were exposed to sublethal (25 μg/L) waterborne Cu and hypoxia (2.3 mg O_2/L) alone, or combined Cu and hypoxia (25 μg Cu/L + 2.3 mg O_2/L) for 96 h. Hypoxia had no effect on Cu accumulation in all measured tissues. Cu alone and in combination with hypoxia significantly increased catalase (CAT) enzyme activity in the gill, associated with increased Cu load. In contrast, Cu exposure alone and combined with hypoxia significantly decreased CAT in liver. Combined Cu and hypoxia exposure significantly decreased liver cytochrome *c* oxidase (COX), suggesting that the combination of the two stressors impaired oxidative capacity of the inner mitochondrial membrane. Hypoxia alone significantly decreased gill protein carbonyl content. In addition, hypoxia appeared to have an antagonistic effect on copper-induced lipid peroxidation in the liver. The results of our study provide important insight in determining the interacting effects of Cu and hypoxia in a model euryhaline teleost.

Introduction

Many coastal ecosystems currently suffer from eutrophication, resulting in hypoxic conditions due to an increased biological oxygen demand. Although natural causes can trigger the eutrophication it has become much more widespread due to anthropogenic influences. Moreover, increased industrial activities in coastal regions can increase waterborne metal concentration. Thus, marine organisms are likely exposed to multiple stressors acting in additive, synergistic or antagonistic ways. For example, it is important to consider Cu exposure in areas experiencing hypoxia, since the interaction of these environmental stressors may result in synergistic physiological and/or toxicological effects.

Copper, a trace metal is an essential micronutrient, but at high concentrations can be toxic to aquatic organisms due to its highly reactive nature. Cu levels, range from 0.06 to 17 μg/L in coastal ecosystems (Klinkhammer and Bender, 1981; van Geen and Luoma, 1993; Kozelka and Bruland, 1998) but has considerably increased in some regions.

Exposure to excess Cu in freshwater fish has been shown to result in loss of appetite, growth suppression, ionoregulation and endocrine disruption, lower aerobic capacity and higher mortality (Lauren and McDonald, 1987; McGeer *et al*., 2000; Taylor *et al*., 2000). The negative effects that occur are in part due to the redox nature of Cu and the metal's high reactivity with hydrogen peroxide (H_2O_2) that can cause rapid generation of reactive oxygen species (ROS) (Harris and Gitlin, 1996). Hypoxia can also induce oxidative stress and under these conditions, fish either: 1) reduce their metabolic rate and/or 2) shift in total metabolism from aerobic to anaerobic metabolism (Hochachka et al 1996, 1997, Storey 1998, Virani and Rees, 2000). Hypoxia-inducible factor (HIF) regulatory proteins are crucially involved in maintaining oxygen homeostasis, including adaptive responses, such as decreased metabolic rate and increased anaerobic metabolism as mentioned previously, against hypoxic stress (Semenza, 2001). Under hypoxic conditions, HIF-1 α is stabilized and translocated to the nucleus where it binds specifically to regulatory regions of over 70 target genes, known as the hypoxia-responsive element (HRE), altering rates of hypoxia-inducible genes and activities of their protein products to help sustain supply of O_2 to tissues (Bunn and Poyton, 1996; Kaluz *et al.*, 2008; Rocha, 2007; Semenza, 1999; Wenger, 2002).

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

Few studies have addressed physiological effects and toxicity of combined Cu and hypoxia in marine organisms. Despite the increasing prevalence of anthropogenic sources contaminating environments with both organic matter and metals, making the combination of Cu and hypoxia a likely phenomenon. Since Cu and hypoxia alone have demonstrated to cause increased ROS generation and induce oxidative stress in fish, the goal of this study was to investigate whether Cu exposure reduces killifish's ability to cope with hypoxia, and whether the combined effects of copper and hypoxia at environmentally relevant concentrations, will act antagonistically or synergistically to induce changes in the oxidative stress response and mitochondrial oxidative

capacity. We use the euryhaline teleost killifish (*F. heteroclitus*) to test the hypothesized that the combination of sublethal Cu and hypoxia would synergistically magnify the oxidative stress response, as well as decrease oxidative capacity in killifish. To evaluate the oxidative stress response we measured protein carbonyl content and TBARS levels, as an index of lipid peroxidation, to indicate the extent of oxidative damage. We also measured changes in the activities of ROS scavenging enzyme catalase and superoxide dismutase to assess antioxidant defenses. Tissue oxidative capacity was determined by changes in enzymatic activity of cytochrome oxidase COX and citrate synthase CS, as well of the ratio of the two to assess mitochondrial dysfunction. The results of this study will also help further our understanding of how stressors may interact with each other.

Materials and Methods

Experimental Animals

Adult killifish of mixed sex (1.11-6.08 g) were collected from the wild (Aquatic Research Organisms, Hampton, NH, USA) and acclimated to freshwater (FW) in static, aerated, 45-liter tanks with water renewed daily, for a minimum of 2-weeks prior to experimentation. During acclimation, fish were fed daily with a commercial tropical fish food (and maintained under natural photoperiod (12:12-h light:dark) at approximately 20°C. Killifish were fasted for 48 hours prior to the start of experiments and throughout the 96-hour exposure.

Copper and Hypoxia Exposure

Killifish (n = 192) were removed from their freshwater tanks and placed in static, aerated, 8-liter tanks (n = 12 per tank). Killifish were either exposed to normoxic water (100% O_2 saturation, n = 48), normoxic water (100% O_2 saturation, n = 48) plus 25 μg L^{-1} Cu, hypoxic water (~25% O_2 saturation, n = 48), or hypoxic water (~25% O_2 saturation, n = 48) plus 25 μg L^{-1} Cu. Approximately 12 fish were tested per treatment, with four replicate tanks, for a total of 48 fish per treatment. A daily 80% renewal of water was performed, with static renewal water prepared 24-hours in advance and thoroughly mixed prior to entering tanks. Water samples were taken prior to each water replacement, filtered through a 0.45 μm filtration disc (Pall Life Sciences, East Hills, NY, USA). Water samples were used to measure the following water chemistry parameters: DOC, cations and pH. Normoxic water was kept fully oxygen-saturated by means of an airstone. For hypoxic treatments, tanks were covered with plastic wrap and dissolved oxygen (DO) concentration of the water was lowered by bubbling a mixture of compressed nitrogen (N_2) gas and air in the correct combination using mass flow controllers to achieve ~25% O_2 saturation (Virani and Rees, 2000). DO concentration was monitored three times a day using a microcathode oxygen electrode (Strathkelvin Model 1302). The oxygen sensor was calibrated with air-saturated water (100% of air saturation) and a zero oxygen solution.

Sampling

After 96-hours, killifish were quickly euthanized by cephalic concussion. Killifish were sampled for gill and liver tissues. Tissue samples and the remaining carcass were weighed and then immediately frozen in liquid nitrogen for further analysis of copper level, enzymatic activity and oxidative damage. blood was collected from the dorsal aorta by severing the tail behind the anal fin and collected in heparinized capillary tubes. Blood samples were kept on ice until centrifuged using a microhematocrit centrifuge (International Equipment Company, Needham Heights, MA, USA) at 14,000 *g* for 2 min to determine hematocrit value.

Tissue and Water Analysis

Copper concentration in tissue and water samples were measured by Graphite Furnace Atomic Absorption Spectroscopy (GFAAS, Spectra AA 220Z, Varian Palo Alto, CA). Tissue and water ion composition were measured by Flame Atomic Absorption Spectroscopy (Spectra AA 220FS, Varian, Inc., Mulgrave, Victoria, Australia) and verified with Na^+, K^+, Mg^{2+} and Ca^{2+} standards (Fisher Scientific, Ottawa, ON, CA). Tissue samples were diluted as necessary with 1% HNO_3 (Na^+), 1% $LaCl_3$ in 1% HNO_3 (Ca^{2+}, Mg^+) or 0.1% $CsCl_2$ in 1% HNO_3. Total DOC concentration of water samples was measured using a Shimadzu TOC-VCPH/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan).

Protein Carbonyl Content

Protein carbonyl content was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI) and performed as described by Craig *et al.* (2007). Protein concentration of the tissue homogenate was assayed according to Bradford (1976), compared with a bovine serum albumin (BSA) standard curve and measured in a 96-well plate at 565 nm.

Thiobarbituric Acid Reactive Substances

Malondialdehyde (MDA) concentrations, a product of lipid peroxidation, was determined using the thiobarbituric acid reactive substances (TBARS) Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Tissue was sonicated in 250 µl of homogenization buffer (100 mM Tris-HCl, 2 mM EDTA, 5 mM $MgCl_2 \cdot 6H_2O$, pH 7.75) containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor, and then centrifuged. Sodium dodecyl sulfate (SDS) solution was added to solutions, followed by a mixture of thiobarbituric acid (TBA), acetic acid and sodium hydroxide. Samples were placed into boiling water for 1 h, immediately removed and incubated on ice, then centrifuged. Then, supernatant was loaded into a black 96-well plate, and fluorescence was measured at an excitation and emission wavelength of 530 and 550 nm, respectively, using a Spectramax fluorescence microplate reader (Molecular Devices, Menlo Park, CA, USA). Protein concentration of the tissue homogenate was assayed as previously stated to yield TBARS levels in micromolar MDA per milligram protein.

Enzyme Activity

Frozen gill and liver tissues were powdered in liquid nitrogen with a mortar and pestle, then diluted 1:20 (mg tissue:µL) 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 SpectraMax Plus 384 spectrophotometer (Molecular Devices, Menlo Park, CA, USA). Assays were performed in triplicate, with an additional negative control well lacking substrate, to correct for background activity, as previously described (McClelland *et al.* 2005). All chemicals used were purchased from Sigma Aldrich (Oakville, ON, CA) and reaction buffers were prepared fresh daily. All enzyme activity is reported as units per milligram protein. Catalase (CAT), superoxide dismutase (SOD), cytochrome oxidase (COX) and citrate synthase (CS) activity was measured, as previously described by Craig *et al* (2007).

Statistical Analysis

All data have been expressed as means ± SEM. One-way analysis of variance (ANOVA) followed by a post hoc Fisher's least significant difference (LSD) test were performed to evaluate potential differences between treatment groups. Gill protein carbonyl content data were normalized by log transformation prior to ANOVA. If conditions

to perform an ANOVA were not fulfilled (i.e., data were not normally distributed or did not have equal variances), the Kruskal-Wallis ANOVA on ranks and Dunn's tests were performed. For all tests, a *p-value* ≤ 0.05 was considered statistically significant. All statistical analysis was performed using SigmaStat 3.5 (Chicago, IL, USA).

Results

Cu accumulation

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

Oxidative damage

Despite increased concentrations of Cu in the gill associated with Cu exposure alone and in combination with hypoxia, no significant increases in gill protein carbonyl content were observed (Figure 1A). Hypoxia significantly decreased gill protein carbonyl content in fish exposed to hypoxia alone (~-61%, $p = 0.018$) compared to controls. When exposed to hypoxia in combination with Cu gill protein carbonyl content tended to decrease (~-38%) compared to controls, but was not significant. Results generally showed protein carbonyl content was higher in the gill, ranging from 0.37 to 7.93 nmol mg protein^{-1} compared to 0.09 to 2.41 nmol mg protein^{-1} in the liver. Neither copper nor hypoxia exposure had any effect on protein carbonyl content in the liver (Figure 1B).

In contrast to protein carbonyl content results, TBARS increased by as much as 2.8-fold in response to single and combined Cu and hypoxia exposure in the gill and liver of killifish, but was not significant (Figure 1). Within the gill, exposure to Cu (2.8-fold) and hypoxia (2.5-fold) alone, as well as in combination (2.9-fold) tended to increase TBARS relative to the controls (Figure 1C). The same trend was not observed in the liver; Cu exposure alone increased TBARS levels 2.1-fold, while hypoxia alone and in combination with Cu induced TBARS levels similar to controls (Figure 1D).

Catalase activity

In the gill, Cu alone and in combination with hypoxia induced a significant two ($p=0.004$) and 2.2-fold ($p=0.002$) increase in catalase activity, respectively, compared to controls (Figure 2A). In contrast, there was a significant decrease in catalase activity in the liver of fish exposed to Cu (-26.06%, $p=0.05$) and a combination of Cu and hypoxia (-36.12%, $p=0.006$, Figure 2B). We observed hypoxia decreased liver catalase activity by 21.34%, but the decrease was not significantly different from controls ($p=0.10$). Liver catalase activity was approximately tenfold higher than in the gill.

Superoxide dismutase activity

Copper, hypoxia nor the combination of the two had an effect on superoxide dismutase activity in the gill and liver of killifish (Figure 2C,D). Although activity was observed to be slightly higher in the gill ranging from 0.36 to 1.13 U mg protein^{-1} compared to 0.09 to 0.78 U mg protein^{-1} in the liver.

Cytochrome c and citrate synthase activity

Copper and hypoxia alone and in combination, did not induce changes in cytochrome *c* or citrate synthase activity in the gill compared to controls (Figure 3A, 3C). Similarly, there was no change in the gill COX-to-CS ratio between treatment groups (not shown). Exposure to hypoxia alone tended to increase liver CS activity, but was

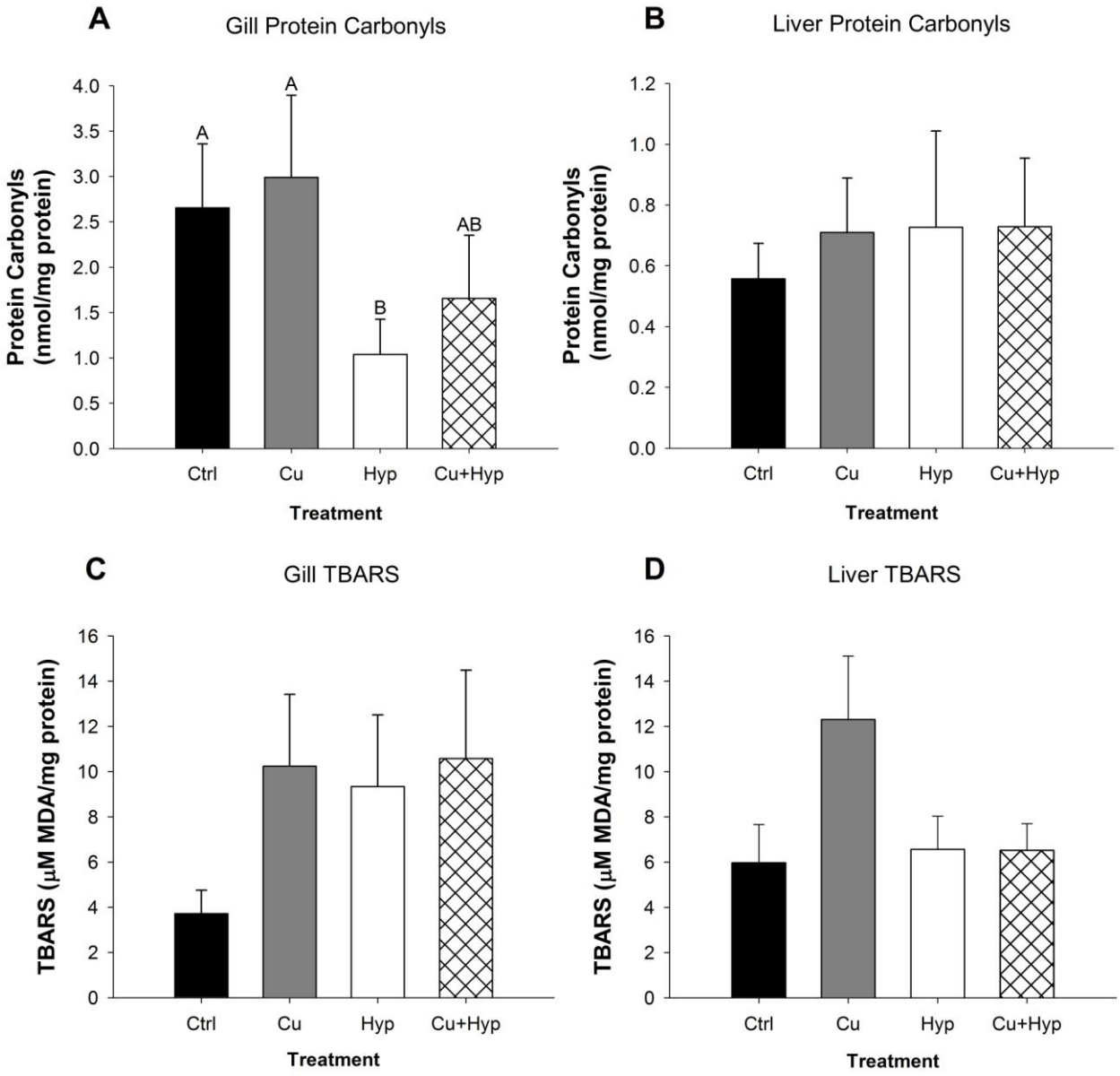

Figure 1. Protein carbonyl content (nmol/mg protein) in the gill (A) and liver (B) and TBARS content (μM MDA/mg protein) in the gill (C) and liver (D) of killifish exposed to copper (Cu), hypoxia (Hyp) or a combination of both (Cu+Hyp). Values are presented as means ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 8$ for all treatments).

not significant (Figure 3D). However, we observed a significant decrease in liver COX activity in fish exposed to combined Cu and hypoxia relative to controls (*p=0.003*, Figure 3C). Despite decreased liver COX activity of killifish exposed to Cu in combination with hypoxia, there was no significant change in the COX-to-CS ratio in the liver between treatment groups (not shown). It should be noted, although not significant, Cu and hypoxia alone tended to decrease the COX-to-CS ratio in the liver, and the combination of Cu and hypoxia showed a synergistic effect in decreasing the COX-to-CS ratio.

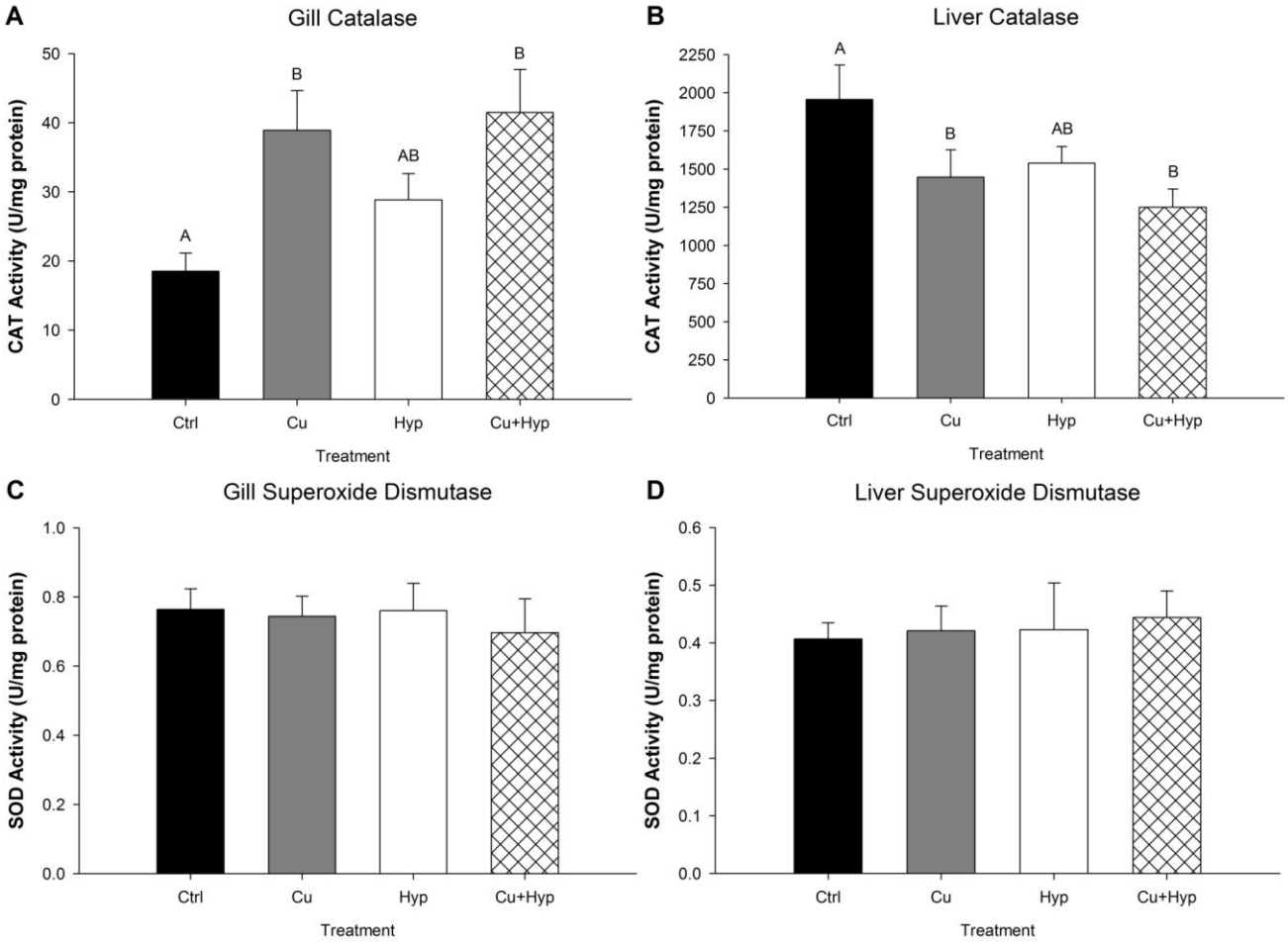

Figure 2. Catalase activity in the gill (A) and liver (B) and superoxide dismutase (SOD) activity (U/mg protein) in the gill (C) and liver (D) of killifish exposed to copper (Cu), hypoxia (Hyp) or a combination of copper and hypoxia (Cu+Hyp). Values are presented as means ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 8$ for all treatments).

Discussion

The present study demonstrated that combined exposure to Cu and hypoxia for 96 h significantly reduced of the inner mitochondrial membrane COx content in the liver of adult killifish. Acute Cu exposure alone and in combination with hypoxia also induced significant increased in catalase activity in the gill. In addition, hypoxia alone significantly decreased gill protein carbonyl content, while Cu alone and in combination with hypoxia resulted in a non-significant increase in gill lipid peroxidation. We observed that hypoxia had an antagonistic effect on copper-induced lipid peroxidation in the liver, however this trend was not substantiated across our tests. Therefore, it remains inconclusive whether Cu and hypoxia work antagonistically or synergistically to induce changes in the oxidative stress response and oxidative capacity in killifish. However, the results of our study provide important insight into the combined effects of Cu and hypoxia and the biochemical and physiological responses they elicit in an euryhaline species.

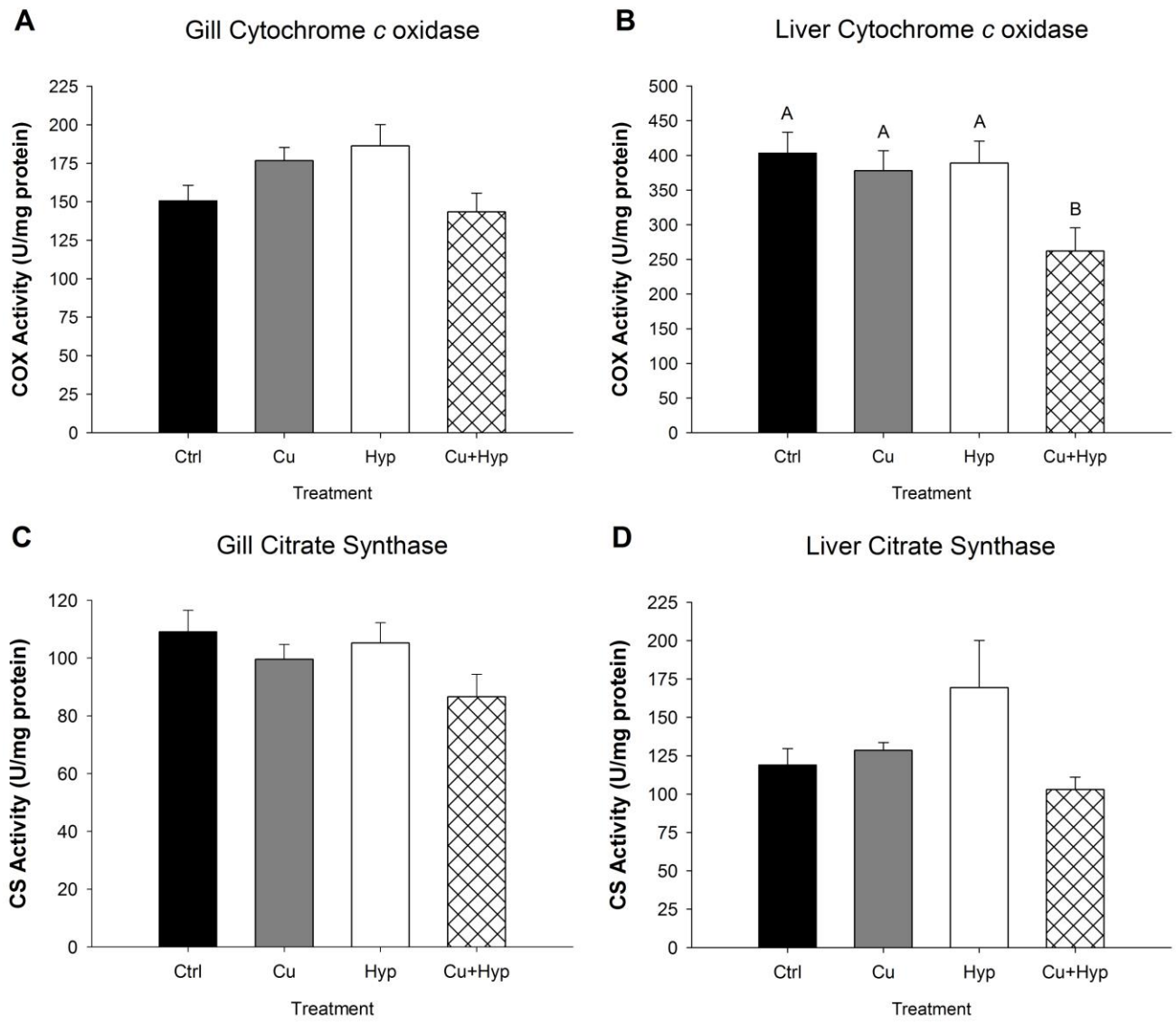

Figure 3. Gill (A) and liver (C) COX activity (U/mg protein) and gill (B) and liver (D) citrate synthase (CS) activity of killifish exposed to copper (Cu), hypoxia (Hyp) or a combination of copper and hypoxia (Cu+Hyp). Values are presented as means ± SEM. Values that do not share the same letter indicate a significant difference ($p \leq 0.05$, $n = 8$ for all treatments).

Antioxidant Responses

Copper-induced ROS formation is well documented among fish species, most notably occurring in the gill (Bopp *et al.*, 2008) and liver cells (Manzl *et al.*, 2004). While counterintuitive, hypoxia has also been observed to increase ROS production (Chandel *et al.*, 2000; Lushchak, 2011, Lushchak *et al.*, 2005). Fish utilize enzymatic antioxidant defense mechanisms, such as CAT and SOD, to combat ROS production eliminating excess ROS, thus preventing oxidative damage. The activities of SOD and CAT are mutually dependent upon one another (Cooper *et al.*, 2002); however, in the present study we did not observe parallel changes in the activity of SOD and CAT.

We found that gill CAT activity

increased in response to Cu alone and in combination with hypoxia, indicating the activation of defensive mechanisms to help mitigate oxidative damage. However, it appears combined Cu and hypoxia did not elicit a synergistic effect, which may suggest that a maximum threshold response of activity exists. In contrast, we observed significant decreases in CAT activity in the gill of killifish exposed to hypoxia and in the liver of killifish exposed to Cu alone and in combination with hypoxia. Although, hypoxia can induce ROS production, hypoxia also tends to suppress metabolism, which has been suggested to affect both protein synthesis and xenobiotic processing, such as reducing CAT activity (Lushchak *et al.*, 2005). Reduced CAT activity in killifish exposed to Cu alone and in combination with hypoxia may be due to excessive ROS, as it has been shown that ROS can inactivate CAT (Halliwell and Gutteridge, 1989).

There were no changes in gill and liver SOD activity observed, however constitutive expressed levels may be sufficient to protect against Cu and hypoxia induced oxidative stress. Sampaio et al. (2008) observed significant increases in SOD activity in the liver of *Piaractus mesopotamicus*, while CAT activity significant decreased in response to Cu exposure alone and in combination with hypoxia. It appears that the relationship between the antioxidant defenses SOD and CAT may be complicated; therefore we would caution the use of single biomarkers as an indicator of oxidative stress response.

Oxidative Damage

Gills are the primary target organ of Cu toxicity, particularly in freshwater, due to the direct contact with the external environment (Brungs et al., 1973; Buckley et al 1982; Stagg and Shuttleworth, 1982). We found significant Cu accumulation in the gill of killifish exposed to Cu alone and in combination with hypoxia. The addition of hypoxia had no effect on accumulation, which agrees with results reported by Pilgaard et al. (1994) and Hughes and Flos (1978). Exposure to Cu alone and in combination with hypoxia in the gill appeared to induce lipid peroxidation, despite the activation of CAT to combat H_2O_2 derived from O_2^-. In contrast, single Cu and combined Cu and hypoxia was insufficient to induce gill protein carbonylation. The accumulation of protein carbonyls in response to Cu appears to be both time- and dose-dependent, as demonstrated in zebrafish (*Danio rerio*, *(*Craig et al (2007). Consequently, the exposure time and level of copper used in the present study may not have been sufficient to induce protein carbonylation. The results of our study suggest lipid peroxidation is a more sensitive indicator of acute Cu-induced oxidative stress than protein carbonyls. In agreement with this observation are the results of Loro et al. (2012), in which gill lipid peroxidation showed the greatest change in response to zinc exposure compared to protein carbonyl levels.

To our surprise, we found that hypoxia had opposite effects in killifish gills in terms of protein carbonylation and lipid peroxidation. Hypoxia significantly decreased gill protein carbonyls, while gill lipid peroxidation tended to increase. Similarly, Lushchak et al. (2005) observed lipid peroxidation assessed as TBARS increased and protein carbonyl content decreased or remained unchanged during hypoxia, and only after normoxic recovery did protein carbonyls accumulate in the liver of common carp, *Cyrinus carpio*. Oxygen concentration has variable effects on the oxidative stress response; hyperoxia clearly induces oxidative stress in different fish species, while hypoxia is known to induce oxidative stress as well as decrease ROS production (review Lushchak, 2011). However, the observe response may be a preparation to cope with oxidative stress

upon recovery rather than the hypoxia per se, a process known as the "preparation to oxidative stress" hypothesis (Hermes-Lima *et al.*, 1998). When Cu exposure was combined with hypoxia, gill protein carbonyls also tended to decrease compare to controls. 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 cope with normoxia is impaired.

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

Oxidative capacity

Due to Cu's essential role in mitochondrial function, the mitochondrion is a major target for Cu-toxicity, acting through oxidative mitochondrial membrane damage and the "poisoning" of enzymes of the tricarboxylic acid cycle (TCA) and energy metabolism (Arciello et al. 2005; Sheline and Choi, 2004). The enzyme activities of CS, a key enzyme in TCA cycle, and COX, a key component of the electron transport chain, can be used as indicators of mitochondrial density and mitochondrial inner membrane, respectively (Capkova et al. 2002). In our study no significant differences in the activities of gill CS and COX were found between single and combined Cu and hypoxia exposure, which was consistent with the results of Cooper *et al.* (2002) where they observed no change in gill CS induced by hypoxia in spot fish (*Leiostomus xanthurus*) and Lauer *et al.* (2012) in which Cu exposure did not induce changes in gill COX activity in crab (*Neohelice granulate*).

We observed a significant decrease in liver COX activity in killifish exposed to Cu in combination with hypoxia. A reduction in COX activity after exposure to hypoxia alone could be explained by the inhibition of electron transport (Chandel *et al.*, 1995). We expected CS activity to decrease in killifish exposed to hypoxia, as a reduction in activity has been observed to correlate with a reduction in oxygen consumption rates in several fish species (Yang and Somero, 1993), as well as the tendencies of hypoxia to increase the usage of glycolytic pathways while decreasing the use of aerobic pathways (Cooper *et al.*, 2002). Conversely, we observed hypoxia increased liver CS activity, which suggests mitochondrial density increased in response to hypoxia. However, no change in liver CS activity has been observed in spot fish (Cooper *et al.* 2002), gilthead sea bream (Perez-Jimenez *et al.*, 2012), carp (Zho *et al.*, 2000). Therefore, it appears the effect of hypoxia on the CS activity is quite variable.

Reductions in the ratio of COX/CS can also be used as a biochemical marker of mitochondrial dysfunction. In the gill, no change in ratio was observed. While in the liver the combination of Cu and hypoxia induced significantly decreased COX activity, it did not significantly affect the COX/CS

ratio compared to control. Therefore, it suggests that neither single nor combined Cu and hypoxia exposure induced mitochondrial dysfunction in adult killifish.

Conclusions

We showed that Cu exposure alone and in combination with hypoxia results in differences in enzymes involved in antioxidant protection and aerobic metabolism in a tissue- specific manner. In addition, hypoxia alone may have an antagonistic effect on copper-induced oxidative damage, as well as decrease oxidative damage in general. It is important to note, however, that the antagonistic or synergistic effect of combined Cu and hypoxia on the oxidative stress response may have been masked by individual biological variation in our data. It is clear from this study along with many others that variation in antioxidant enzyme activity is common in fish (e.g. Lushchak, 2011; Virani and Rees, 2000). Therefore, we suggest future studies will need to expose fish chronically to environmentally relevant concentrations versus acutely, as well as increase Cu and hypoxia exposure levels that surpass environmentally relevant concentrations to clearly elucidate the combined effects of Cu and hypoxia on biochemical and physiological markers of oxidative stress.

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An amoeba with glasses: o.o

And one watching the tele: . []