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Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*)

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Craig PM, Wood CM, McClelland GB. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). *Am J Physiol Regul Integr Comp Physiol* 293: R1882–R1892, 2007. First published September 12, 2007; doi:10.1152/ajpregu.00383.2007.—In fish, environmental pollution is one factor that induces oxidative stress, and this can disturb the natural antioxidant defense system. Oxidative stress has been well characterized in vitro, yet the in vivo effects of metal-induced oxidative stress have not been extensively studied. In two experiments we examined the impacts of copper (Cu) on gene expression, oxidative damage, and cell oxidative capacity in liver and gill of zebrafish. In the first experiment, soft water-acclimated zebrafish were exposed to 8 and 15 $\mu\text{g/l}$ Cu for 48 h. This exposure resulted in significant increases in gene expression of cytochrome *c* oxidase subunit 17 (COX-17) and catalase, associated with both increased Cu load and protein carbonyl concentrations in the gill and liver after 48 h. In addition, we examined the potential protective effects of increased waterborne Ca^{2+} (3.3 mM) and Na^+ (10 mM) on acute Cu toxicity. While both treatments were effective at reducing liver and/or gill Cu loads and attenuating oxidative damage at 48 h, 10 mM Na^+ was more protective than 3.3 mM Ca^{2+} . There were variable changes in the maximal activities of COX and citrate synthase (CS), indicating possible alterations in cell oxidative capacity. Moreover, Cu affected COX-to-CS ratios in both gill and liver, suggesting that Cu alters normal mitochondrial biogenic processes, possibly through metallochaperones like COX-17. Overall, this study provides important steps in determining the transcriptional and physiological endpoints of acute Cu toxicity in a model tropical species.

gene expression; oxidative capacity; catalase; COX-17; protein-carbonyls

ZEBRAFISH HAVE LONG BEEN AN excellent model for developmental and reproductive biology due to their rapid rate of growth and clear, well-characterized embryonic development. Zebrafish are now becoming a popular model for aquatic toxicological and physiology studies (e.g., 8, 14, 43) due to their publicly available genome and ability to tolerate soft water (6, 14), a key requirement in testing toxic effects of metals without the interfering effects of other cations (47, 48). They also answer the need for a tropical species in comparative toxicology. Zebrafish are endemic to the Ganges River which flows through the Indian subcontinent where they are reported to face a wide variety of water chemistries (including moderately soft water) and a range of Cu concentrations from 6–39 $\mu\text{g/l}$ (2, 32, 59).

Copper (Cu) is an essential micronutrient for all organisms and, in the case of fish, is acquired by the gills from the surrounding water, as well as from the diet by the digestive

tract (29, 30). Naturally occurring levels of Cu found in lakes and rivers range from 0.2 to 30 $\mu\text{g/l}$, whereas areas influenced by anthropogenic sources (e.g., mining, industrial discharge, passage through Cu pipes) can exhibit levels ranging from 100 to 200,000 $\mu\text{g/l}$ in heavily mined areas (26, 54). Elevated aquatic Cu levels cause a range of negative effects on fish, including reduced growth (52), interference with whole body ionoregulation (34, 41), and endocrine disruption (60). Many of these responses are in part due to Cu's high reactivity with H_2O_2 and potential to undergo redox reactions to form reactive oxygen species (ROS), a process known as the Fenton reaction. The resulting cellular damage can be in the form of membrane lipid peroxidation, DNA damage, and protein carbonyl production (18). Like other organisms, fish combat elevated levels of ROS with protective ROS-scavenging enzymes, such as superoxide dismutase (SOD) and catalase (CAT) that convert the superoxide anions into H_2O_2 and further into H_2O and O_2 , respectively. Once these enzymes are overwhelmed by excessive ROS production, irreversible cellular damage and death can occur.

It is well established that Cu toxicity is most aggressive in the absence of any competitive ions, such as in soft water (45, 47, 48). Current theory suggests that the mechanisms for acute Cu toxicity in fish occurs mainly at the gills, which involves ionic Cu^{2+} outcompeting Na^+ at apical Na^+ channels and ultimately inhibiting the basolateral $\text{Na}^+\text{-K}^+\text{-ATPase}$, effectively inhibiting normal Na^+ uptake (23, 34, 66). With increased waterborne Na^+ concentrations, gill Cu uptake is reduced most likely due to this competition at the Na^+ uptake sites (23, 47, 48). However, there is evidence that elevated ambient Ca^{2+} concentrations can also effectively inhibit Cu accumulation (47, 58). Ca^{2+} appears to bind to the apical surface of the gill, thereby reducing membrane permeability and stabilizing tight junctions (28). Indeed the biotic ligand model (BLM; reviewed in Refs. 44 and 46), a relatively new regulatory model that is now being used to determine the allowable levels of Cu in freshwater, takes into account these protective effects of water chemistry, and is based on a fixed relationship between short-term gill Cu accumulation and ultimate toxicity. Many studies have examined the effects of metal toxicity on zebrafish (e.g., Refs. 1, 16, 35), yet none have examined the combined effects of protective ions and oxidative stress when fish are exposed to elevated copper. However, in preliminary experiments, we have found that 3.3 mM Ca^{2+} and 10 mM Na^+ have identical protective effects (80% inhibition) against short-term (3 h) gill Cu binding in zebrafish (as assessed by the accumulation of radiolabeled Cu in the gill

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tissue). Yet they had differential protective effects against lethality, where Cu in soft water + 3.3 mM Ca^{2+} is 11-fold more protective and Cu in soft water + 10 mM Na^+ is 25-fold more protective than Cu in soft water alone. There is, therefore, a need to explore the physiological, biochemical, and gene expression effects of these exposures to understand the nature of the toxic effects and this differential protection.

Although the gills are the main site of Cu uptake from the ambient medium, very little Cu accumulates within the gills after the initial exposure (22). In fact, Cu is exported from the gill cells to the blood, transported to the liver where it is incorporated into ceruloplasmin transcuprein, and also is bound to albumin. It is then exported in these forms for use by other parts of the body. In addition, in the liver, Cu is also sequestered by metal chaperone proteins, such as metallothionein, detoxified, and finally excreted via the bile (22, 39). With high ambient Cu concentrations, the pathological accumulation of Cu within the liver is inevitable, ultimately increasing ROS production and leading to hepatocyte death (36, 37). Prior to cell death, trout hepatocytes demonstrating elevated Cu levels exhibited cellular respiration rates, which were increased by 50% *in vitro*, although there was no impact on energy (ATP) production (36). Cu's impact on cellular respiration may be, in part, due to its role in the formation of cytochrome *c* oxidase (COX), the terminal enzyme of the electron transport chain. In vertebrates, COX is composed of 13-subunits, and a further 30 proteins are required for proper COX assembly (61). One of these proteins, COX-17, is of interest in particular, since it aids in the assembly of the Cu center of COX (12, 20). COX-17 is a metal chaperone that binds to free cytoplasmic-Cu, further transporting Cu to the mitochondria intermembrane space where it delivers Cu to Sco1 (another mitochondrial copper chaperone) for COX assembly (27). Excess Cu may have an impact on this pathway; however, to date no studies have examined this as a potential mechanism of Cu toxicity.

The goal of this study was to examine the *in vivo* effects of short-term sublethal Cu exposure on zebrafish to elucidate the physiological and transcriptional endpoints of acute toxicity. Additionally, we hypothesized that increased Ca^{2+} (3.3 mM) and Na^+ (10 mM) in the ambient medium would mitigate the deleterious effects of Cu on zebrafish but in a differential fashion. We examined the mRNA expression and enzyme activity patterns of SOD and CAT to assess the antioxidant defenses with Cu exposure. COX-17 mRNA expression and enzymatic activity of COX and citrate synthase (CS) were examined as indices of changes in the oxidative capacity in liver and gills with respect to increased Cu exposure. The results of this study will further our understanding of acute Cu exposure, especially in terms of gene expression and oxidative stress, and in a tropical species of genomic importance. These data can also be applied to improve regulatory models, such as the BLM (44, 46) that can predict both acute and chronic toxicity levels of Cu.

MATERIALS AND METHODS

Animals. Adult zebrafish of mixed sex (*Danio rerio*) were purchased from a local pet supply store (PetsMart, Canada) and acclimated to soft water (Na^+ , $115 \pm 3.3 \mu\text{M}$; Ca^{2+} , $51 \pm 1.0 \mu\text{M}$; Mg^{2+} , $26 \pm 1.6 \mu\text{M}$, Cu^{2+} , $1.8 \pm 0.5 \mu\text{g/l}$; pH 6.8) over a 7-day period in an aerated 40-liter aquarium as described previously (14). After acclimation, zebrafish were housed in multiple 3-liter self-cleaning

Aquatic Habitats (Apopka, FL) tanks racked in a soft water, recirculating, stand-alone Aquatic Habitats filtration system. Fish were fed daily with a commercial tropical fish food (Topfin, Phoenix, AZ) and maintained on a 12:12-h light-dark photoperiod regimen. Zebrafish were fasted for 24 h prior to the beginning of experimentation. All procedures used were approved by the McMaster University Animal Research Ethics Board and conform to the principles of the Canadian Council for Animal Care.

Experiment 1. Zebrafish ($n = 120$) were removed from their recirculating tanks and placed in static, aerated, 3-liter Aquatic Habitats tanks ($n = 10$ per tank). Fish were either exposed to control soft water ($n = 40$), soft water plus $8 \mu\text{g/l}$ Cu ($n = 40$), or soft water plus $15 \mu\text{g/l}$ Cu ($n = 40$) using a concentrated Cu solution made from CuSO_4 dissolved in 0.05% HNO_3 , which had no measurable impact on water pH. Zebrafish were quickly euthanized by cephalic concussion (rather than terminal anesthesia, so as to avoid changes in indices of oxidative stress) after 1-, 4-, 24-, and 48-h exposure. Fish were sampled for gill and liver tissues, which were collected and immediately frozen in liquid N_2 for further analysis of protein carbonyls, Cu load, enzyme activity, and gene expression levels. At the time of sampling, a 10-ml water sample was taken from each tank, filtered through a $0.45\text{-}\mu\text{m}$ filtration disc (Pall, East Hills, NY), added to a plastic tube containing $100 \mu\text{l}$ HNO_3 and kept at 4°C for analysis of ion content and Cu concentration.

Experiment 2. After acclimation to soft water, zebrafish ($n = 40$) were then acclimated to soft water plus 3.3 mM CaCl_2 , or soft water plus 10 mM NaCl in separate 40-liter aquaria over a period of 14 days before the Cu exposures. Fish were placed in 3-liter static Aquatic Habitats tanks containing either soft water + 3.3 mM CaCl_2 or soft water + 10 mM NaCl , and exposed to $15 \mu\text{g/l}$ Cu for 48 h. Control tanks contained soft water, soft water + 3.3 mM CaCl_2 , or soft water + 10 mM NaCl , yet had no additional Cu added. Fish were sampled in the same manner as before, and tissues were immediately frozen in liquid N_2 for analysis. Water samples were also taken at each time point. Additionally, liver and gill samples were analyzed for Na^+ and Ca^{2+} tissue load.

Water and tissue ion and Cu levels. Gill and liver tissue was first digested in 1 ml of 1 N HNO_3 for 48 h at 60°C . Gill and liver digests were diluted 10 times, and dissolved Cu levels were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z; Varian, Palo Alto, CA) compared with a $40 \mu\text{g/l}$ Cu standard (Fisher Scientific, Ottawa, ON). Water samples were measured undiluted. Both tissue and water ion composition were measured by flame atomic absorption spectroscopy (Spectra AA 220FS; Varian) after 10 times dilutions were made with 1% HNO_3 (Na^+) or 0.5% $\text{LaCl}_3/1\%$ HNO_3 (Ca^{2+}), and verified using certified Na^+ and Ca^{2+} standards (1 mg/l diluted in 1% HNO_3 or 0.5% $\text{LaCl}_3/1\%$ HNO_3 ; Fisher Scientific).

Protein carbonyl content. Protein carbonyls were quantified using a commercial kit (Cayman Chemical, Ann Arbor, MI). Briefly, tissue was homogenized in 1 ml of homogenization buffer (50 mM phosphate buffer, 1 mM EDTA, pH 6.7) and centrifuged at $10,000 g$ for 15 min at 4°C . Two separate tubes were then prepared (assay and control). To both tubes, $200 \mu\text{l}$ of supernatant was added plus $800 \mu\text{l}$ of 2,4-dinitrophenylhydrazine to the assay tube and $800 \mu\text{l}$ of 2.5 M HCl to the control tube, and both were incubated in the dark for 1 h. After incubation, 1 ml of 20% TCA was added, and the tubes were centrifuged at $10,000 g$ for 15 min at 4°C . The supernatant was removed, 1 ml of 10% TCA was added, and the tubes were again centrifuged. The supernatant was removed, and the pellet was washed with three sequential washes of 1 ml 1:1 ethanol/ethyl acetate mixture followed by centrifugation. After the final wash, the pellet was resuspended in $500 \mu\text{l}$ guanidine hydrochloride (2.5 M). Then, $220 \mu\text{l}$ from each tube was placed in a 96-well plate, and the absorbance was measured between 360 and 385 nm. The peak absorbance from the control value was subtracted from the assay value, and this corrected absorbance was used to calculate protein carbonyl content. Protein

Table 1. Forward (F) and reverse (R) primers used for real-time PCR

Gene	GeneBank Accession No.	Primer	Amplicon
COX-17	NM001004652	F-gcagcgcagaaaaagccact R-acgcaagcagtcacacacat	200
CAT	AF170069	F-agggaactgggatcttaca R-tttatgggaccagaccttgg	499
Cu/Zn-SOD	Y12236	F-ggccaaccgatagtgtag R-ccagcgttgccagtttttag	157
EF1 α	NM131263	F-gtgctgtgctgattgttgc R-tgatgctgacttctctg	201

COX-17, cytochrome *c* oxidase-17; CAT, catalase; SOD, superoxide dismutase; EF1 α , elongation factor 1 α .

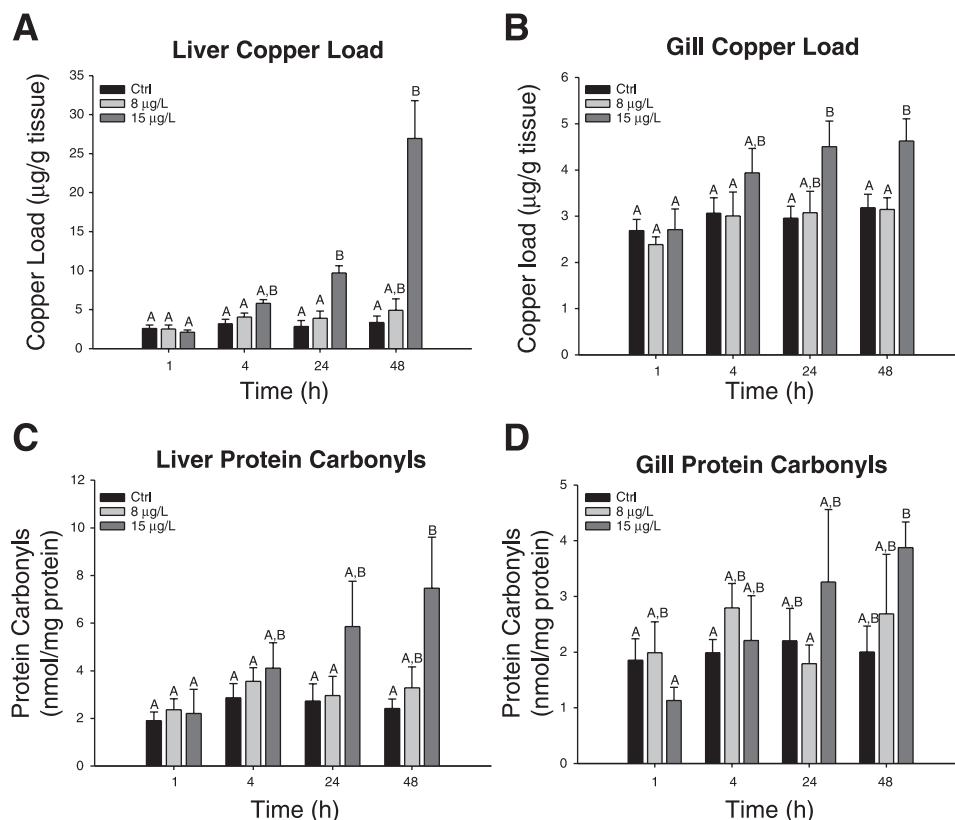
content of the control sample was then measured in a quartz 96-well plate at 280 nm to yield protein carbonyl content in nanomol per milligram protein. Samples were compared with a BSA standard curve (0.125–1 mg/ml) dissolved in guanidine hydrochloride (2.5 M).

Enzyme activities. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and reaction buffers were prepared fresh daily. For all enzyme assays, both gill and liver tissues were homogenized in ice-cold 20 mM HEPES, 1 mM EDTA, 0.1% Triton X-100, pH 7.0. All enzymes were assayed in 96-well format on a SpectraMAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA). SOD (CuZn-SOD and Mn-SOD) was measured by using a modified assay based on the method of Crapo et al. (15). The assay exploits the ability of SOD to outcompete cytochrome *c* for superoxide generated by xanthine oxidase. One unit of SOD is defined as the amount required to inhibit the cytochrome *c* reduction reaction by 50%. The reaction mixture comprised 50 mM K-phosphate buffer, 0.1 mM EDTA, 100 μ M xanthine, 40 mM cytochrome *c*, and 20 U/ml xanthine oxidase. Various amounts of homogenate

were added to the reaction buffer to determine the amount required to inhibit activity by 50%. Absorbance was measured at 550 nm. COX activity was measured by the addition of homogenate to a reaction buffer containing 50 mM Tris·HCl, pH 8, and 50 μ M reduced cytochrome *c*, as described previously (40). After mixing, the absorbance was measured at 550 nm and the reaction was followed for 90 s. Homogenate volumes were chosen to ensure cytochrome *c* was at saturating concentrations. For CS and CAT assays, remaining homogenates were frozen overnight and then sonicated for 30 s (Misonix, New York, NY). For the CS assay, the reaction buffer contained 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.3 mM acetyl CoA, and 0.5 mM oxaloacetate in 20 mM Tris·HCl buffer, pH 8.0. Absorbance was measured at 412 nm over 3 min, and a control well lacking oxaloacetate was used to correct for background thiolase activity. CAT activity was assayed by using a modified method based on Claiborne (13). The reaction buffer consisted of 20 mM K-phosphate, pH 7.0, and 20 mM H₂O₂. The reaction was recorded as the decomposition of H₂O₂ at 240 nm over 1 min. Protein concentrations for all samples were determined using Bio-Rad kit reagents according to Bradford (7), using BSA as a standard.

Quantification of mRNA by real-time RT-PCR. Total RNA from the gill and liver tissue (frozen in liquid N₂) was extracted within 24 h of sampling using Trizol reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by UV spectrophotometry at 260 nm, and RNA purity was verified by the 260/280 nm ratios of 1.8 or greater. First-strand cDNA was synthesized from 1 μ g of total RNA treated with DNase I (Invitrogen) and reverse transcribed to cDNA by using SuperScript II RNase H-reverse transcriptase (Invitrogen). mRNA expression was quantified in duplicate on a Stratagene MX3000P real-time PCR machine by using SYBR Green with ROX as reference dye (Bio-Rad, Mississauga, ON). Each reaction contained 12.5 μ l SYBR Green mix, 1 μ l of each forward and reverse primer (5 μ M), 5.5 μ l RNase/DNase free H₂O, and 5 μ l

Fig. 1. Liver (A) and gill (B) Cu load (μ g/g tissue) and liver (C) and gill (D) protein carbonyl levels (nmol/mg protein) from soft water-acclimated zebrafish exposed to 8 μ g/l or 15 μ g/l Cu for 1, 4, 24, or 48 h. Values are presented as means \pm SE. Treatments that do not share a common letter are significantly different from each other ($n = 6$ zebrafish for all treatments, $P < 0.05$).



cDNA template. Cycling conditions were as follows: 3-min initial denaturation at 95°C, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. This was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of stock liver cDNA. To account for differences in cDNA production and loading differences, all samples were normalized to the expression level of the housekeeping gene elongation factor 1 α (EF1 α), which did not change over the course of the experiment. Both water and non-reverse-transcribed RNA were assayed on each plate to ensure there was no contamination present in reagents or primers used. Primers were designed by using Primer3 (56), and targets were verified by gel electrophoresis and sequencing. Target genes of interest are as follows and primers used can be found in Table 1: COX-17, CAT, Cu/ZnSOD, and EF1 α . Genes were normalized to EF1 α , and each value was expressed as a percentage of the soft water control, which allowed for the calculation of means \pm SE.

Statistical analysis. Statistical analysis was performed by using Sigma Stat (SPSS, Chicago, MI). All data have been expressed as means \pm SE. For *experiment 1*, a two-way ANOVA and a Tukey's test were performed ($P < 0.05$). For *experiment 2*, a one-way ANOVA and a Dunnett's test were used to test for significance relative to the soft water control value, and a Student's unpaired *t*-test was used to compare within a treatment ($P < 0.05$).

RESULTS

Experiment 1. After 48-h Cu exposure, we saw no morbidity or mortality in any of the experimental or control tanks. Average measured concentrations of dissolved Cu for each treatment were 2.1 ± 0.2 (control), 8.1 ± 0.4 (moderate), and 15.1 ± 0.5 $\mu\text{g/l}$ (high). Average ion concentrations did not fluctuate from original soft water acclimation values. Associated with the high Cu exposure, we saw a significant increase in the liver Cu load after 24 and 48 h, with a corresponding threefold increase in protein carbonyl levels after 48 h (Fig. 1, A and C). However, there was no change in either Cu load or protein carbonyls associated with a moderate Cu exposure (Fig. 1, A and C). Similarly, we saw significant increases in gill Cu load after 24 and 48 h with the high Cu exposure and increased gill protein carbonyl content after 48 h, compared with 1- and 4-h exposures (Fig. 1, B and D).

Upon examination of mRNA expression, no changes in SOD expression in the liver were detected despite the elevated Cu load and protein carbonyl levels. However, we did see a significant 2.5-fold increase in liver CAT mRNA expression after 48-h exposure to high Cu levels (Fig. 2, A and B). Additionally, we saw a progressive increase in liver COX-17 mRNA expression with a high Cu exposure, with a significant eightfold increase in expression after 48 h (Fig. 2C).

Experiment 2. Since the majority of significant changes occurred after 48-h exposure to the higher Cu level (15 $\mu\text{g/l}$), we examined the potential protective effect of Ca²⁺ and Na⁺ under this treatment. Zebrafish were exposed to either high Ca²⁺ (nominally 3.3 mM, measured chemistry: 3.4 ± 0.1 mM Ca²⁺, 71.1 ± 3.7 μM Na⁺, 23.2 ± 2.2 μM Mg²⁺, 14.5 ± 0.1 $\mu\text{g/l}$ Cu, pH 6.9 ± 0.2) or high Na⁺ (nominally 10 mM, measured chemistry: 59.6 ± 1.9 μM Ca²⁺, 10.6 ± 0.4 mM Na⁺, 27.4 ± 3.5 μM Mg²⁺, 14.7 ± 0.2 $\mu\text{g/l}$ Cu²⁺, pH 7.0 ± 0.1). Control tanks were maintained with the same water chemistry, yet had baseline levels of Cu (1.8 ± 0.6 and 1.5 ± 0.9 $\mu\text{g/l}$ Cu for high Ca²⁺ and Na⁺ exposures, respectively). With two exceptions, total Na⁺ and Ca²⁺ concentrations in

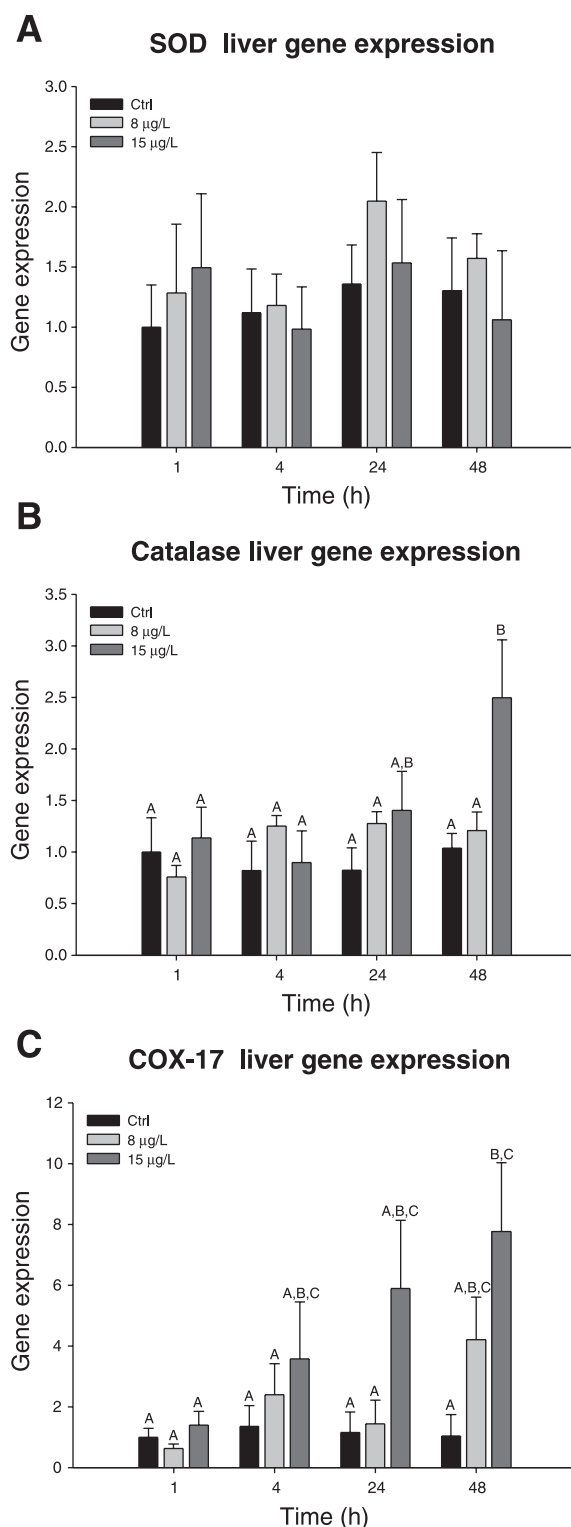


Fig. 2. mRNA expression of superoxide dismutase (SOD; A), catalase (CAT; B), and cytochrome *c* oxidase-17 (COX-17; C) from the liver of soft water-acclimated zebrafish exposed to 8 $\mu\text{g/l}$ or 15 $\mu\text{g/l}$ Cu for 1, 4, 24, or 48 h. Gene expression values were normalized to housekeeping gene elongation factor 1 α (EF1 α), expressed as a ratio of the control (control = 1) and are presented as means \pm SE. Treatments that do not share a common letter are significantly different from each other ($n = 6$ zebrafish for all treatments, $P < 0.05$).

Table 2. Liver and gill tissue concentrations of Na^+ and Ca^{2+} for soft water acclimated control zebrafish and after exposure to 3.3 mM Ca^{2+} , and 10 mM Na^+ for a 48-h period with and without 15 $\mu\text{g}/\text{l}$ Cu

Treatment	Liver		Gill	
	Control	15 $\mu\text{g}/\text{l}$ Cu	Control	15 $\mu\text{g}/\text{l}$ Cu
<i>Na⁺ Load</i>				
Soft water	38.9±6.5	40.4±8.5	45.6±3.6	48.6±2.7
3.3 mM Ca	54.1±1.6	62.6±18.5	54.1±1.6	58.4±6.3
10 mM Na	43.9±3.5	38.0±9.8	55.5±1.3	60.7±2.8*
<i>Ca²⁺ Load</i>				
Soft water	11.6±3.7	15.5±4.5	126.8±10.3	122.3±13.5
3.3 mM Ca	8.1±0.8	21.9±6.7*	125.1±16.1	136.4±11.8
10 mM Na	12.5±4.8	8.3±3.7	125.3±18.5	123.2±12.7

Values are means \pm SE; $n = 6$ zebrafish for all treatments. *Significant difference from respective control ($P < 0.05$).

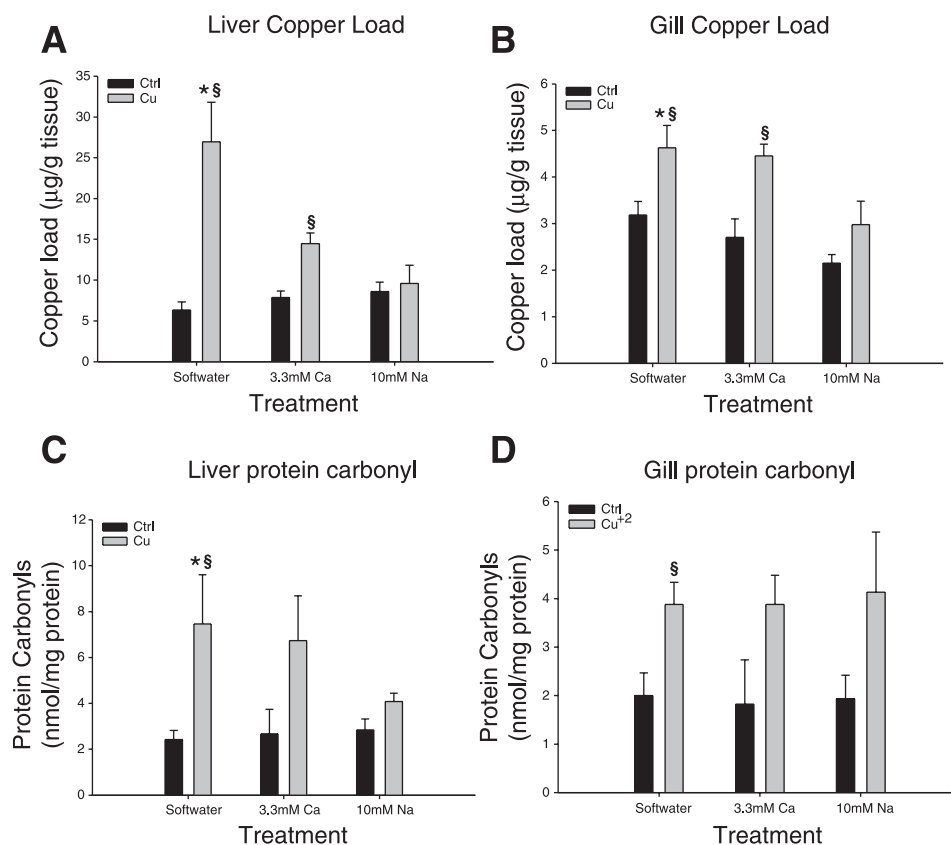
both the liver and gills did not change as a result of the various exposures (Table 2); however, liver Ca levels increased in the Cu-exposed fish under high Ca^{2+} , and gill Na^+ level increased in the Cu-exposed fish under high Na^+ . In general, these results indicate that the zebrafish had acclimated to the elevated ion levels and that ionoregulation was not significantly impacted by Cu during these 48-h exposures, as there were no falls in tissue ion levels. (Table 2).

When Cu exposure was performed in the presence of high Ca^{2+} , we saw diminished Cu load in the liver at 48 h and the associated increase in protein carbonyl levels was no longer significantly different from the control, although it was of

similar magnitude (Fig. 3, A and C). When Cu exposure was performed in the presence of high Na^+ , we saw no accumulation of Cu above control levels in the liver and no change in protein carbonyl levels (Fig. 3, A and C). The high Ca^{2+} + Cu exposure did not significantly reduce the 48-h Cu load in the gills, but again, the associated increase in protein carbonyl levels was no longer significant, although of similar magnitude. However, the high Na^+ + Cu exposure virtually eliminated the accumulation of Cu in the gills, and the increase in gill protein carbonyl level was no longer significant (Fig. 3, B and D).

Overall, these results suggest that 10 mM Na^+ offered greater protection than 3 mM Ca^{2+} against Cu uptake and associated oxidative damage. However, we saw changes in mRNA expression levels that we had not predicted. It appeared that elevated Na^+ plus Cu increased SOD mRNA expression in the liver by 2.5-fold over its respective high Na^+ control (Fig. 4A). Likewise, we saw an increase in liver CAT mRNA expression after exposure to 10 mM Na^+ plus 15 $\mu\text{g}/\text{l}$ Cu (Fig. 4B), which was slightly greater than in control fish exposed to high Cu in soft water alone. However, this increase did not occur when Cu exposure was combined with elevated Ca^{2+} levels (Fig. 4B). The general effects of both high Ca^{2+} and high Na^+ exposures were to greatly attenuate the increases in liver COX-17 mRNA expression caused by high Cu exposure with respect to their controls (Fig. 4C). Indeed, both high Ca^{2+} and high Na^+ exposures alone appeared to reduce COX-17 expression levels, although there was no significant difference from the soft water control (Fig. 4C). There were no significant changes in gill mRNA expression levels for any of the genes tested over the 48-h experimental period (Table 3).

Fig. 3. Liver (A) and gill (B) Cu load ($\mu\text{g}/\text{g}$ tissue) and Liver (C) and gill (D) protein carbonyl levels (nmol/mg protein) from soft water-acclimated zebrafish exposed to 15 $\mu\text{g}/\text{l}$ Cu for 48 h in either soft water, soft water + 3.3 mM Ca^{2+} , or soft water + 10 mM Na^+ . *Significance from the soft water control; §significance within a treatment. Values are presented as means \pm SE ($n = 6$ zebrafish for all treatments, $P < 0.05$).



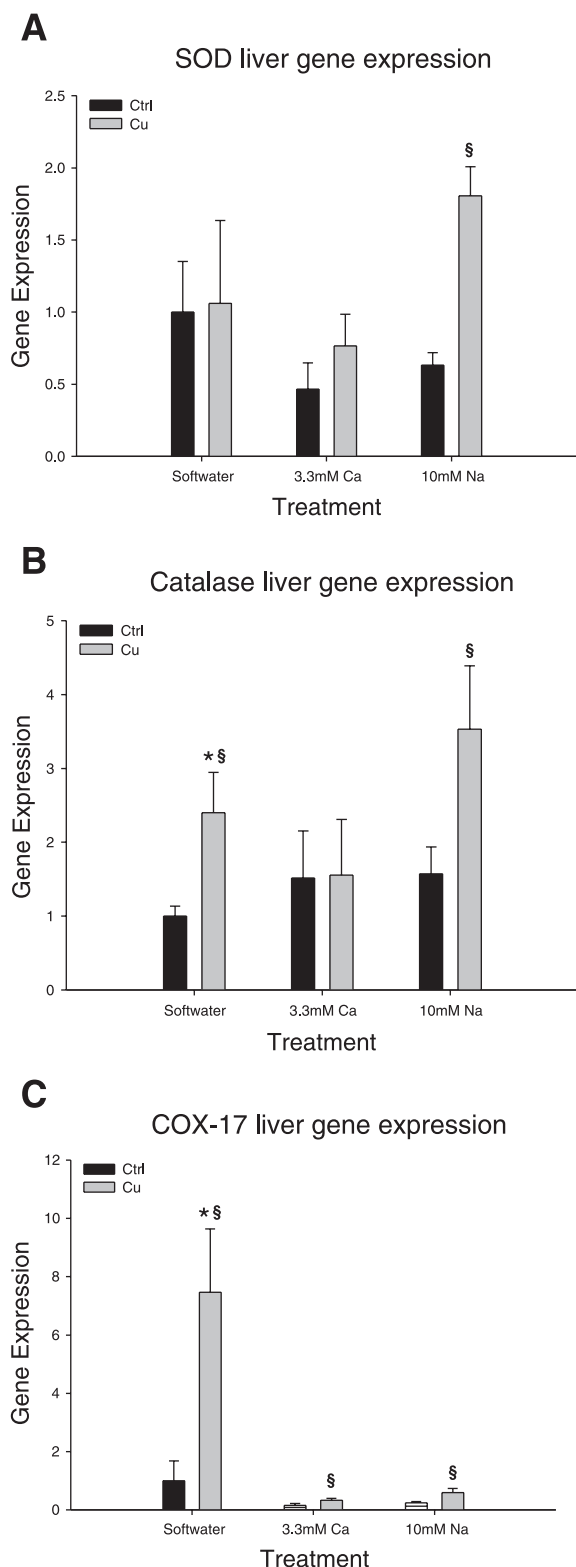


Fig. 4. mRNA expression of SOD (A), Cat (B), and COX-17 (C) from the liver of soft water-acclimated zebrafish exposed to 15 µg/l Cu for 48 h in either soft water, soft water + 3.3 mM Ca²⁺, or soft water + 10 mM Na⁺. Gene expression values were normalized to EF1α expressed as a ratio of the control (control = 1), and are presented as means ± SE. *Significance from the soft water control; §significance within a treatment. Values are presented as means ± SE (n = 6 zebrafish for all treatments, P < 0.05).

Table 3. mRNA expression of SOD, CAT, and COX-17 in the gills of soft water-acclimated zebrafish exposed to 15 µg/l Cu for 48 h in either soft water, soft water + 3.3 mM Ca²⁺, or soft water + 10 mM Na⁺

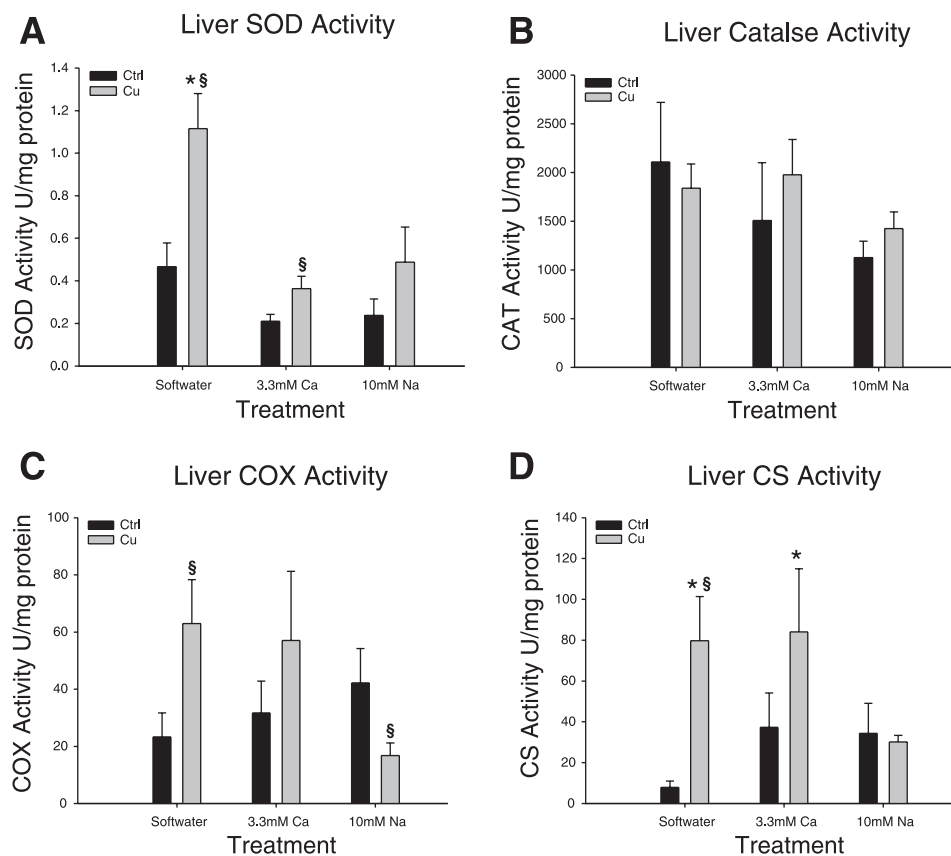
Treatment	Gill	
	Control	15 µg/l Cu
<i>SOD</i>		
Soft water	1.00±0.37	1.55±0.48
3.3 mM Ca	1.00±0.56	0.67±0.23
10 mM Na	1.00±0.23	0.81±0.24
<i>CAT</i>		
Soft water	1.00±0.25	0.67±0.18
3.3 mM Ca	1.00±0.41	1.28±0.35
10 mM Na	1.00±0.37	1.15±0.48
<i>COX-17</i>		
Soft water	1.00±0.11	1.11±0.08
3.3 mM Ca	1.00±0.18	0.96±0.12
10 mM Na	1.00±0.16	0.95±0.11

Values are means ± SE; n = 6 zebrafish for all treatments. Gene expression values were normalized to EF1α expressed as a ratio of the control (control = 1). There were no significant differences when compared to control values (P > 0.05).

Despite no changes in liver SOD mRNA expression in response to Cu in either the soft water or high Ca²⁺ treatments (Fig. 4A), there was a significant increase in SOD enzymatic activity in the liver of zebrafish exposed to 15 µg/l Cu (Fig. 5A) in these exposures. In contrast, there was no increase in liver SOD activity (Fig. 5A), despite an increase in liver SOD mRNA expression in zebrafish exposed to high Na⁺ and Cu (Fig. 4A). In the gill, SOD activity was sixfold higher in the soft water plus Cu exposure, and activities declined to levels not significantly different from controls with high Na⁺ or Ca²⁺ plus Cu exposures (Fig. 6A). Although there were no changes in CAT enzyme activity in the liver (Fig. 5B), there was a significant inhibition of CAT in the gill associated with Cu exposure both in soft water and in combination with high Ca²⁺ (Fig. 6B).

The induction of COX-17 mRNA expression by Cu (Figs. 2C and 4C) warranted the examination of the potential changes in oxidative capacity by examining COX and CS enzymatic activity. In the soft water plus Cu exposure, we saw significant increases in liver COX and CS activity (Fig. 5, C and D), yet only a significant increase in gill COX activity (Fig. 6C) with no change in gill CS activity (Fig. 6D). We found that Cu exposure decreased COX-to-CS ratios in liver and increased the COX-to-CS ratio in gill (Fig. 7, A and B). Zebrafish exposed to high Ca²⁺ plus Cu, exhibited only increased CS activity in the liver (Fig. 5D). Those fish exposed to high Na⁺ demonstrated decreased liver COX activity (Fig. 5C). Interestingly, gill CS activity decreased in the Na⁺ control compared with soft water values, although CS activity did increase in the Na⁺ plus Cu group, compared with its respective control (Fig. 6D). Similar to both the soft water and high Ca²⁺ treatments, high Na⁺ plus Cu exposure group showed a decreased COX-to-CS ratio (Fig. 7A).

Fig. 5. Liver enzyme activity (U/mg protein) for SOD (A), Cat (B), COX (C), and citrate synthase (CS; D) from the liver of soft water-acclimated zebrafish exposed to 15 $\mu\text{g/l}$ Cu for 48 h in either soft water, soft water + 3.3 mM Ca^{2+} , or soft water + 10 mM Na^+ . *Significance from the soft water control; §significance within a treatment. Values are presented as means \pm SE ($n = 6$ for all treatments, $P < 0.05$).



DISCUSSION

This is the first study to examine the effects of acute Cu exposure on adult zebrafish with respect to markers of oxidative stress. Exposure to sublethal Cu concentrations (15 $\mu\text{g/l}$) for 48 h in soft water elicited significant changes in markers of oxidative damage, enzymes in the oxidative stress response pathways, and altered mitochondrial properties. Acute Cu exposure also induced changes in genes involved in oxidative stress response and in a key metal chaperone. Furthermore, results of this study can be used to extend current predictive toxicity models (e.g., the BLM) to a tropical species. In addition, it will contribute the framework for future extension of this model to exposures using chronic, environmentally realistic Cu levels that will be necessary for constructing a chronic BLM and lifetime species protection.

Acute Cu exposure in soft water. In the absence of any protective ions, there was a significant increase after 48 h in the Cu load and oxidative damage, as indicated by increased protein carbonyls in both the gill and liver of zebrafish (Fig. 1). Cu accumulation data are congruent with a majority of freshwater Cu exposure studies where elevated Cu levels have been found to increase Cu load in the gill and liver (17, 24, 29, 41). There were significant reductions in the Cu load in the liver and/or gills at 48 h, as well as in the degree of oxidative damage at this time associated with high ambient Cu (15 $\mu\text{g/l}$) when Ca^{2+} and Na^+ were added back to the soft water. However, 3.3 mM Ca^{2+} did not seem to provide the same level of protection as 10 mM Na^+ in this respect (Fig. 3). While this conflicts with the observation that these levels of Ca^{2+} and Na^+ have identical protective effects against short-term (3 h)

gill Cu accumulation, it is congruent with the finding that 10 mM Na^+ has a greater protective effect against lethality (25-fold increase) than does 3.3 mM Ca^{2+} (11-fold increase).

It is commonly agreed that both acute and chronic Cu exposures result in elevated ROS production in both mammals and fish (9, 36, 37). For this reason, we examined representative genes that encode proteins involved in combating oxidative stress (SOD, CAT) to determine whether these might serve as molecular endpoints of acute Cu toxicity. We found significant changes in liver CAT expression (Fig. 2B), indicative that transcription of this gene is stimulated by oxidative stress with soft water Cu exposure. Surprisingly, we saw no changes in SOD mRNA expression with acute Cu exposure in soft water (Fig. 2A). Contrary to these transcriptional changes, we found a significant increase in liver SOD enzymatic activity but no change in CAT enzymatic activity with Cu in soft water (Fig. 5, A and B). This suggests that some antioxidant enzymes are not initially controlled by transcriptional means, yet rather by enzyme activation, at least in the case of SOD (Fig. 2A). Moreover, it is known that superoxide radicals can directly inhibit CAT activity (29). Therefore, if the initial Cu effect is an increase in superoxide radicals, then CAT activity might be inhibited, and this in turn could stimulate transcript expression. In contrast, other studies seem to indicate increased expression of antioxidant enzyme transcripts in the liver occurring 1–2 wk postexposure (24, 25). We saw no changes in the gene expression of SOD, CAT, or COX-17 gene expression in the gills (Table 3). However, in zebrafish gills, SOD activity is increased and CAT activity decreased at 48 h of Cu exposure (Fig. 6, A and B). This pattern, along with protein carbonyl

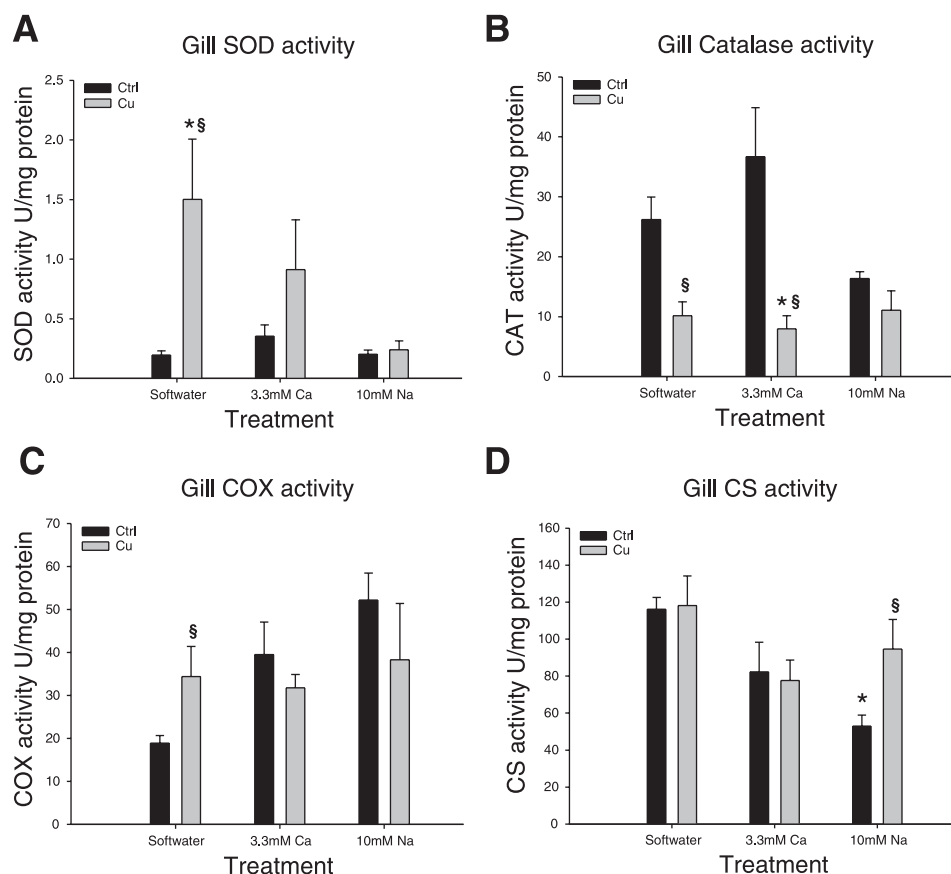


Fig. 6. Gill enzyme activity (U/mg protein) for SOD (A), CAT (B), COX (C), and CS (D) from the liver of soft water-acclimated zebrafish exposed to 15 $\mu\text{g/l}$ Cu for 48 h in either soft water, soft water + 3.3 mM Ca^{2+} , or soft water + 10 mM Na^+ . *Significance from the soft water control; §significance within a treatment. Values are presented as means \pm SE ($n = 6$ zebrafish for all treatments, $P < 0.05$).

production, suggests a rapid rise in oxidative stress and comparably rapid response. Future studies should examine the response to chronic Cu and oxidative stress to elucidate the long-term changes in gene expression and enzyme activities of the ROS scavenging system.

Protective effects of Ca^{2+} and Na^+ . Since it is well documented in other fish species that certain cations can have a protective effect against both metal uptake and metal toxicity (5, 42, 45, 46), we added Na^+ and Ca^{2+} back into the soft water medium. Our results confirm in a tropical species the protective effect of these cations against Cu toxicity. While these were added as chloride salts, there is extensive evidence that protection against Cu toxicity is provided by the cations, rather than by Cl^- anions (46).

It has long been known that Cu disrupts Na^+ homeostasis through competition for apical uptake sites and inhibition of basolateral $\text{Na}^+-\text{K}^+-\text{ATPase}$ in the gills (23, 34, 66), which explains the protective effects of elevated Na^+ . However, the protective effects of Ca^{2+} to Cu toxicity are poorly understood, since disruption in Ca^{2+} uptake is mainly attributed to other metals that are direct calcium analogs, such as cadmium and lead (10, 55, 62, 63, 65, 66). Certainly this study indicates that Ca^{2+} , and therefore water hardness, does lessen acute Cu toxicity, but the mechanisms by which this occurs are currently unclear. Potentially unreduced Cu^{2+} may travel across the gill apical surface via epithelial calcium channels in the same fashion as Cd^{2+} (62, 63). With soft water acclimation alone, epithelial calcium channels mRNA expression and protein levels increase four- and threefold, respectively, and this change in transporter abundance may accentuate Cu sensitivity

in zebrafish (14). However, the partial protection in the current study by elevated Ca^{2+} may have effectively increased competition with Cu at Ca^{2+} uptake sites, yet still allowed Cu to travel through other pathways, such as apical Na^+ channels. However, in zebrafish, a primary uptake pathway for Cu appears to be the apical Na^+ channels, similar to the situation in trout (23), since we saw a significant reduction in both Cu load and oxidative damage in both the gill and liver when 10 mM Na^+ was included in the high Cu exposure (Fig. 3).

With the Ca^{2+} plus Cu treatment, we saw no change in SOD or CAT gene expression in the liver, indicating that increased Ca^{2+} limited the impact of Cu on expression of these genes (Fig. 4, A and B). However, in gill, the apparent repression of CAT activity was still present in this exposure, possibly indicative of ROS-induced inhibition (Fig. 6B), although there were no transcriptional changes (Table 3). A surprising result was the large increase in both SOD and CAT transcription levels in the liver of fish exposed to high Na^+ plus Cu (Fig. 4, A and B) despite the fact that Cu load and oxidative damage is reduced (Fig. 3). Furthermore, there was no association between increased transcription and enzyme activity, highlighted by the lack of change in SOD and CAT activity in both tissues (Figs. 5, A and B and 6, A and B, respectively). One possible explanation is that high Na^+ levels by themselves resulted in increased oxidative stress, which was exacerbated by the addition of Cu, although elevated tissue Na^+ levels were only apparent in the gill, not the liver (Table 2). High NaCl has been shown to induce oxidative stress in the renal medullary cell cultures (67) and isolated perfused rat livers (57). Moreover, there was a significant depression of gill CAT activity in the 10

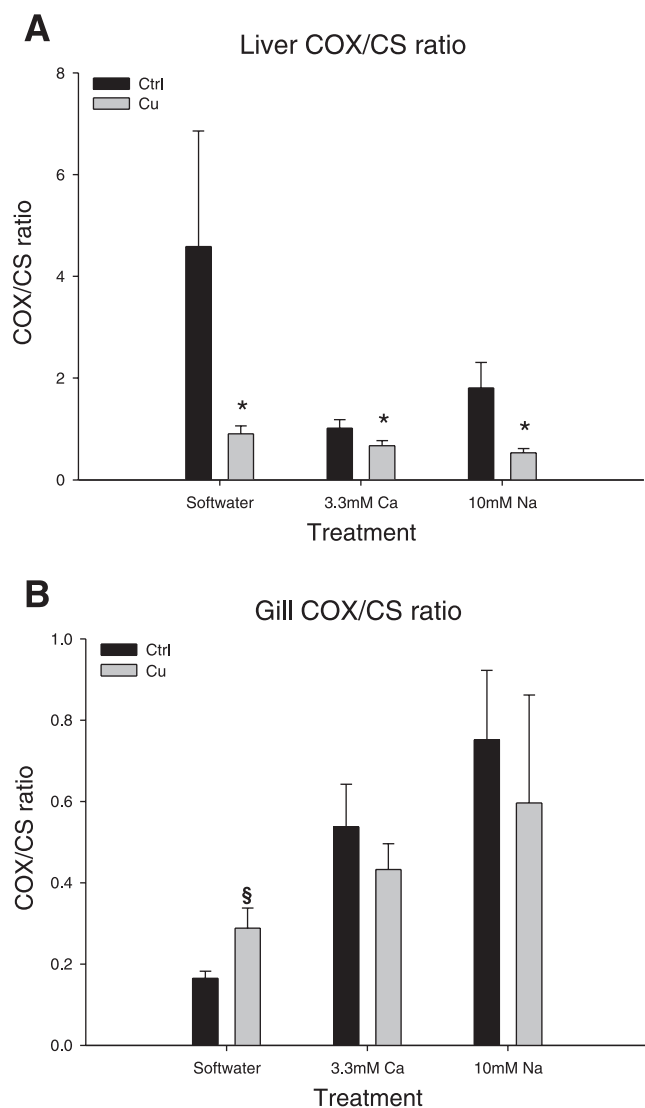


Fig. 7. Liver (A) and gill (B) COX-to-CS ratios for soft water-acclimated zebrafish exposed to 15 $\mu\text{g/l}$ Cu for 48 h in either soft water, soft water + 3.3 mM Ca^{2+} , or soft water + 10 mM Na^+ . *Significance from the soft water control; §significance within a treatment. Values are presented as means \pm SE ($n = 6$ for all treatments, $P < 0.05$).

mM Na^+ control group compared with the soft water control (t -test, $P = 0.048$). Again, this may be oxidative stress related, although to a lesser extent than in soft water, since we did not see any significant increase in protein carbonyls in gills (Fig. 3D). In total, Ca^{2+} and Na^+ did provide protection against Cu toxicity, with Na^+ effectively reducing Cu load by 50%, and protein carbonyl levels 60% over reductions due to Ca^{2+} in the liver. Likewise, Na^+ provided 40% greater protection than Ca^{2+} when gill Cu load was examined. By reducing the Cu load, both liver and gill tissues are protected from the acute effects of high Cu exposure.

Cu toxicity and mitochondrial targets. One of the main targets of metal toxicity is the mitochondrion, and there is a close relationship between metal-induced oxidative stress and proper mitochondrial function, as seen in mammals and fish (33, 49, 53). Many studies have examined the in vitro impact of metals on mitochondrial respiration and energetics in fish

(36, 37), but to our knowledge, this is the first look at the in vivo effects of Cu exposure on markers of mitochondrial density (CS) and oxidative capacity of the inner mitochondrial membrane (COX). After 48-h exposure to Cu, we saw a significant increase in the mRNA expression of COX-17 (Fig. 2C), which is a key metal chaperone essential in the proper assembly of COX (12, 20). Upon addition of Ca^{2+} and Na^+ , the increased COX-17 expression persisted but at a reduced level, despite the apparent protective effects of Ca^{2+} and Na^+ (Fig. 4C). Whether this is due to the interactions between Cu levels and COX-17, Cu handling and stimulation of transcription are unclear and further investigation into the changes in protein abundance and amount of COX-17-bound Cu with varying cytosolic Cu is warranted. However, we did see an increase in maximal COX activity in both the gill and liver of zebrafish exposed to soft water plus Cu (Figs. 5C and 6C). COX is a key enzyme in the electron transport chain, located in inner mitochondrial membrane. An increase in V_{max} for COX suggests an increase in COX protein and both elevated oxygen consumption potential but also an increase in oxidative ATP production capacity. Manzl et al. (36) found that in isolated trout hepatocytes, Cu can increase cellular O_2 consumption, yet there is no change in ATP production compared with basal values, indicating mitochondria were in a partially uncoupled state. It has been shown in mammals that uncoupling respiration from ATP production leads to a reduction in ROS production in the mitochondria (3, 19), since there is a relationship between membrane potential and ROS production (33). Potentially, Cu-exposed fish may use uncoupled respiration as an adaptive response to Cu-induced ROS production, since increased proton leak lowers the membrane potential and leads to a significant reduction of ROS production (38). Answering these questions represents a fruitful area for future research.

We also measured CS activity as a marker of mitochondrial volume density (51), which significantly increased in liver with the soft water plus Cu exposure (Fig. 5). Increases in the COX-to-CS ratio can be used as an indicator of relative oxidative capacity or a decrease in this ratio that can be used to indicate general mitochondrial dysfunction (11). We saw a significant increase in the COX-to-CS ratio in the gill, yet a substantial decrease in this ratio within liver with acute Cu toxicity. These changes may, in part, be due to the gill morphological changes associated with soft water acclimation, which might be further stimulated with Cu exposure. For instance, in soft water-exposed rainbow trout, there is a distinct increase in the number of mitochondrial-rich chloride cells, impacting the blood-to-water diffusion distance, thereby impacting gas transfer (21). This may explain why the control Ca^{2+} and Na^+ treatments lowered liver COX-to-CS ratios compared with the soft water control. As for the impact of Cu on the COX-to-CS ratio, we saw a significant drop in liver compared with the soft water control (Fig. 7A). This may be indicative of mitochondrial dysfunction as the increased Cu may inhibit proper COX assembly, although this is only apparent in the soft water + Cu exposure group. An alternate explanation is the impact that ROS can have on the lipid membrane of mitochondria. ROS are known to increase lipid peroxides (4, 50), thereby altering the lipid membrane of the mitochondria. Changes in lipid membrane composition have been shown to alter the activity of membrane-bound COX in carp (64). This may explain the increases found in COX

activity in both the gill and liver of Cu-exposed zebrafish (Figs. 5C and 6C). In the gills, there was no change in the relative oxidative capacity between the Ca^{2+} and Na^{+} treatments, again demonstrating potential protective effects of water hardness and ions on Cu toxicity.

Perspectives

We show here that zebrafish are a powerful model for the study of oxidative stress and metal toxicology in vivo. A relatively low, environmentally relevant level of Cu in soft water (15 $\mu\text{g/l}$) has immediate impacts on oxidative damage and antioxidant protection pathways in the zebrafish. Furthermore, by increasing either the water Ca^{2+} or Na^{+} concentration, we can protect against the acute toxic effects of elevated waterborne Cu, although 3.3 mM Ca^{2+} is not as effective as 10 mM Na^{+} . Interestingly, with the exception of COX-17, we are able to abolish the transcription expression effects of Cu by 3.3 mM Ca^{2+} , and it appears that 10 mM Na^{+} allows for a transcriptional response to occur. Furthermore, 10 mM Na^{+} exposure with Cu decreased the oxidative damage and overall Cu load in both the gills and liver. With increased Cu exposure, there is an increase in the relative oxidative capacity at the gills but indications of mitochondrial dysfunction in the liver, indicating these as possible physiological endpoints of acute toxicity. Even more apparent is the relationship between increasing ambient Cu concentration and induction of COX-17 mRNA expression ($R^2 = 0.851$; $P = 0.009$), which provides a key molecular determinant of acute toxicity. Combined, the rapid induction of these physiological and transcriptional endpoints with acute exposures suggests that they should be applied further to chronic Cu studies. Moreover, as a representative tropical species, zebrafish will provide essential information for the development of water quality guidelines.

GRANTS

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