Am J Physiol Regul Integr Comp Physiol 296: R362–R373, 2009.
First published November 5, 2008; doi:10.1152/ajpregu.90581.2008.

Dietary iron alters waterborne copper-induced gene expression in soft water acclimated zebrafish (*Danio rerio*)

Paul M. Craig, Michal Galus, Chris M. Wood, and Grant B. McClelland
Department of Biology, McMaster University, Hamilton, Ontario, Canada

Submitted 6 July 2008; accepted in final form 28 October 2008

Craig PM, Galus M, Wood CM, McClelland GB. Dietary iron alters waterborne copper-induced gene expression in soft water acclimated zebrafish (*Danio rerio*). *Am J Physiol Regul Integr Comp Physiol* 296: R362–R373, 2009. First published November 5, 2008; doi:10.1152/ajpregu.90581.2008.—Metals like iron (Fe) and copper (Cu) function as integral components in many biological reactions, and, in excess, these essential metals are toxic, and organisms must control metal acquisition and excretion. We examined the effects of chronic waterborne Cu exposure and the interactive effects of elevated dietary Fe on gene expression and tissue metal accumulation in zebrafish. Softwater acclimated zebrafish exposed to 8 μg/l Cu, with and without supplementation of a diet high in Fe (560 vs. 140 mg Fe/kg food) for 21 days demonstrated a significant reduction in liver and gut Cu load relative to waterborne Cu exposure alone. Gene expression levels for divalent metal transport (DMT)-1, copper transporter (CTR)-1, and the basolateral metal transporter ATP7A in the gills and gut increased when compared with controls, but the various combinations of Cu and high-Fe diet revealed altered levels of expression. Further examination of the basolateral Fe transporter, ferroportin, showed responses to waterborne Cu exposure in the gut and a significant increase with Fe treatment alone in the liver. Additionally, we examined metallothionein 1 and 2 (MT1 and MT2), which indicated that MT2 is more responsive to Cu. To explore the relationship between transcription and protein function, we examined both CTR-1 protein levels and gill apical uptake of radiolabeled Cu^{64}, which demonstrated decreased Cu uptake and protein abundance in the elevated Cu treatments. This study shows that high dietary Fe can significantly alter the genetic expression pattern of Cu transporters at the level of the gill, liver, and gastrointestinal tract.

divalent metal transport; copper transporter; metallothionein

TRACE ELEMENTS SUCH AS COPPER (Cu) and iron (Fe) are essential micronutrients for all organisms because of their high redox potential, and importance as cofactors for a variety of metabolic proteins, such as cytochrome *c* oxidase and hemoglobin (45). However, because of increased anthropogenic activities, exogenous concentrations of these trace elements are tending to increase in natural ecosystems, which can be harmful, if not fatal, to aquatic organisms. Because of their persistence in the aquatic environment, it is important to examine the chronic biological impacts of these metals, especially in tropical water systems, where focus has been lacking. Zebrafish, a tropical species, have become an excellent model to study the physiological and genetic impact of increased metal contamination because of their publicly available genome and ability to tolerate soft water, which is key in examining metal impacts without interference of other cations (12, 13, 49, 55, 56). Furthermore, zebrafish are an endemic species to the Indian subcontinent and may be found in waters that can contain Fe and Cu at levels 100 and 15 times greater, respectively, than those dictated by Indian environmental protection rules (Fe: 3 mg/l vs. 326 mg/l; Cu: 3 mg/l vs. 48 mg/l; see Ref. 57) or United States Environmental Protection Agency regulations (66). It is essential to understand the impact metals can have on tropical species in such situations to assess the potential risk of morbidity and mortality that would lead to a population decline.

The primary uptake pathway of trace metals in fish is the diet, although considerable evidence suggests that Cu and Fe can also be taken up by the gills (10, 23, 28). Several transport mechanisms associated with Cu and Fe uptake are found in the gill, liver, and gastrointestinal tract of zebrafish. Although principally characterized as a Fe transporter, divalent metal transporter-1 (DMT-1) appears to function as a carrier for most divalent metal ions across the apical surface of the cell (24). Its expression has been detected in both the gills and gastrointestinal tract of zebrafish (11, 31). Ferroportin, a known Fe exporter, transports Fe from the cell into circulation and has been characterized in both mammalian and fish models (1, 17). Interestingly, in macrophages, ferroportin gene expression has been shown to increase in a dose-dependent manner upon increased Cu exposure, which stimulates the release of Fe (9). Other transporters involved are specifically related to Cu: the apical copper transporter-1 (CTR-1) and the basolateral Cu-ATPase (ATP7A or Menkes gene). CTR-1 is ubiquitously expressed in all tissues in both mammals and other vertebrates, although the largest concentration of CTR-1 in mammals is found in the small intestine, where 90% of total body Cu is absorbed (61). In zebrafish, the CTR-1 gene was first cloned and characterized in studies focusing on embryonic development and the importance of Cu for growth (39). It was found that altering ambient levels of Cu inversely affected CTR-1 transcripts, indicating CTR-1 expression changes are crucial for normal zebrafish development (39). However, the CTR-1 gene has not been examined under elevated waterborne Cu in adult zebrafish. Craig et al. (13) found that zebrafish had fluctuations in transcript levels of CTR-1 in the gill during soft water acclimation, indicating that waterborne Cu is required for normal physiological homeostasis. ATP7A ("Menkes" protein) is an essential Cu transport protein involved in both the packaging and transport of cellular Cu in the plasma. Essentially, ATP7A can transfer Cu to the Golgi apparatus to be incorporated into Cu-dependent enzymes such as lysyl oxidase or allow for direct secretion of Cu in the circulation (see review in Ref. 33). Characterization of this transporter is well estab-

Address for reprint requests and other correspondence: P. M. Craig, Dept. of Biology, McMaster Univ., 1280 Main St. West, Hamilton, ON L8S 4K1 Canada (e-mail: craigpm@mcmaster.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

lished in mammals because of its importance in human Cu deficiency diseases such as Menkes disease (36). To date, no characterization of ATP7A expression has been performed in adult zebrafish exposed to elevated waterborne Cu. Changes in expression of this protein may be important for the regulation of basolateral Cu transport with changing environmental Cu exposure.

Because metals accumulate within cells above ambient levels, several mechanisms are invoked to prevent cellular damage, particularity metal chaperone proteins such as metallothioneins (MT). MTs are metal-binding proteins of low molecular mass that play essential biological roles in metal homeostasis, cytoprotection, and detoxification (27). Furthermore, they are increasingly used as biomarkers of metal pollution in the environment (14, 30, 51). Two different isoforms of MTs have been cloned and characterized in zebrafish (MT1 and MT2) and are found in the majority of tissues (7, 21). These isoforms are particularly sensitive to cadmium (Cd) and Cu, but they are known to bind to various other metals (27). However, the relative responsiveness of the two isoforms has not been studied previously in zebrafish. It is of particular interest to examine which isoform is most sensitive to Cu, since this may aid in determining biomarkers of Cu toxicity.

There is a wealth of data on single metal exposures in fish (4, 5, 8), including zebrafish (12). However, it is more relevant, both environmentally and physiologically, to examine exposure to metal mixtures. We chose to examine the interactive effects of Fe and Cu, since in a pilot study, we found that chronic exposure to 15 μg/l waterborne Cu caused a surprising increase in expression of DMT-1 in the gills, which was contrary to our predictions of zebrafish maintaining homeostatic control over Cu. There was also a significant reduction in the Fe load in the liver, indicating a potential competition between Cu and Fe, although interpretation was confounded by some mortality (unpublished observations). Many studies have shown that the best-characterized link between Cu and Fe is the Cu-derived protein ceruloplasmin, which has been shown to act as a serum ferrioxidase and is required for Fe mobilization and metabolism in Fe storage tissues (see review in Ref. 62). There is a link between decreased total body Cu and decreased ceruloplasmin levels, which in turn lead to an impairment of Fe metabolism, resulting in anemia (47, 52). Furthermore, under conditions of excess Cu in mammals, hemolytic anemia occurs because of Cu interruption of glycolysis in erythrocytes, which denatures the hemoglobin (20). However, this may not occur in fish since their erythrocytes have a greater aerobic capacity (19). If excess Cu in zebrafish leads to altered Fe homeostasis, then supplementing the fishes' diet with Fe should alleviate any Cu-induced anemia.

In the present study, we employed a level of chronic waterborne Cu exposure (8 μg/l) that is environmentally relevant for soft water environments (66), examining its effects on gill, liver, and gut expression of CTR-1, DMT-1, ATP7A, ferroportin, MT1, and MT2. To further characterize the expressional pattern of Cu uptake, we examined how a Fe-supplemented diet can alter the gene expression of these transcripts at the gills and the liver. Several studies on fish have demonstrated how a metal- or ion-amended diet can affect uptake of nonessential ions from both the water and food. For example, dietary Ca or Fe can affect branchial Cd uptake (2, 11) (in trout and zebrafish, respectively). The goal of this study was to identify the transcription profile of Cu transporters in the gill, liver, and gut of soft water-acclimated zebrafish chronically (21 days) exposed to moderate levels of waterborne Cu and to determine how dietary Fe can affect both the transcription profile and gill apical uptake of an essential micronutrient. Additionally, as a functional test, short-term uptake of radiolabeled Cu (^{64}Cu) was employed to complement these data to clarify the relationship between transcript levels, protein levels, and functional Cu transport activity.

MATERIALS AND METHODS

Fish care. Adult zebrafish of mixed sex (*Danio rerio*) were purchased from a local pet fish distributor (DAP International) and acclimated to soft water over a 7-day period in an aerated 40-liter aquarium, as described previously (10; Table 2). After acclimation, zebrafish were housed in multiple 3-liter self-cleaning AHAB tanks racked in a soft water recirculating stand-alone AHAB filtration system (Aquatic Habitats, Apopka, FL). Fish were fed two times daily with a commercial tropical fish food (Topfin, Phoenix, AZ) and maintained on a 12:12-h light-dark photoperiod regime until experiments began. 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.

Exposure to waterborne Cu and Fe diet. Zebrafish (n = 224; 40–48/treatment) were weighed and placed in 8-liter aerated, flow-through soft water tanks set to 25 ml/min. There were four experimental treatments: 0 μg Cu/l control (Ctrl), 0 μg Cu/l (Ctrl + Fe diet), 8 μg Cu/l or 8 μg Cu/l + Fe diet. For the two elevated Cu treatments, Mariotte bottles were used to dose tanks with a concentrated Cu solution made from $CuSO_4 \cdot 5H_2O$ dissolved in 0.05% HNO_3, nitric acid, which had no measurable impact on water pH. Fish were fed 2% body weight one time per day of commercial tropical fish food or a diet supplemented with Fe. The high-Fe diet was formulated by the addition of 240 mg $FeSO_4 \cdot 7H_2O$ to 40 g of powdered tropical food. Food and Fe were mixed with the addition of a small amount of water, spread on a baking sheet, dried at 35°C for 1 h, and then collected and repowdered. A sample of the normal diet and elevated Fe diet was taken and dissolved in HNO_3 and measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z; Varian, Palo Alto, CA) to verify Fe concentrations. The normal diet contained ~140 mg Fe/kg food, whereas the high-Fe diet contained ~560 mg Fe/kg food. Tanks were monitored daily for mortality and cleaned of any food or waste that had accumulated. Daily, a 10-ml water sample was taken from each tank, filtered though a 0.45-μm filtration disc (Pall, East Hills, NY), added to a plastic tube containing 100 μl HNO_3, and kept at 4°C for analysis of Cu and Fe concentrations. At the end of the exposure period, fish were quickly killed by cephalic concussion and sampled for gill, gut, and liver tissue, which were immediately frozen in liquid N_2 for further analysis of Cu and Fe burdens and gene expression. Additional gill samples were taken for Western blotting.

Water and tissue ion levels. All tissues (and food) were first digested in 1 ml of 1 N HNO_3 for 48 h at 60°C. Digests were diluted 10×, and dissolved Cu levels were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z; Varian) and compared with a 40 μg/l Cu standard (Fisher Scientific, Ottawa, ON). Water Cu levels were read without dilution. Water Fe levels were measured by graphite furnace and compared with a diluted 1 mg/l Fe standard (Fisher Scientific). Both tissue digests (Na^+, Mg^{2+}, Fe^{2+}, and Ca^{2+}) and water ion composition (Na^+, Mg^{2+}, and Ca^{2+}) were measured by flame atomic absorption spectroscopy (Spectra AA 22 0FS; Varian) after 10× dilutions were made with 1% HNO_3 (Na^+) or 0.5% $LaCl_3$/1% HNO_3 (Mg^{2+}, Ca^{2+}, Fe^{2+}), and verified using certified Na^+, Mg^{2+}, Fe^{2+} and Ca^{2+} standards (1 mg/l diluted in 1% HNO_3 or 0.5% $LaCl_3$/1% HNO_3; Fisher Scientific).

Quantification of mRNA by real-time RT-PCR. Total RNA from the gill, gut, and liver tissues was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by ultraviolet spectrophotometry at 260 nm, and RNA purity was verified by the 260- to 280-nm ratio (Nanodrop ND-1000; Fisher Scientific, Wilmington, DE). First-strand cDNA was synthesized from 1 μg of total RNA treated with DNase I (Invitrogen) and reverse transcribed to cDNA using SuperScript II RNase H-RT (Invitrogen). mRNA expression was quantified in duplicate on a Stratagene MX3000P real-time PCR machine using SYBR green with ROX as reference dye (Bio-Rad, Mississauga, ON). Each reaction contained 12.5 μl SYBR green mix, 1 μl of each forward and reverse primer (5 μM), 5.5 μl RNase- and/or DNase-free H_2O, and 5 μl of 5× diluted 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 45 s, and 72°C for 30 s. This was followed by a melting curve analysis to verify the specificity of the PCR products within and between tissues. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of stock gill, gut, and 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-1α (EF1α), which did not change over the course of the experimental treatments. Gene expression data were calculated using the $2^{-\Delta\Delta C_t}$ method (38). Both DNase- and RNase-free water and nonreverse transcribed RNA were assayed on each plate to ensure there was no contamination present in reagents or primers used. Primers were designed using Primer3 (59). Target genes of interest are as follows: Menkes transporter (ATP7a), CTR-1, DMT-1, EF1α, ferroportin, MT1, and MT2. Primers and accession numbers can be found in Table 1.

Apical gill uptake of ^{64}Cu. Following final sampling on *day 21* (i.e., at the end of the exposure period), an acute (20 min) Cu gill uptake study was performed on fish remaining from each of the experimental treatments ($n = 8$/treatment). ^{64}Cu was prepared from dried $Cu(NO_3)_2$ (300 μg) and irradiated at the McMaster nuclear reactor to achieve a radioactivity level of 0.6 mCi (half-life = 12.9 h). After irradiation, the $Cu(NO_3)_2$ was dissolved in 0.1 mM HNO_3 (400 μl), 0.01 mM $NaHCO_3$ (400 μl), and Nanopure water (1.7 ml). The resuspended ^{64}Cu stock was added to 1.5-liter tanks containing aerated soft water 30 min before the addition of fish. Water samples (10 ml) were taken in duplicate at the beginning and end of the exposure for the measurement of dissolved and radioactive Cu. After exposure, fish were removed, rinsed in a concentrated solution of Cu to remove any loosely bound radioisotope by displacement, and were terminally anesthetized with an overdose of MS-222 (1 g/l). Previous tests have validated this procedure (23). The gills were excised from the body, and both gills and carcass were blotted and weighed before gamma counting. The gamma radioactivities of ^{64}Cu of the gills (representative of apical uptake; see Ref. 23) and carcass were measured on a Minaxi-γ Auto gamma 5530 counter (Canberra Packard, Mississauga, ON) using energy windows of 433–2,000 KeV for ^{64}Cu. ^{64}Cu was corrected for decay to a common reference time because of a short half-life (12.9 h).

Western blot of gill CTR-1. Whole gill arches were homogenized in buffer (100 mM imidazole, 5 mM EDTA, 200 mM sucrose, and 0.1% deoxycholate, pH 7.6) and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was collected and diluted to 30 μg of protein in 4× loading buffer (48 mM Tris·HCl, pH 6.8, 4% glycerol, 3.2% SDS, 600 mM β-mercaptoethanol, and 1.6% bromphenol blue). Samples were denatured in boiling water for 5 min and loaded on a 7.5% SDS-polyacrylamide gel. Proteins were separated by electrophoresis for 1 h at 150 volts. Samples were transferred to a polyvinylidene fluoride membrane (Bio-Rad), and blots were incubated overnight at 4°C in 7.5% skim milk + 10 mM phosphate buffer, 0.09% NaCl, and 0.05% Tween 20, pH 7.5 (PBST). Blots were washed three times for 5 min in PBST and incubated at room temperature with the primary antibody diluted in 5% skim milk + PBST. The primary antibody (1:200) used was a human CTR-1 antibody (ab30907; Abcam, Cambridge, MA) that was designed on a highly conserved region of the human (h) CTR protein. The percent amino acid homology of the conserved region between the hCTR-1 (accession no. NM_001855) and the zebrafish CTR-1 (accession no. NM_205717) was 90%. Tubulin was used because of availability and good reactivity with zebrafish tissues and did not change with our treatments (13). Membranes were washed three times for 5 min in PBST and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit IgG (CTR-1, 1:25,000; PerkinElmer, Boston, MA) or anti-mouse IgG (tubulin, 1:50,000). After three 5-min washes with PBST, proteins were visualized with a Western Lightning chemiluminescence kit, following the manufacturer's protocol (PerkinElmer). Blots and band density analysis were completed on a ChemiImager (AlphaInnotech, San Leandro, CA), which used pixel density to quantify band intensity. Bands were normalized to tubulin, and expressed as a ratio of the control.

Statistical analysis. Statistical analysis was performed using Sigma Stat (SPSS, Chicago, IL). In particular, a one-way ANOVA and a Student-Newman Keul's post hoc test was used to test for significance for all pairwise treatments ($P < 0.05$). All data have been expressed as means ± SE.

Table 1. *Forward and reverse primers used for real-time qPCR*

Gene	Primer	Acession No.	Amplicon size, bp
ATP7a	F: 5′-GGCTCGACTTCTCGCAGCT-3′ R: 5′-ATTCCGCATTTTCACTGCCT-3′	NM_001042720	50
CTR-1	F: 5′-GAATCAGGTGAACGTGCGCT-3′ R: 5′-CCATCAGATCCTGGTACGGG-3′	AY077715	51
DMT-1	F: 5′-CAATCGACACTACCCCACGG-3′ R: 5′-TCCACCATAAGCCACAGGATG-3′	AF529267	51
Ferroportin	F: 5′-ACATTCTGGCACCGATGCTT-3′ R: 5′-AGTGTGAGCCAAATGCCATG-3′	NM_131629	51
MT-1	F: 5′-CGTCTAACAAAGGCTAAAGAGGGA-3′ R: 5′-GCAGCAGTACAAATCAGTGCATC-3′	AY514790	51
MT-2	F: 5′-GGAGGAGGGTCAGAGGAACC-3′ R: 5′-AGCAACTGAAGCTCCATCCG-3′	NM_194273	51
EF1-α	F: 5′-GTGCTGTGCTGATTGTTGCT-3′ R: 5′-TGTATGCGCTGACTTCCTTG-3′	NM131263	201

ATP7a, basolateral Cu-ATPase or Menkes gene; CTR, Cu transporter; DMT, divalent metal transport; MT, metallothionein; EF, elongation factor; F, forward; R, reverse.

RESULTS

Water ion composition and fish weights. Water ion analysis verified that all experiments were conducted in a soft water environment (hardness as $CaCO_3$ equivalents = 6.9 ± 0.3 mg/l; Table 2). This reduced the protective effects that would be exerted by ions normally present in hard water against metal toxicity. Statistical analysis revealed that a significant difference exists only when comparing Cu concentrations with that of controls (Table 2). The elevated Fe diet in *experiment 2* did not cause any change in waterborne Fe levels (Table 2). Zebrafish were weighed before and after exposure to all treatments, with the control and control + Fe diet increasing in weight after 21 days and all other treatments decreasing in weight, by up to 17% for fish exposed to 8 μg/l Cu alone (Table 3). There were no mortalities in any of the treatments.

Exposure to waterborne Cu and Fe diet. Significant increases in Cu load were found in all tissues examined in zebrafish exposed to 8 μg/l Cu (Fig. 1). Interestingly, there was a significant accumulation of Cu in the gills of zebrafish fed the high-Fe diet only, which was further exacerbated in fish exposed to 8 μg/l Cu + high-Fe diet (Fig. 1*A*). There was a tendency toward a decrease in Fe tissue burden in both the gut and gill of fish exposed to 8 μg/l Cu alone ($P = 0.06$ and $P = 0.07$, respectively) and, surprisingly, a significant decrease in liver Fe of zebrafish exposed to 8 μg/l Cu + high-Fe diet (Fig. 2). There was little change in expression of CTR-1 except in zebrafish treated with 8 μg/l Cu + high-Fe diet where there was a 13- and 7-fold significant increase in CTR-1 gene expression in both the gill and gut (Fig. 3, *A* and *B*). Likewise, we saw a fivefold increase in DMT-1 gene expression in the gill under the same conditions (Fig. 4*A*), but no changes were detected in the gut or liver. However, DMT-1 expression did increase in both the gill and gut of zebrafish fed a high-Fe diet in the absence of additional Cu (Fig. 4, *A* and *B*). Interestingly, DMT-1 was upregulated in the liver when zebrafish were exposed to 8 μg/l Cu (Fig. 4*C*), as was ATP7A (Fig. 5*C*). Zebrafish exposed to 8 μg/l Cu + high-Fe diet had significant increases in ATP7A expression in both the gill and gut. There was also a significant increase in gut ATP7A expression but no change in the gill with Cu alone (Fig. 5, *A* and *B*). Ferroportin expression increased significantly in the gut and gill of fish exposed to 8 μg/l Cu alone, but the gut was not responsive to other treatments (Fig. 6, *A* and *B*). Furthermore, we saw a 2.5- and 4-fold increase in gene expression of ferroportin in the gill and liver of fish fed a high-Fe diet in the absence of Cu. However, there were no increases in ferroportin gene expression in liver of fish exposed to 8 μg/l Cu + high-Fe diet (Fig. 6*C*). When we compared the expression profiles of MT1 vs. MT2 in the gill and liver, we found that MT2 had a greater response to Cu than MT1, but MT1 did significantly increase when fish were exposed to both 8 μg/l Cu + Fe diet (Fig. 7, *A* and *C*). Neither MT1 nor MT2 showed changes in mRNA expression in the gut with any of the experimental treatments (Fig. 7*B*).

We examined CTR-1 protein expression changes using Western blotting. Band intensities were normalized to tubulin and expressed as a ratio of the control. We saw a significant decrease in protein expression of CTR-1 in zebrafish exposed to 8 μg/l Cu only. Feeding zebrafish a high-Fe diet resulted in a partial reversal of the Cu-induced decline in CTR-1 protein expression (Fig. 8). Functional measurements of Cu transport revealed a significant decrease in the gill apical and whole body uptake of ^{64}Cu in zebrafish exposed for 21 days to 8 μg/l Cu and only a decrease in the whole body uptake of zebrafish exposed to 8 μg/l Cu + high-Fe diet (Fig. 9).

Table 2. *Concentrations of water ions, Cu, and Fe for all experimental exposures*

Ion	Ctrl	Ctrl + Fe diet	Cu (8 μg/l)	Cu (8 μg/l) + Fe diet
Na^+	65.3±3.6	53.4±3.8	49.0±3.0	51.6±3.2
Mg^{2+}	20.5±3.6	12.5±0.9	12.4±1.4	12.8±1.9
Fe^{2+}	2.5±0.7	2.2±0.6	2.1±0.8	2.2±0.7
Ca^{2+}	49.2±3.8	42.2±2.9	38.0±2.8	42.2±2.8
Cu^{2+}	1.8±0.2	1.2±0.0.2	8.0±0.4*	8.7±0.3*

Values are presented as means ± SE; $n = 21$ zebrafish. Units are as follows: water ions (μM), copper (μg/l), and iron (μg/l). *Significant difference from respective control ($P < 0.05$).

Table 3. *Mean weight of fish before and after respective 21-day treatment, including percent change in weight*

Treatment	Before, g	After, g	Change, %
Ctrl	0.35±0.01	0.50±0.05	28.5
Ctrl + Fe diet	0.43±0.04	0.55±0.13	21.6
Cu (8 μg/l)	0.45±0.11	0.38±0.02	−17.7
Cu (8 μg/l) + Fe diet	0.50±0.06	0.45±0.03	−10.1

Values are presented as means ± SE; n = 40-48 zebrafish/treatment. Ctrl, control.

DISCUSSION

This study shows that both chronic waterborne Cu exposure and a high-Fe diet can on their own significantly alter the genetic expression pattern of Cu transporters at the level of the gill, liver, and gastrointestinal tract but that there are also unique interaction effects. With a moderate Cu exposure (8 μg/l), in confirmation of an initial pilot study at a higher Cu level (15 μg/l, unpublished), we found there was a decrease in tissue Fe levels in the gill and gut (Fig. 2, *A* and *B*). This prompted us to expose zebrafish to an elevated Fe diet, since we assumed Cu could have a competitive effect with Fe because of a shared uptake pathway (DMT-1). With the addition of Fe to the diet, we did not see any changes in Fe tissue levels, despite an increased Cu load in the gills, gut, and liver (Fig. 1). Upon examination of the gene expression profile, we found 8 μg/l Cu + Fe diet significantly increased expression of CTR-1 in the gill and gut (Fig. 3, *A* and *B*). Additional increases in DMT-1 were found in the gill and liver with waterborne Cu, with the Fe diet exacerbating the increase in gill DMT-1 expression over the 8 μg/l Cu exposure (Fig. 4, *A* and *C*). Interestingly, we found that a high-Fe diet without waterborne Cu significantly increased DMT-1 expression in the gut (Fig. 4*B*). With respect to the basolateral Cu transporter ATP7A, we found that moderate Cu exposure increased expression levels in the gut and liver, and, with the addition of an Fe diet, we saw increased expression in the gills and gut (Fig. 5). Contrary to our gene expression profile, the protein expression of CTR-1 and the apical uptake of Cu in the gills tell a different story. Protein expression actually decreased concurrent with a decrease in Cu uptake. This may hint at an

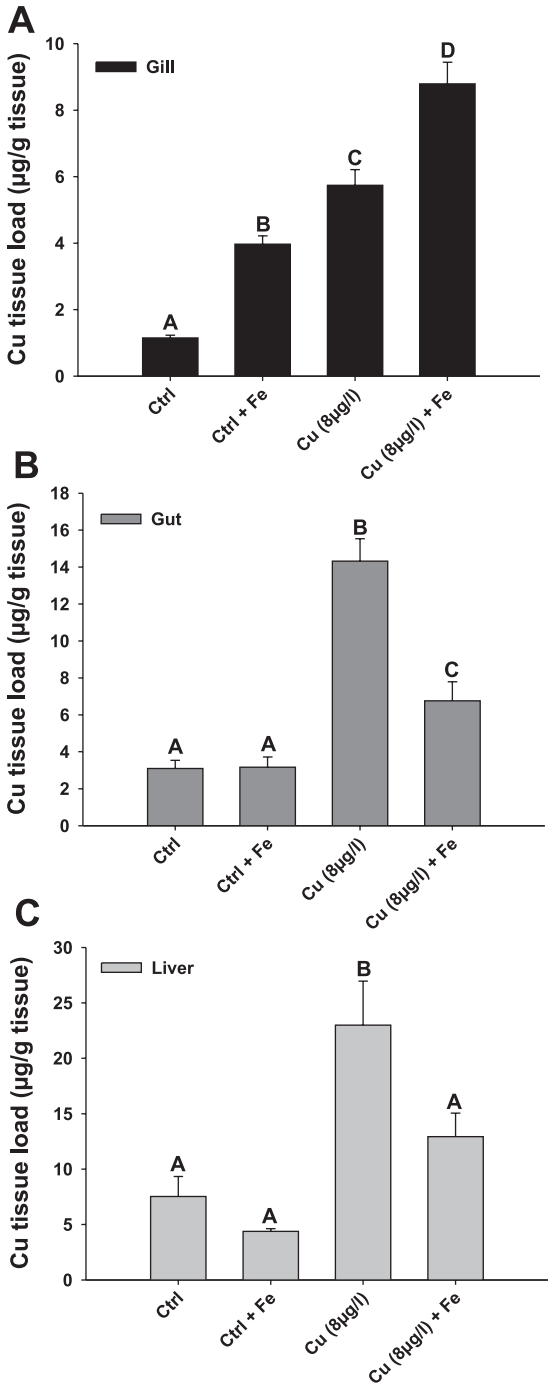

Fig. 1. Copper (Cu) load (μg/g tissue) in gill (*A*), gut (*B*), and liver (*C*) tissue from soft water acclimated zebrafish exposed to control, control + Fe diet, 8 μg/l waterborne Cu, and 8 μg/l waterborne Cu + Fe diet for 21 days. Values are presented as means ± SE, and treatments that do not share a common letter are significantly different from each other ($n = 7$ zebrafish for all treatments, $P < 0.05$).

increased protein turnover rate under stressful conditions (Figs. 6 and 7).

Cu homeostasis in fish is tightly regulated, and as in higher vertebrates, such as mammals, excess Cu is accumulated in the liver and excreted in the bile (22). Likewise, in zebrafish, we saw an elevated Cu load in the liver of fish exposed to increased waterborne Cu (Fig. 1*C*). However, we also saw increases in Cu load in the gut under these conditions (Fig. 1*B*). Waterborne Cu has two possible modes of uptake, either through the gills or the gut, and excess Cu is excreted through the bile (22). Increased Cu load in the gut may be primarily because of increased biliary excretion of Cu. Furthermore, at 8 μg/l waterborne Cu, we saw elevated Cu load in the gills. Grosell and Wood (23) demonstrated that gill Cu load rapidly

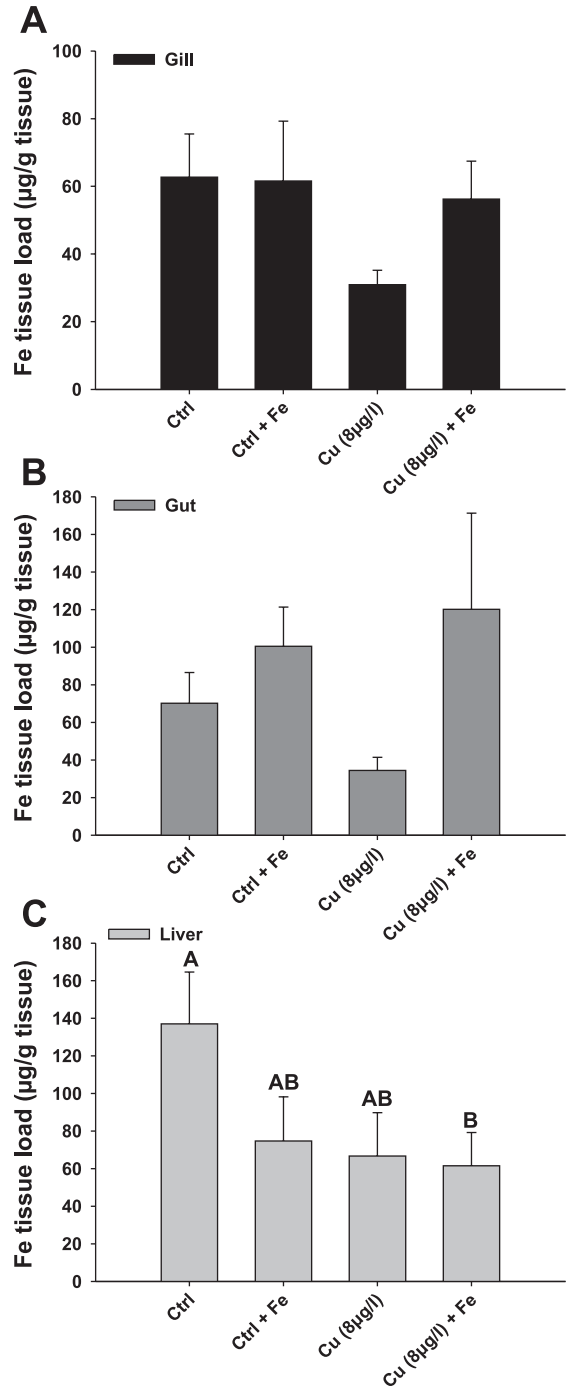

Fig. 2. Iron (Fe) load (μg/g tissue) in gill (*A*), gut (*B*), and liver (*C*) tissue from soft water acclimated zebrafish exposed to control, control + Fe diet, 8 μg/l Cu, and 8 μg/l Cu + Fe diet for 21 days. Values are presented as means ± SE, and treatments that do not share a common letter are significantly different from each other ($n = 7$ for all treatments, $P < 0.05$).

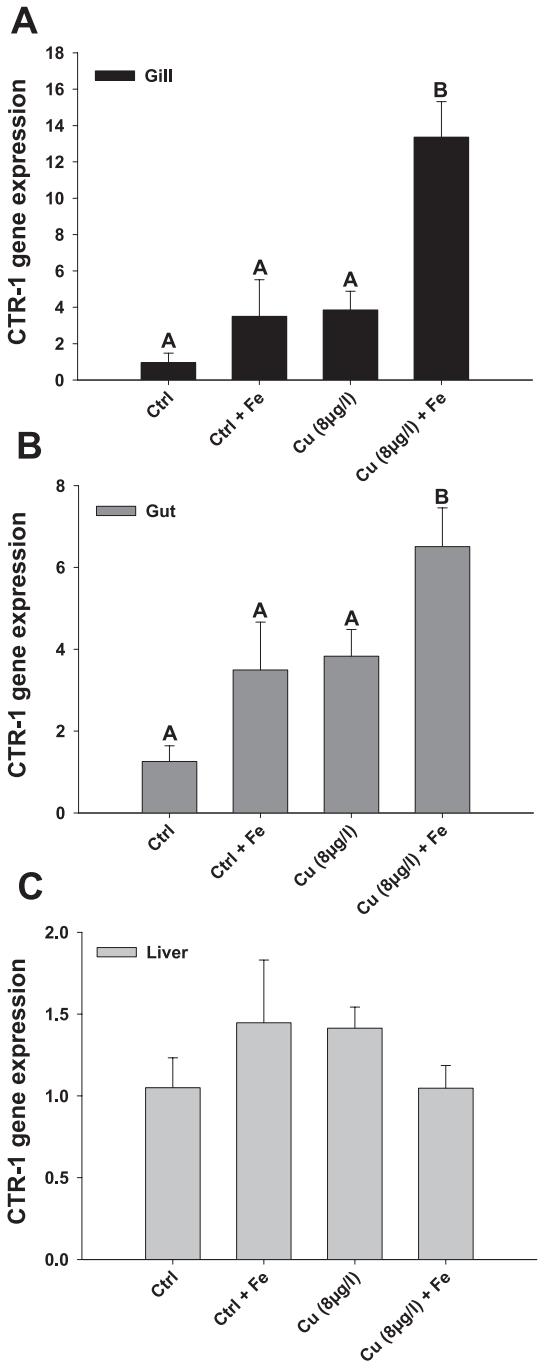

Fig. 3. Gene expression of copper transporter (CTR)-1 in the gill (*A*), gut (*B*), and liver (*C*) tissue from soft water acclimated zebrafish exposed to control, control + Fe diet, 8 μg/l Cu, and 8 μg/l Cu + Fe diet for 21 days. Gene expression values were normalized to elongation factor (EF)-1α, and are presented as means ± SE [arbitrary units (AU)]; treatments that do not share a common letter are significantly different from each other ($n = 6$ for all treatments, $P < 0.05$).

peaks with waterborne Cu exposure after the first 3 h and then gradually reaches equilibrium, which supports our findings at a moderate Cu exposure.

In our initial pilot experiment of exposing zebrafish to 15 μg/l Cu (unpublished observation), we found evidence that fish exhibited significantly lower tissue Fe load in both the gill and liver. Although there is little prior evidence in fish to suggest Cu would have such an effect, there is the likelihood that high waterborne Cu can outcompete Fe for apical uptake at the gill, since they can use the same transporter, DMT-1 (24). With zebrafish exposed to both moderate waterborne Cu (8 μg/l) and Fe diet, we were able to reverse the diminished Fe tissue levels, although a high-Fe diet significantly increased the Cu load in the gills both with and without waterborne Cu (Fig. 1*A*). Potentially, this is an adaptive response to an elevated Fe load,

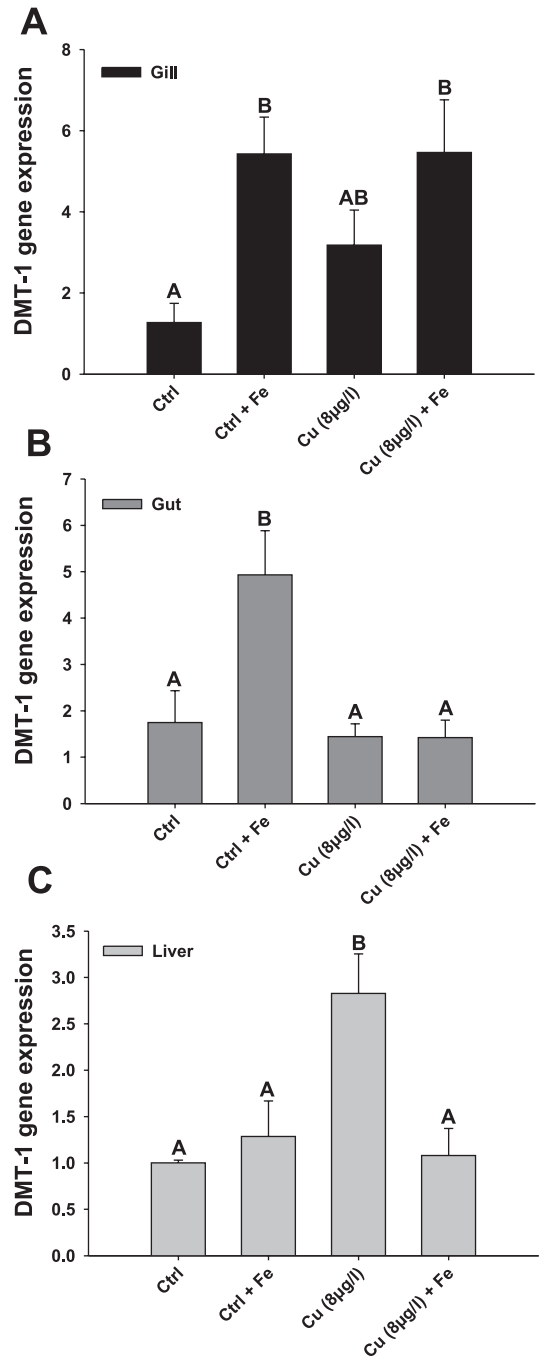

Fig. 4. Gene expression of divalent metal transporter (DMT-1) in the gill (*A*), gut (*B*), and liver (*C*) tissue from soft water acclimated zebrafish exposed to control, control + Fe diet, 8 μg/l Cu, and 8 μg/l Cu + Fe diet for 21 days. Gene expression values were normalized to EF1α and are presented as means ± SE (AU); treatments that do not share a common letter are significantly different from each other ($n = 6$ for all treatments, $P < 0.05$).

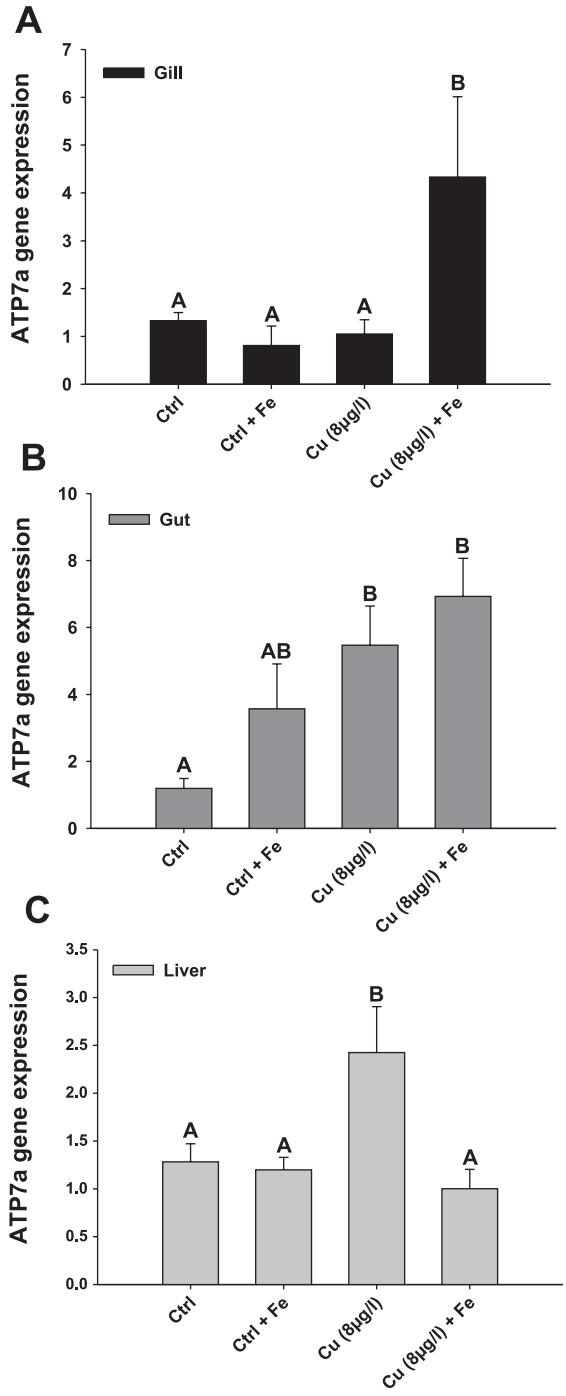

Fig. 5. Gene expression of Cu^{2+}-transporting ATPase, α-polypeptide (ATP7A) in the gill (*A*), gut (*B*), and liver (*C*) tissue from soft water acclimated zebrafish exposed to control, control + Fe diet, 8 μg/l Cu, and 8 μg/l Cu + Fe diet for 21 days. Gene expression values were normalized to EF1α and are presented as means ± SE (AU); treatments that do not share a common letter are significantly different from each other ($n = 6$ for all treatments, $P < 0.05$).

since there are Cu-essential transmembrane ferroxidases, like hephaestin, that function in addition to ferroportin, to translocate Fe across the basolateral membranes in the general circulation, although this protein has not as yet been localized in the zebrafish gill (68).

In an effort to identify potential genetic endpoints of chronic waterborne toxicity, we examined both metal sequestering proteins and several key transporters thought to regulate the uptake of Cu at both the level of the gills and gut. Furthermore, we examined gene expression changes for these transporters in the liver to identify any compensatory responses to toxicity and characterize detoxification methods. Upon examination of the two known MT isoforms, we found that MT2 was more

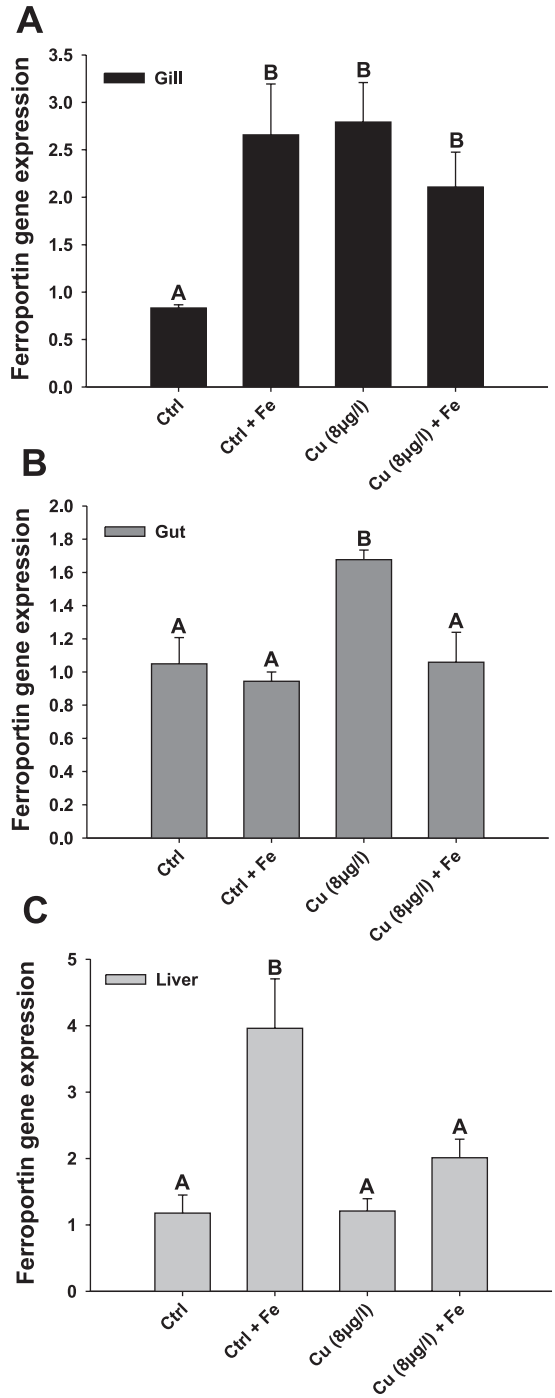

Fig. 6. Gene expression of ferroportin in the gill (*A*), gut (*B*), and liver (*C*) tissue from soft water acclimated zebrafish exposed to control, control + Fe diet, 8 μg/l Cu, and 8 μg/l Cu + Fe diet for 21 days. Gene expression values were normalized to EF1α and are presented as means ± SE (AU); treatments that do not share a common letter are significantly different from each other ($n = 6$ for all treatments, $P < 0.05$).

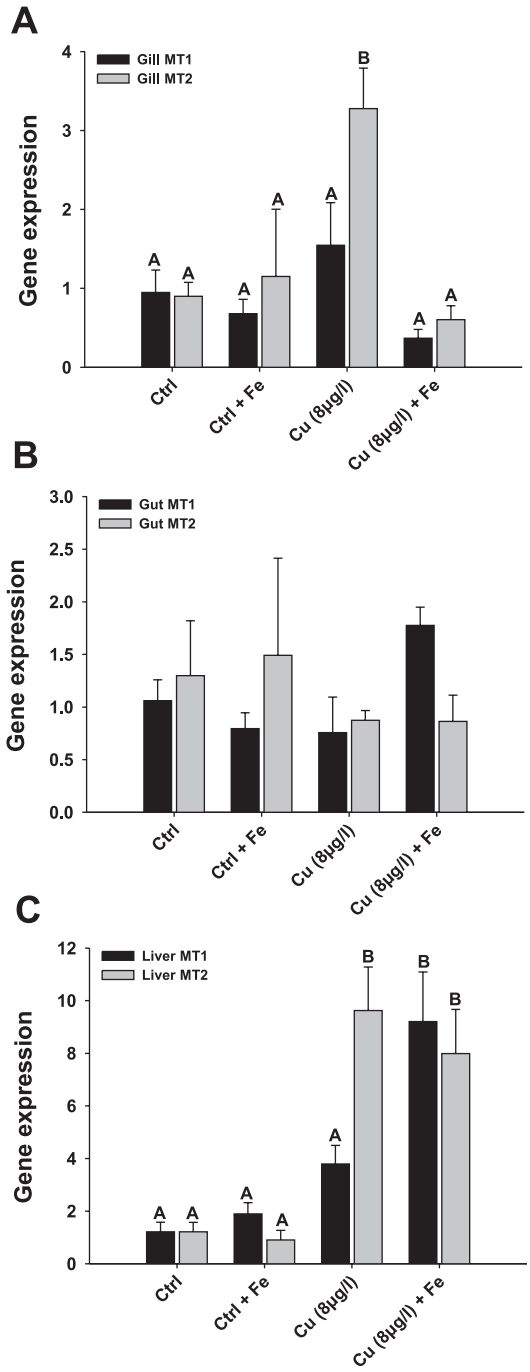

Fig. 7. Gene expression of metallothionein 1 and 2 (MT1 and MT2) in the gill (*A*), gut (*B*), and liver (*C*) tissue from soft water acclimated zebrafish exposed to control, control + Fe diet, 8 μg/l Cu, and 8 μg/l Cu + Fe diet for 21 days. Gene expression values were normalized to EF1α and are presented as means ± SE (AU); treatments that do not share a common letter are significantly different from each other ($n = 6$ for all treatments, $P < 0.05$).

responsive to Cu exposure than MT1 in both gill and liver tissue (Fig. 7, *A* and *C*), where there is the highest accumulation of Cu (Fig. 1, *A* and *C*). Previous studies examining increased zinc exposure demonstrated that MT-1 is more responsive to excessive Zn in rats (40), whereas MT-2 has demonstrated specificity to Cu in blue crabs, although this has not yet been identified in fish species (63). We additionally saw a ninefold increase in expression of MT1 in the liver of zebrafish exposed to both 8 μg/l Cu + high-Fe diet (Fig. 7*C*). Aside from a role in metal sequestration, MTs have been shown to scavenge both hydroxyl radicals and superoxides in mouse and rat liver cells (46). Free cytoplasmic Cu^{2+} and Fe^{2+} are highly reactive ions and can result in the production of superoxide hydroxyl radicals if left unchecked. In the liver of zebrafish, there is an excessive buildup of Cu that may promote increased reactive oxygen species production (12), and this may be further exacerbated by Fe flux through the liver. However, the bottom line is that MT2 has been identified as a Cu-related biomarker, opposite to the current thinking of examining all forms of MTs.

CTR-1 is a high-affinity Cu transporter first cloned in zebrafish by Mackenzie et al. (39). Interestingly, there is no evidence for transcriptional regulation of CTR-1 by Cu in mammals, although protein levels undergo changes in subcellular location and stabilization (35, 54, 65). Our study suggests that CTR-1 is transcriptionally regulated in the gills and gut of zebrafish by moderate Cu + high-Fe diet (Fig. 3, *A* and *B*). Although increased transcription of CTR-1 in a situation of elevated waterborne Cu seems counterintuitive, it again could

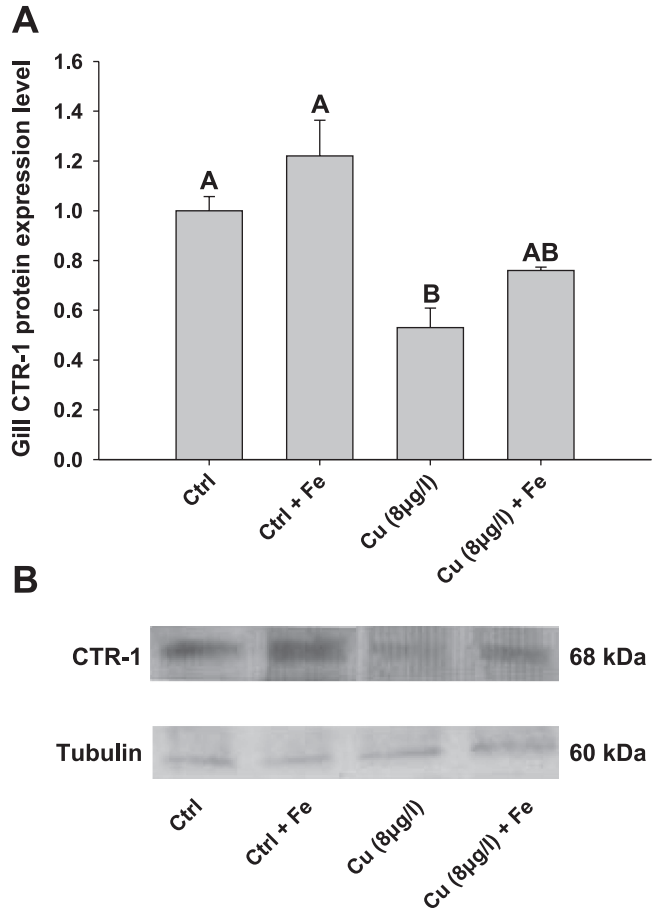

Fig. 8. *A*: relative protein expression of gill CTR-1 from soft water acclimated zebrafish exposed to control, control + Fe diet, 8 μg/l Cu, and 8 μg/l Cu + Fe diet for 21 days. Tubulin was measured to account for differences in protein loading. *B*: representative Western blot of CTR-1 (68 kDa) and protein normalizer tubulin (60 kDa). Values are represented as means ± SE, and treatments that do not share a common letter are significantly different from each other ($n = 8$ for all treatments, $P < 0.05$).

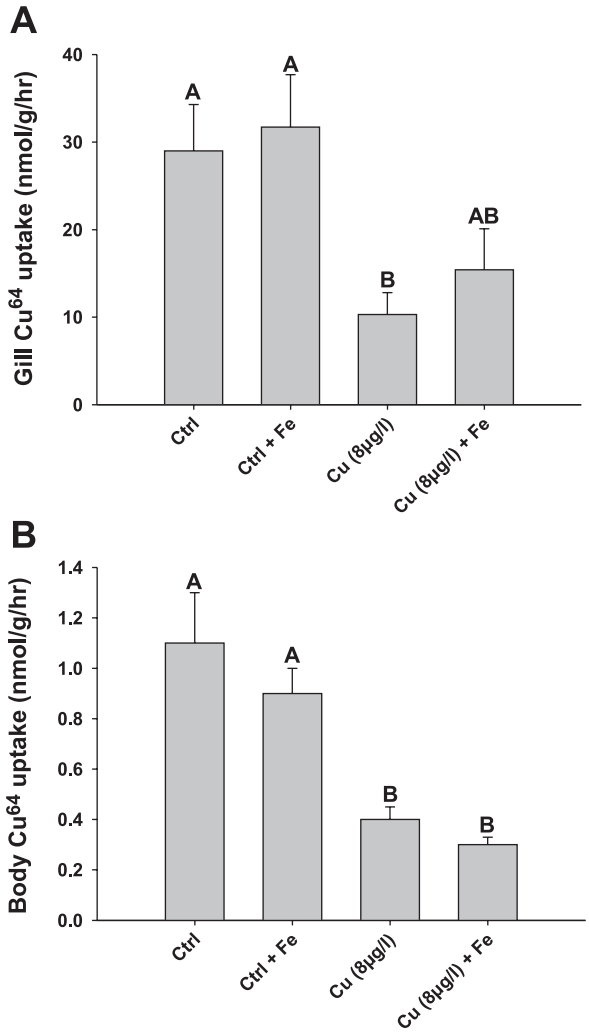

Fig. 9. Gill apical (*A*) and whole body (*B*) uptake rate of ^{64}Cu (20 min uptake exposure) from soft water acclimated zebrafish exposed to control + Fe diet, 8 μg/l Cu, 8 μg/l Cu + Fe diet, and 15 μg/l Cu for 21 days. Gene expression values were normalized to EF1α and are presented as means ± SE (AU). *Treatments that do not share a common letter are significantly different from each other ($n = 6$ for all treatments, $P < 0.05$).

be a compensatory response to promote cellular assimilation and translocation of essential metals. Cu is transported from the cell in the bloodstream through two potential routes, either direct secretion via ATP7A or incorporation into ceruloplasmin (44, 67). Ceruloplasmin can act as a ferroxidase, oxidizing Fe^{2+} to Fe^{3+}, which allows for Fe delivery to peripheral organs for use or detoxification (26, 29). Therefore, to respond to an increased Fe diet, more Cu needs to be absorbed to aid in transport and oxidization of Fe.

DMT-1 has primarily been identified as an Fe transporter, although it does have the potential to transport other divalent metals (24, 60) and has been identified in numerous fish species, including zebrafish (18). In *Xenopus* oocytes, DMT-1 appears to transport a diverse range of metals such as Pb^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} (24). Recently, it has been implicated in the dietary uptake of Cu and zinc in rainbow trout intestine (48, 50). In similar fashion to CTR-1, we saw a significant increase in DMT-1 expression in the gills and gut of zebrafish exposed to a high-Fe diet (Fig. 4, *A* and *B*), suggesting increased Fe uptake, although we did not see any substantial increase in tissue Fe load (Fig. 2, *A* and *B*). Cooper et al. (11) have shown that a low-Fe diet results in increased DMT-1 expression as an adaptive response to absorb more Fe from a deficient diet. In our study, we found that DMT-1 expression increased with a high-Fe diet in the gut and gills (Fig. 4, *A* and *B*). There was also a significant increase in DMT-1 expression in zebrafish exposed to 8 μg/l Cu + high-Fe diet in the gills, although there were no changes in expression in the gut in this treatment (Fig. 4, *A* and *B*). Potentially, zebrafish exposed to high waterborne Cu are reducing the uptake of Cu in the gut by decreasing the number of uptake pathways. In the liver, we see a significant increase in DMT-1 only in zebrafish exposed to 8 μg/l waterborne Cu (Fig. 4*C*). In fish, Cu is transported through the blood via ceruloplasmin (53), and, as seen in mammalian liver cell suspensions, Cu bound to ceruloplasmin is taken up in hepatocytes by a ceruloplasmin receptor (64). However, in cases of excessive Cu exposure, free Cu ion levels can increase in the blood serum, although this phenomenon has only been identified in mammals (6, 15). Although there was no change in CTR-1 expression in the liver (Fig. 3*C*), the increase in DMT-1 expression (Fig. 4*C*) may allow for the uptake of excessive Cu ions from the blood serum in the liver hepatocytes for sequestration and excretion.

In a manner similar to DMT-1, ferroportin, a known Fe exporter, is modulated by both Cu and Fe (Fig. 6). The function of ferroportin as a Fe exporter has been studied in mammalian and amphibian models and has shown increased expression levels upon exposure to excessive exogenous Fe and Cu (9, 17, 42). Likewise, we saw increased expression in the gut when zebrafish were exposed to 8 μg/l Cu alone and a fourfold increased expression in the liver of zebrafish fed a high-Fe diet in the absence of Cu (Fig. 6, *B* and *C*). Furthermore, there were significant increases in ferroportin expression in the gills of zebrafish exposed to either 8 μg/l Cu alone or Fe diet alone (Fig. 6*A*). In *Xenopus* oocytes and mammalian macrophages, excessive Fe results in increased ferroportin expression and protein levels to offload excessive Fe, which can increase levels of reactive oxygen species (9, 17, 42). However, in our fish, we saw that, in the liver, there was an increased ferroportin expression when fish were exposed to a high-Fe diet alone, even though there was no accumulation of Fe in the liver (Fig. 2*C*). Indeed, under all experimental conditions, there was a depression of Fe levels in the liver, which indicates that the liver functions to offload excessive Fe in these situations, yet we did not see the respective changes in gene expression, except under exposure of a high-Fe diet (Fig. 6*C*). Furthermore, we saw that Cu can stimulate ferroportin expression in the gills and gut (Fig. 6, *A* and *B*), as previously demonstrated (9, 17, 42). In mammalian macropahges, increased Cu not only increased expression levels of ferroportin gene and protein, it also produced a dose-dependent excretion of Fe (9). This supports the model that Cu plays an essential role in Fe cycling and transport and also eludes to ferroportin having the potential to transport Cu and other divalent metals as well.

Cu metabolism and export from the cell rely on important Cu-transporting ATPases. In mammals, there are two types of ATPases [ATP7A (Menkes gene) and ATP7B (Wilson's gene)] that are associated with human genetic disorders of the same name (37). In mammals, ATP7A is found through all

tissues, except the liver, where only ATP7B is found (32). Barnes et al. (3) demonstrated that ATP7A has high turnover rates and can transport more Cu per minute than ATP7B. In fish, little is known about that localization and characterization of Cu-transporting ATPases, although only the ATP7A gene has been identified in the zebrafish (43). Furthermore, although the ATP7B gene has been identified in the zebrafish genome through predicted computational sequencing, there is no published RNA sequence in Genbank, which hints at a functional loss of the ATP7B gene. Our study suggests that ATP7A is important in basolateral transport of Cu out of the cells of the gill and gut under conditions of moderate Cu + high-Fe diet (Fig. 5, *A* and *B*). In addition, there were significant increases in ATP7A in the gut and liver under conditions of moderate Cu exposure (Fig. 5, *B* and *C*). Two plausible explanations for the increase in ATP7A are *1*) Cu plays a vital role in the oxidation of Fe ions, and this relates to the increased expression of ATP7A, since this transporter also acts to shuttle Cu to the Golgi apparatus to be incorporated in secreted cuproenzymes (44); and *2*) increased cellular Cu is known to increase reactive oxygen species, and this results in increased oxidative damage (12, 16). Therefore, increased basolateral transport of Cu would help remove Cu from the cell and, in the case of the liver, allow for the excretion of excessive Cu, although this requires further investigation.

Contrary to our findings that CTR-1 expression remained stable following exposure to 8 μg/l Cu and actually increased after treatment with 8 μg/l Cu + high-Fe diet (Fig. 3*A*), we found a significant reduction in both apical uptake (in the 8 μg/l Cu + high-Fe diet exposure) and whole body incorporation of waterborne Cu (in both treatments) and reduced protein expression levels of CTR-1 in the gill (Figs. 8 and 9). Note also that the expression levels of the other two genes likely involved in Cu uptake at the gills (DMT1; Fig. 4*A*) and ATP7A (Fig. 5*A*) remained stable or increased in one or both of these treatments. This is the first study to investigate both the transcription profile and functional uptake of CTR-1 and provides significant insight into predictions of chronic endpoints of Cu toxicity, since increased expression of target genes does not necessarily translate into increased protein levels or increased function.

However, the importance of CTR-1 proteins in Cu transport cannot be disputed, since they are an essential route of Cu uptake for all vertebrates (62). There is obviously some post-transcriptional regulation occurring that downregulates the protein abundance under periods of excessive Cu; however, this requires further investigation. Outside of the above explanations for increased transcription rates of our genes of interest, an alternate explanation is that, under conditions of environmental stress, there is an increased protein turnover rate, which allows for the faster production of new proteins, and faster degradation of harmful proteins (see review in Ref. 25). Furthermore, it has been shown that growth rate is sacrificed as protein turnover rate increases in rainbow trout (41). In all of our fish exposed to waterborne Cu, we see a significant reduction in growth rate compared with those fish under normal circumstances (Table 3). Increased gene expression could in part be explained by increased turnover rates, although this was not directly examined.

Perspectives

Overall, we have demonstrated that alterations in the diet can affect uptake and accumulation of Cu in zebrafish when exposed to elevated waterborne Cu, as well as the gene expression of transport and binding proteins that may be involved. This study provides further evidence that Fe can enhance the uptake of an essential metal Cu (as well as nonessential metals), which has been demonstrated in both fish and mammalian species (11, 34). We have also provided evidence that MT2 can be used as a Cu-specific genetic endpoint of chronic exposure and furthered our knowledge that Cu plays an essential role in Fe cycling and transport by modifying the expression level of ferroportin. Furthermore, this is the first study to examine the CTR-1 protein from both a transcriptional and functional point of view in zebrafish and highlights the importance of functional studies in future research because of the disconnect between message, protein, and functional responses seen, and this has been identified in other studies (e.g., see Ref. 58). Our results suggest that, while transcriptional responses may be of diagnostic importance, they may not relate directly to protein abundance and function.

ACKNOWLEDGMENTS

We thank Peter Chapman (Golder Associates) and 3 anonymous reviewers for providing constructive comments.

GRANTS

This study was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Collaborative Research and Development Grant Program, the International Copper Association, the Copper Development Association, the Nickel Producers Environmental Research Association, the International Lead Zinc Research Organization, the International Zinc Association, Teck Cominco, Xstrata, and Vale Inco. Infrastructure for this work was made possible though Canadian Foundation for Innovation Grants to G. B. McClelland and C. M. Wood. C. M. Wood is supported by the Canada Research Chair Program; G. B. McClelland is supported by an Early Researcher Award from the Ontario Ministry of Research and Innovation. PMC is the recipient of an NSERC postgraduate scholarship.

REFERENCES

1. **Abboud S, Haille DJ.** A novel iron-regulated protein involved in intracellualer iron metabolism. *J Biol Chem* 275: 19906–19912, 2000.
2. **Baldisserotto B, Kamunde C, Matsuo A, Wood CM.** A protective effect of dietary calcium against acute waterborne cadmium uptake in rainbow trout. *Aquat Toxicol* 67: 57–73, 2004.
3. **Barnes N, Tsivkovskii R, Tsivkovskaia N, Lutsenko S.** The copper-transporting ATPases, menkes and wilson disease proteins, have distinct roles in adult and developing cerebellum. *J Biol Chem* 280: 9640–9645, 2005.
4. **Bury NR, Wood CM.** Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. *Am J Physiol Regul Integr Comp Physiol* 277: R1385–R1391, 1999.
5. **Bury NR, Grosell M.** Waterborne iron acquisition by a freshwater teleost fish, zebrafish *Danio rerio*. *J Exp Biol* 206: 3529–3535, 2003.
6. **Cauza E, Maier-Dobersberger T, Polli C, Kaserer K, Kramer L, Ferenci P.** Screening for Wilson's disease by serum ceruloplasmin, *J. Hepatol* 27: 358–362, 1997.
7. **Chen WY, John JA, Lin CH, Chang CY.** Expression pattern of metallothionein, MTF-1 nuclear translocation, and its dna-binding activity in zebrafish (*Danio rerio*) induced by zinc and cadmium. *Environ Toxicol Chem* 26: 110–117, 2007.
8. **Chowdhury MJ, Bucking C, Wood CM.** Pre-exposure to waterborne nickel downregulates gastrointestinal nickel uptake in rainbow trout: indirect evidence for nickel essentiality. *Environ Sci Technol* 42: 1359–64, 2008.
9. **Chung J, Haile DJ, Wessling-Resnick M.** Copper-induced ferroportin expression in J774 macrophages is associated with increased iron efflux. *Proc Natl Acad Sci USA* 101: 2700–2705, 2004.

10. **Clearwater SJ, Farag AM, Meyer JS.** Bioavailability and toxicity of dietborne copper and zinc to fish. *Comp Biochem Physiol C Toxicol Pharmacol* 132: 269–313, 2002.
11. **Cooper CA, Handy RD, Bury NR.** The effects of dietary iron concentration on gastrointestinal and branchial assimilation of both iron and cadmium in zebrafish (*Danio rerio*). *Aquat Toxicol* 79: 167–175, 2006.
12. **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.
13. **Craig PM, Wood CM, McClelland GB.** Gill membrane remodeling with soft-water acclimation in zebrafish (*Danio rerio*). *Physiol Genomics* 30: 53–60, 2007.
14. **Dallinger R, Lagg B, Egg M, Schipflinger R, Chabicovsky M.** Cd accumulation and Cd-metallothionein as a biomarker in *Cepaea hortensis* (Helicidae, Pulmonata) from laboratory exposure and metal-polluted habitats. *Ecotoxicology* 13: 757–772, 2004.
15. **DiDonato Sarkar BM.** Copper transport and its alterations in Menkes and Wilson disease, *Biochim Biophys Acta* 1360: 3–16, 1997.
16. **DiGiulio R, Washburn P, Wenning R, Winston G, Jewell C.** Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environ Toxicol Chem* 8: 1103–1123, 1989.
17. **Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, Law TC, Brugnara C, Lux SE, Pinkus GS, Pinkus JL, Kingsley PD, Palls J, Fleming MD, Andrews NC, Leonard IZ.** Positional cloning of zebrafish ferroportin 1 idnetifies a conserved vertebrate iron exporter. *Nature* 403: 776–781, 2000.
18. **Donovan A, Brownlie A, Dorschner MO, Zhou Y, Pratt SJ, Paw BH, Phillips RB, Thisse C, Thisse B, Zon LI.** The zebrafish mutant gene chardonnay (cdy) encodes divalent metal transporter 1 (DMT1). *Blood* 100: 4655–4659, 2002.
19. **Eddy FB, Lomholt JP, Weber RE, Johansen K.** Blood respiratory properties of rainbow trout (*Salmo gairdneri*) kept in water of high CO_2 tension. *J Exp Biol* 67: 37–47, 1977.
20. **Fairbanks VF.** Copper-induced hemolysis (Abstract). *N Engl J Med* 276: 1209, 1967.
21. **Gonzalez P, Baudrimont M, Boudou A, Bourdineaud JP.** Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (*Danio rerio*). *Biometals* 19: 225–235, 2006.
22. **Grosell M, Hansen HJ, Rosenkilde P.** Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne Cu exposure. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 120: 295–305, 1998.
23. **Grosell M, Wood CM.** Copper uptake across rainbow trout gills: mechanisms of apical entry. *J Exp Biol* 205: 1179–1188, 2002.
24. **Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA.** Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388: 482–488, 1997.
25. **Hawkins AJS.** Protein turnover: a functional appraisal. *Funct Ecol* 5: 222–233, 1991.
26. **Hellman NE, Gitlin JD.** Ceruloplasmin metabolism and function. *Annu Rev Nutr* 22: 439–458, 2002.
27. **Kagi JH, Schaffer A.** Biochemistry of metallothionein. *Biochemistry* 27: 8509–8515, 1988.
28. **Kamunde C, Clayton C, Wood C.** Waterborne versus dietary copper uptake in rainbow trout and the effects of previous waterborne copper exposure. *Am J Physiol Regul Integr Comp Physiol* 283: R69–R78, 2002.
29. **Kaplan J.** Mechanisms of cellular iron acquisition: another iron in the fire. *Cell* 111: 603–606, 2002.
30. **Knapen D, Reynders H, Bervoets L, Verheyen E, Blust R.** Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. *Aquat Toxicol* 82: 163–172, 2007.
31. **Knöpfel M, Schulthess G, Funk F, Hauser H.** Characterization of an integral protein of the brush border membrane mediating the transport of divalent metal ions. *Biophys J* 79: 874–884, 2000.
32. **Kuo YM, Gitschier J, Packman S.** Developmental expression of the mouse mottled and toxic milk genes suggests distinct functions for the Menkes and Wilson disease copper transporters. *Hum Mol Genet* 6: 1043–1049, 1997.
33. **La Fontaine S, Mercer JF.** Trafficking of the copper-ATPases, ATP7A and ATP7B: role in copper homeostasis. *Arch Biochem Biophys* 463: 149–167, 2007.
34. **Leazer TM, Liu Y, Klaassen CD.** Cadmium absorption and its relationship to divalent metal transporter-1 in the pregnant rat. *Toxicol Appl Pharmacol* 185: 18–24, 2002.
35. **Lee J, Prohaska JR, Dagenais SL, Glover TW, Thiele DJ.** Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. *Gene* 254: 87–96, 2000.
36. **Lee J, Peña MM, Nose Y, Thiele DJ.** Biochemical characterization of the human copper transporter Ctr1. *J Biol Chem* 277: 4380–4387, 2002.
37. **Linz R, Lutsenko S.** Copper-transporting ATPases ATP7A and ATP7B: cousins, not twins. *J Bioenerg Biomembr* 39: 403–407, 2007.
38. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2[-Delta Delta C(T)] method. *Methods* 25: 402–408, 2001.
39. **Mackenzie NC, Brito M, Reyes AE, Allende ML.** Cloning, expression pattern and essentiality of the high-affinity copper transporter 1 (ctr1) gene in zebrafish. *Gene* 328: 113–120, 2004.
40. **Mamdouh MA, Frei E, Straub J, Breuer A, Wiessler M.** Induction of metallothionein by zinc protects from daunorubicin toxicity in rats. *Toxicology* 179: 85–93, 2002.
41. **McCarthy ID, Houlihan DF, Carter CG.** Individual variation in protein turnover and growth efficiency in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Proc R Soc Lond B* 257: 141–147, 1994.
42. **McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW, Simpson RJ.** A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell* 5: 299–309, 2000.
43. **Mendelsohn BA, Yin C, Johnson SL, Wilm TP, Solnica-Krezel L, Gitlin JD.** Atp7a determines a hierarchy of copper metabolism essential for notochord development. *Cell Metab* 4: 155–162, 2006.
44. **Mercer JF, Llanos RM.** Molecular and cellular aspects of copper transport in developing mammals. *J Nutr* 133, *Suppl* 1: 1481S–1484S, 2003.
45. **Mertz W.** The essential trace elements. *Science* 213: 1332–1338, 1981.
46. **Min KS, Nishida K, Onosaka S.** Protective effect of metallothionein to ras DNA damage induced by hydrogen peroxide and ferric ion-nitrilotriacetic acid. *Chem Biol Interact* 122: 137–152, 1999.
47. **Miyajima H, Nishimura Y, Mizoguchi K, Sakamoto M, Shimizu T, Honda N.** Familial apoceruloplasmin deficiency associated with blepharospasm and retinal degeneration. *Neurology* 37: 761–767, 1987.
48. **Nadella SR, Grosell M, Wood CM.** Mechanisms of dietary Cu uptake in freshwater rainbow trout: evidence for Na-assisted Cu transport and a specific metal carrier in the intestine. *J Comp Physiol [B]* 177: 433–446, 2007.
49. **Neumann NF, Galvez F.** DNA microarrays and toxicogenomics: applications for ecotoxicology? *Biotechnol Adv* 20: 391–419, 2002.
50. **Ojo AA, Nadella SR, Wood CM.** In vitro examination of interactions between copper and zinc uptake via the gastrointestinal tract of the rainbow trout (*Oncorhynchus mykiss*). *Arch Environ Contam Toxicol* 89: 55–64, 2008.
51. **Olsvik PA, Hindear K, Zachariassen KE, Andersen RA.** Brown trout (*Salom trutta*) metallothioneins as biomarkers for metal exposure in two Norwegian rivers. *Biomarkers* 6: 274–288, 2001.
52. **Osaki S, Johnson DA.** Mobilization of liver iron by ferroxidase (ceruloplasmin). *J Biol Chem* 244: 5757–5758, 1969.
53. **Parvez S, Sayeed I, Pandey S, Ahmad A, Bin-Hafeez B, Haque R, Ahmad I, Raisuddin S.** Modulatory effect of copper on nonenzymatic antioxidants in freshwater fish *Channa punctatus* (Bloch.). *Biol Trace Elem Res* 93: 237–248, 2003.
54. **Petris MJ, Voskoboinik I, Cater M, Smith K, Kim BE, Llanos RM, Strausak D, Camakaris J, Mercer JF.** Copper-regulated trafficking of the Menkes disease copper ATPase is associated with formation of a phosphorylated catalytic intermediate. *J Biol Chem* 277: 46736–46742, 2002.
55. **Playle R, Gensemer R, Dixon D.** Copper accumulation on the gills of fathead minnows: Influence of water hardness, complexation and pH of the gill micor-environment. *Environ Toxicol Chem* 11: 381–391, 1992.
56. **Playle R, Dixon D, Burnison K.** Copper and cadmium binding to fish gills: estimates of metal-stability constants and modelling of metal accumulation. *Can J Fish Aquat Sci* 50: 2678–2687, 1993.
57. **Rawat M, Moturi MCZ, Subramanian V.** Inventory compilation and distribution of heavy metals in wastewater from small-scale industrial areas of Delhi, *India. J Environ Monit* 5: 906–912, 2003.

58. **Richards JG, Wang YS, Bauner CJ, Gonzalez ML, Patrick PM, Schulte PM, Chippari-Gomes Almeida-Val VM, Val AL.** Metabolic and ionoregulatory responses of the Amazonian cichlid, Astronotus ocellatus, to severe hypoxia. *J Comp Physiol B* 177: 361–374, 2007.
59. **Rozen S, Skaletsky HJ.** Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, edited by Krawetz S and Misener S. Totowa, NJ: Humana, 132: 365–386. 2000.
60. **Savigni DL, Morgan EH.** Transport mechanisms for iron and other transition metals in rat and rabbit erythroid cells. *J Physiol* 508: 837–850, 1998.
61. **Sharp PA.** CTR1 and its role in body copper homeostasis. *J Biochem Cell Bio* 35: 288–291, 2002.
62. **Sharp PA.** The molecular basis of copper and iron interactions. *P Nutr Soc* 63: 563–569, 2004.
63. **Syring RA, Hoexum Brouwer T, Brouwer M.** Cloning and sequencing of cDNAs encoding for novel copper-specific metallothionein and two cadmium-inducible metallothioneins from the blue crab *Callinectes sapidus. Comp Biochem Physiol C* 125: 325–332, 2000.
64. **Tavassoli M, Kishimoto T, Kataoka M.** Liver endothelium mediates the hepatocyte's uptake of ceruloplasmin. *J Cell Biol* 102: 1298–1303, 1986.
65. **Tennant J, Stansfield M, Yamaji S, Srai SK, Sharp P.** Effects of copper on the expression of metal transporters in human intestinal Caco-2 cells. *FEBS Lett* 527: 239–244, 2002.
66. **USEPA.** *Aquatic Life Ambient Freshwater Quality Criteria-Copper 2007 Revision*. EPA-822-R-07–001, 2007. http://www.epa.gov/waterscience/criteria/copper/2007/index.htm.
67. **Voskoboinik I, Camakaris J.** Menkes copper-translocating P-type ATPase (ATP7A): biochemical and cell biology properties, and role in Menkes disease. *J Bioenerg Biomembr* 34: 363–371, 2002.
68. **Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ.** Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nat Genet* 21: 195–199, 1999.