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# Gene expression endpoints following chronic waterborne copper exposure in a genomic model organism, the zebrafish, *Danio rerio*

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**Craig PM, Hogstrand C, Wood CM, McClelland GB.** Gene expression endpoints following chronic waterborne copper exposure in a genomic model organism, the zebrafish, *Danio rerio*. *Physiol Genomics* 40: 23–33, 2009. First published September 29, 2009; doi:10.1152/physiolgenomics.00089.2009.—Although copper (Cu) is an essential micronutrient for all organisms, in excess, waterborne Cu poses a significant threat to fish from the cellular to population level. We examined the physiological and gene expression endpoints that chronic waterborne Cu exposure (21 d) imposes on soft-water acclimated zebrafish at two environmentally relevant concentrations: 8 µg/l (moderate) and 15 µg/l (high). Using a 16,730 65-mer oligonucleotide customized zebrafish microarray chip related to metal metabolism and toxicity to assess the transcriptomic response, we found that 573 genes in the liver responded significantly to Cu exposure. These clustered into three distinct patterns of expression. There was distinct upregulation of a majority of these genes under moderate Cu exposure and a significant downregulation under high Cu exposure. Microarray results were validated by qPCR of eight genes; two genes, metallothionein 2 (mt2) and Na<sup>+</sup>-K<sup>+</sup>-ATPase 1a1 (atp1a1), displayed increased expression under both Cu exposures, indicative of potential genetic endpoints of Cu toxicity, whereas the remaining six genes demonstrated opposing effects at each Cu exposure. Na<sup>+</sup>-K<sup>+</sup>-ATPase enzyme activity decreased during Cu exposure, which may be linked to Cu's competitive effects with Na<sup>+</sup>. Whole body cortisol levels were significantly increased in Cu-exposed fish, which prompted an analysis of the promoter region of all significantly regulated genes for glucocorticoid (GRE) and metal (MRE) response elements to dissociate metal- and stress-specific gene responses. Of the genes significantly regulated, 30% contained only a GRE sequence, whereas 2.5% contained only a consensus MRE. We conclude that the indirect effects of Cu exposure regulate gene expression to a much greater degree than the direct effects.

microarray; cortisol; glucocorticoid response element; metal response element

NOW COMMONPLACE, MICROARRAYS are used to identify the effects of environmental stressors on both human and nonhuman organisms to ascertain the global genetic response to a particular threat (1, 39, 50). When used effectively, microarrays can elucidate major and minor biological pathways that define environment influences on an organism, and the enormous amount of data generated can be amalgamated into databases for use in legislative and environmental protective standards. Genetic markers of environmental pollution are essential to identify detrimental effects to populations, where phenotypic responses such as reduced reproductive capacity or altered morphology are not immediately evident (60, 62).

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Organisms can react to environmental stressors with a biphasic response, as first identified by Selye (52) and recently confirmed through meta-analysis of dose-response curve studies (4). Even though this phenomenon is known to exist, chronic effects at low and high doses of particular toxicants have been little studied. Our primary objective was to examine the differential genetic expression between environmentally relevant moderate and high chronic waterborne doses of copper (Cu) in zebrafish.

Zebrafish are particularly useful in the study of metal contaminants, due to their softwater tolerance, key for metal-specific effects without the interference of competitive cations (8, 9, 40, 43, 44). Although an essential metal, in excess Cu has a variety of detrimental effects, impacting ionoregulation in the shorter term, growth in the longer term, and endocrine disruption (13, 38, 66). Furthermore, excess Cu can cause a cascade of cellular damage through Fenton-like reactive oxygen species (ROS) generating reactions. Given such a broad range of possible effects, microarrays may offer an ideal tool to identify specific targets of chronic Cu toxicity at the level of gene transcription. To date, no study has examined the chronic effects on zebrafish of varying toxic doses of waterborne Cu using a microarray approach.

Cu, in similar fashion to many other environmental toxicants, elicits a general stress response, indicated by increased circulating cortisol levels (see review Ref. 18). In fact, many of the physiological responses to Cu may be due, in part, to the elevated circulating cortisol levels that can alter enzyme activity and protein synthesis rates (33, 57–59). Although beneficial in protecting an organism against an oncoming threat, chronic stress is known to be deleterious (45). Cortisol acts on target cells by binding to cytosolic receptors, which are translocated into the nucleus and bind to glucocorticoid response elements (GRE) with the consensus sequence 5'-GGTCANNNTGT-TCT-3' (27, 28). Subsequent consensus sequences, including a mineralocorticoid response element, have been identified in various nonmammalian species (e.g., *Carassius cuvieri*; Ref. 48) with response to cortisol stimulation.

Along with the general stress response, transcription can be stimulated by a direct metal response. Cu is known to stimulate metal-regulatory transcription factor 1 (MTF1), which, in turn, activates the DNA-binding region to specifically bind metal response elements (MRE), containing a consensus sequence of 5'-TGCRCNC-3' and initiating a transcriptional response (20, 51). Very recently, Hogstrand et al. (22) performed an analysis of MREs in the promoter region of zebrafish genes significantly altered by zinc exposure and found >44% of significantly regulated genes contained one or more putative MREs. This analysis was based on results obtained using the same custom-designed zebrafish arrays used in this study. Therefore,

our second objective was to identify gene transcription responses to Cu associated with potential GRE and MRE activation. The overall aim of this study was to investigate both direct and indirect effects of Cu exposure by using promoter region analysis of both GRE and MRE motifs, to isolate genetic endpoints solely related to chronic Cu stress, as opposed to combinational endpoints of chronic stress and metal exposure.

Naturally occurring levels of Cu found in lakes and rivers range from 0.2 to 30  $\mu\text{g/l}$ , while 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 (19, 49). We assessed the transcriptomic changes of softwater-acclimated zebrafish associated with chronic exposure to two environmentally relevant waterborne Cu levels, a moderate ( $\sim 8 \mu\text{g/l}$ ) and high ( $\sim 15 \mu\text{g/l}$ ) Cu dose, for a period of 21 days. The present study employed a custom designed 16,399 65-mer oligonucleotide zebrafish microarray chip with an additional 331 customized oligonucleotides related to metal metabolism and toxicity. Significantly regulated genes were further analyzed using web-based bioinformatics tools to ascertain whether the physiological and genetic changes due to excessive waterborne Cu were due to metal effects, a general stress response, or a combination of these two pathways. Although not the first study to investigate a biphasic response to contaminants (see review Ref. 5), this study does provide a cautionary message when interpreting results based on single contaminant exposure in whole organisms. Transcriptional responses may only in part be a direct result of the specific threat, and further pathway analysis is required.

## MATERIALS AND METHODS

**Animals.** Adult zebrafish of mixed sex (*Danio rerio*) were purchased from a local distributor (DAP International) and acclimated to soft-water ( $\text{Na}^+ 75.5 \pm 3.6 \mu\text{M}$ ,  $\text{Ca}^{2+} 49.2 \pm 3.7 \mu\text{M}$ ,  $\text{Mg}^{2+} 20.5 \pm 1.5 \mu\text{M}$ , total Cu  $0.044 \pm 0.001 \mu\text{M}$  or  $2.8 \pm 0.1 \mu\text{g/l}$ ; pH 6.9) over a 7 day period in an aerated 40-liter aquarium as described previously (8). The hardness of this water, expressed as  $\text{CaCO}_3$  equivalents, was  $\sim 8 \text{ mg/l}$ . After acclimation, zebrafish were housed in multiple 3-liter self-cleaning tanks racked in a soft-water recirculating stand-alone filtration system (AHAB; Aquatic Habitats, Apopka, FL). Fish were fed daily with a commercial tropical fish food (Topfin, Phoenix, AZ; ion content:  $8.3 \pm 0.2 \text{ mg Cu/kg food}$ ,  $147.0 \pm 2.3 \text{ mmol Na/kg food}$ ,  $191.0 \pm 6.2 \text{ mmol Ca/kg food}$ ,  $45.4 \pm 0.9 \text{ mmol Mg/kg food}$ ) and maintained on a 12-h light, 12-h dark photoperiod regime. 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.

**Experimental protocol.** Zebrafish ( $n = 120$ , 40/treatment) were weighed and placed in 8-liter aerated, flow-through tanks served with a softwater flow of 25 ml/min. Mariotte bottles were used to dose tanks with Cu (concentrated Cu solution made from  $\text{CuSO}_4$  dissolved in 0.05%  $\text{HNO}_3$ ; control tanks were also dosed with 0.05%  $\text{HNO}_3$  only) to three treatment regimes of either control (background Cu levels of  $1.8 \pm 0.3 \mu\text{g/l}$ ), low Cu ( $8.0 \pm 0.4 \mu\text{g/l}$ ), or high Cu ( $14.4 \pm 0.6 \mu\text{g/l}$ ). The dilute nitric acid had no measurable impact on water pH. Fish were fed 2% body weight once per day of the commercial tropical fish food. Tanks were monitored daily for mortality and cleaned of any food or waste that had accumulated. Each day, a 10-ml water sample was taken from each tank, filtered through a 0.45  $\mu\text{m}$  filtration disc (Pall, East Hills, NY), added to a plastic vial containing

100  $\mu\text{l}$   $\text{HNO}_3$ , and kept at  $4^\circ\text{C}$  for analysis of ion content and Cu concentration. At the end of the exposure period, fish were quickly euthanized by cephalic concussion and sampled for gill, liver, and gut, which were immediately frozen in liquid  $\text{N}_2$  for further analysis of Cu burden and gene expression. Whole fish were also snap-frozen in liquid  $\text{N}_2$  for analysis of whole body cortisol concentration.

**Water and tissue ion and Cu levels.** All tissues ( $n = 7$ /treatment) were first digested in 1 ml of 1 N  $\text{HNO}_3$  for 48 h at  $60^\circ\text{C}$ . Tissue digests were diluted  $10\times$ , and dissolved Cu levels were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z; Varian, Palo Alto, CA) using an appropriately diluted commercial Cu standard (40  $\mu\text{g/l}$ ; Fisher Scientific, Ottawa, ON, Canada). 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%  $\text{HNO}_3$  ( $\text{Na}^+$ ) or 0.5%  $\text{LaCl}_3/1\% \text{HNO}_3$  ( $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ), and verified using certified  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$  standards (1 mg/l diluted in 1%  $\text{HNO}_3$  or 0.5%  $\text{LaCl}_3/1\% \text{HNO}_3$ ; Fisher Scientific).

**Whole body cortisol extraction and measurement.** Whole body cortisol ( $n = 7$ /treatment) was extracted based on combining methods described by Ramsay et al. (47) and Sink et al. (56). In brief, whole frozen zebrafish were weighed, cut into small pieces, and placed in a  $15 \times 85 \text{ mm}$  screw cap test tube. Deionized  $\text{H}_2\text{O}$  (3 ml) was added, and the mixture was homogenized on ice for 45 s using a PowerGen homogenizer (Fisher Scientific). The homogenizer was rinsed with 1 ml diethyl ether (Sigma, St. Louis, MO). To the mixture, 200  $\mu\text{l}$  of food-grade vegetable oil per gram body weight was added prior to the addition of another 7 ml of diethyl ether. The samples were vortexed vigorously for 30 s and then centrifuged at 2,700 rpm for 3 min to separate the aqueous and ether (cortisol containing) phases. Tubes were snap-frozen in liquid nitrogen for 30 s, and the ether phase was poured into a new test tube. This extraction was repeated again, and ether was collected in a tube which was evaporated under a gentle stream of nitrogen for 30 min. The remaining oil + cortisol mixture was stored at  $4^\circ\text{C}$  for no more than 24 h prior to measuring cortisol.

A competitive cortisol radioimmunoassay (RIA) assay was purchased from MP Biomedicals (Solon, OH) to quantify cortisol in all of the fish extracts. We measure 10  $\mu\text{l}$  samples in duplicate following the manufacturer's instructions. As suggested by Sink et al. (56), the vegetable oil used to boost extract volume was tested by RIA to ensure that no cortisol was present. Additional spiked samples were extracted with known cortisol concentrations (50, 25, 12.5 ng/ml), which yielded greater than a 90% recovery. Linearity and parallelism were tested using serial dilutions (0, 25, 50, 75% dilutions) of extracted samples, which gave an  $R^2 = 0.92$ . With the use of pooled samples, intra- and interassay variability was 6.8% ( $n = 9$ ) and 9.7% ( $n = 9$ ), respectively.

**$\text{Na}^+ - \text{K}^+ - \text{ATPase}$  activity.** Tissue  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  (NKA;  $n = 6$ ) activity was determined using the microassay method of McCormick (36). All liver samples were homogenized in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) containing 0.3% Na deoxycholic acid. Both NKA activity and Bradford protein assays (Bio-Rad, Hercules, CA) were run in 96-well format on a Spectra-MAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA).

**Zebrafish oligonucleotide arrays.** The zebrafish oligonucleotide arrays were spotted on UltraGAPS Coated Slides (Corning Life Sciences, Promega), using a Qarray2 robot (Genetix) in the Genomics Centre, King's College London, UK. The 16,399 65-mer oligonucleotides were designed and synthesized by Compugen and Sigma Genesys as a Zebrafish OligoLibrary ready set, which represent 15,806 LEADST clusters plus 171 controls. In addition, 331 customized oligonucleotides were added to the array set, and the array design was submitted to public archive ArrayExpress (<http://www.ebi.ac.uk/microarray-as/ae/>). Also included within the array set were 23 controls from Amersham Lucidea Universal ScorecardTM (Amersham Bio-

sciences, GE Healthcare), which were used to perform a quality control test.

Reporter annotation was adopted from that by Zheng et al. (67). For this reannotation, the oligonucleotide reporters were mapped at the sequence level (using megablast allowing 2 bp mismatches) to Unigene and Ensembl (mapped and predicted), and this allowed implied linkage to ZFIN, Gene Ontology (GO), and human orthologs. Because there is a shortage of functional information for zebrafish genes, and zebrafish share a similar gene set as humans, the functional analysis of genes of interest was performed using a combination of corresponding human orthologs extracted from National Center for Biotechnology Information HomoloGene database using Unigene IDs (with sequence similarity >45%) and functionally identifiable zebrafish genes. Microarray design was based on comparison to a common pooled reference. Treatment samples ( $n = 5/\text{treatment}$ , hence, 5 microarray slides/treatment) were labeled with Cy3 dye, and pooled liver RNA from softwater acclimated zebrafish (the reference pool) was labeled with Cy5.

**RNA extraction, cDNA production, and Cy3 and Cy5 coupling.** Frozen liver tissue (10–20 mg tissue) was homogenized in 800  $\mu\text{l}$  TRIzol reagent (Invitrogen, Carlsbad, CA), incubated at room temperature, mixed with 200  $\mu\text{l}$  chloroform, incubated for a further 3 min, then centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was removed and further purified using the QIAgen RNeasy kit (Mississauga, ON, Canada), following the manufacturer's instructions. A DNase I digest was performed on the column as outlined in the QIAgen protocol to remove any contaminating genomic DNA. RNA concentration was determined on a ND-1000 Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA) to ensure that the concentration was >0.625  $\mu\text{g}/\mu\text{l}$  and that there was no contamination based on A260/A280. Amino-allyl cDNA was synthesized (two tubes: one for each Cy dye) by the addition of 10  $\mu\text{g}$  total RNA, 0.5  $\mu\text{l}$  of both Oligo-dT (3  $\mu\text{g}/\mu\text{l}$ , Invitrogen) and random hexamers (3  $\mu\text{g}/\mu\text{l}$ , Invitrogen), and the volume topped up to 17  $\mu\text{l}$  with DEPC H<sub>2</sub>O. The mix was incubated at 70°C for 15 min, snap cooled, and pulse spun. A master mix of 6  $\mu\text{l}$  5 $\times$  first-strand buffer, 3  $\mu\text{l}$  0.1 M DTT, 0.6  $\mu\text{l}$  50 $\times$  dNTP (no aa-dUTP), 0.9  $\mu\text{l}$  10 mM aa-dUTP, 2  $\mu\text{l}$  Superscript II RT, and 0.5  $\mu\text{l}$  RNase OUT was added to each tube for a final volume of 30  $\mu\text{l}$ , and was incubated at 42°C for 3 h. The RNA was then hydrolyzed with 10  $\mu\text{l}$  1 M NaOH and incubated for 15 min at 65°C. The pH was then neutralized with 10  $\mu\text{l}$  1 M HCl. cDNA was purified by the addition of 40  $\mu\text{l}$  H<sub>2</sub>O, 10  $\mu\text{l}$  3 M NaOAc pH 5.2, and 300  $\mu\text{l}$  of 100% EtOH and allowed to precipitate overnight at –20°C. Samples were spun at 12,000 rpm for 20 min and washed twice with 75% ethanol before continuing. The aa-cDNA was coupled to Cy3 and Cy5 dyes (PA23001 and PA25001; Amersham, GE Healthcare, Piscataway, NJ) in separate tubes by resuspending the air-dried aa-cDNA pellet in 10  $\mu\text{l}$  fresh 0.1 M NaHCO<sub>3</sub> pH 9.0 and added to each ready-made dye aliquot. Samples were incubated in the dark at room temperature for 1 h with shaking at 15 min intervals. Ester-dye coupled aa-cDNA was further purified using QIAquick PCR purification columns (Qiagen) as per the manufacturer's instructions and was eluted in 35  $\mu\text{l}$  of PCR quality H<sub>2</sub>O. Incorporation of dye molecules into aa-cDNA was measured on the Nanodrop spectrophotometer, ensuring an incorporation rate of 30[(cDNA in ng/ $\mu\text{l}$ )/(CyDye in pmol/ $\mu\text{l}$ )\*3.45] and that a concentration >70 pmol of dye was achieved. A final volume of 20  $\mu\text{l}$  (70 pmol of each dye + H<sub>2</sub>O) was used to hybridize to the microarray slides.

**Microarray hybridization.** Prehybridization solution (5 $\times$  SSC, 0.1% SDS, 1% BSA) was prewarmed to 42°C, and microarray slides were immersed for 60 min. Slides were washed twice in 0.1 $\times$  SSC for 30 s, and slides were dried by centrifugation at 1,600 rpm for 2 min. Arrays were kept dust-free and immediately used for hybridization. We combined 20  $\mu\text{l}$  of fresh 2 $\times$  hybridization solution (50% formamide, 10 $\times$  SSC, 0.2% SDS, 0.2 mg/ml salmon sperm) with the 20  $\mu\text{l}$  of combined Cy3 and Cy5 aa-cDNA samples from above, heated to 95°C for 5 min, and pipetted onto the microarray slides. A glass

coverslip was placed on top to ensure even distribution of the hybridization sample. The slides were placed in a closed humidified chamber containing 3 $\times$  SSC and allowed to hybridize overnight in the dark at 42°C. Coverslips were removed and slides serially washed in a progressively diluted solution of 2 $\times$  SSC and 0.1% SDS until a final rinse in H<sub>2</sub>O (5 min each wash with vigorous shaking). After the final wash, slides were dried by centrifugation and stored in light-protected slide boxes until ready to scan.

**Microarray scanning and analysis.** Hybridized slides were scanned at 5  $\mu\text{m}$  with a GenePix 4100A microarray scanner (Axon Instruments, Redwood City, CA). Standard parameters in the GenePix Pro 6.0 software were modified to optimize cut-off signal values while retaining the maximum number of informative spots. Signal data was exported to GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA) software package for Lowess signal normalization after the subtraction of the median background. A total of five slides per treatment were used (5 biological replicates, 15 slides total). The  $P$  value cut-off was  $\alpha = 0.01$  for the ANOVA and post hoc Tukey's tests, and the Benjamini and Hochberg multiple corrections was used, for a resulting 0.05 false discovery rate. A minimal change threshold set to 1.5 was employed to maximize the detection of significant genes in a situation of high biological variability (outbred fish). Significant genes were mapped to their respective human ortholog, and the list was submitted to the Database for Annotated, Visual, and Integrative Discovery (DAVID; <http://david.abcc.ncifcrf.gov/home.jsp>; Refs. 12, 23) for ontological analysis of the significantly overrepresentation of GO biological (general descriptive function) and molecular (detailed descriptive process) terminology. Genes were further clustered into similar patterns of expression based on treatment using the K-mean Pearson centered algorithm found within the Genespring software package. We conducted a GRE and MRE analysis of the promoter region of up- and downregulated Cu-responsive genes. Using the zebrafish Ensembl ID, we were able to extract a 2 kb region upstream of the start codon using the BioMart tool from the Ensembl zebrafish Genome server Zv7. Using consensus sequence matrices found in NUBIScan software (<http://www.nubiscan.unibas.ch/>), we analyzed the 2 kb promoter region for significant GRE hits. There were a total of 257 genes that contained at least one consensus GRE, and significant hits were ranked based on the number of hits and the proximity to the start codon to maximize the likelihood that the gene, in part, is regulated by glucocorticoids. Sequences were also searched for the MRE core consensus, 5'-TGCRNC-3', as well as other MREs contained within the Transfac database (2007) using GCG version 11.1 (Accelrys) software. A total of 99 sequences contained at least one consensus MRE, and significant hits were ranked in the same fashion as GRE sequences. Unique and overlapping genes were submitted to DAVID for ontological analysis of their respective molecular functions. Complete results of this microarray experiment were submitted to the public archive ArrayExpress (<http://www.ebi.ac.uk/microarray-as/ae/>) in accordance with Microarray Gene Expression Data Society recommendations (accession #A-MEXP-1580).

**Microarray validation via qPCR.** Microarray results were validated by real-time quantification of nine genes of interest (primer sequences found in Table 1): Na<sup>+</sup>-K<sup>+</sup>-ATPase isoform 1a (atp1a1), elongation factor 1 alpha (ef1 $\alpha$ ), estrogen receptor 1 (esr1), heat shock transcription factor 2 (hsf2), heat shock protein 60 (hsp60), heat shock protein 90a (hsp90 $\alpha$ ), metallothionein 2 (mt2), vascular endothelial growth factor a (vegfa), and the zinc transporter, zip1 (slc39a1). First-strand cDNA was synthesized from 1  $\mu\text{g}$  of liver total RNA (from above) treated with DNase I (Invitrogen) and reverse transcribed to cDNA using SuperScript II RNase H- reverse transcriptase (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, Canada). Each reaction contained 12.5  $\mu\text{l}$  SYBR green mix, 1  $\mu\text{l}$  of each forward and reverse primer (5  $\mu\text{M}$ ), 5.5  $\mu\text{l}$  RNase-/DNase-free H<sub>2</sub>O, and 5  $\mu\text{l}$  of 5 $\times$  diluted cDNA template. Cycling conditions were as follows: 3 min

Table 1. Forward and reverse primers used for real-time qPCR validation of microarray results

Gene	Primer	Accession No.	Amplicon Size, bp
ATP1a1	F: 5'-CTGCCACTTCAATCTTCCCG-3' R: 5'-TGAAGTGGAAAGTCTCAGGA-3'	NM_131686	51
ESR1	F: 5'-AGAAACACAGCCGGCCCTA-3' R: 5'-TGGTGAGCAGGGACATCATG-3'	NM_152959	51
HSF2	F: 5'-ACGTCCCGCATTTCTTACC-3' R: 5'-TATCCGAATCCTCGACGAGG-3'	NM_131867	50
HSP60	F: 5'-CGCTGTGGAGGAAGGAATTG-3' R: 5'-CAGCGCAGTAAAGCACATCC-3'	NM_181330	50
HSP90a	F: 5'-ATCCGCAAAAACCTGGTCAA-3' R: 5'-TGCCAGTTCCGTTGAAGAGATC-3'	NM_131328	51
MT2	F: 5'-CCCATCTGGTTGCAGCAAGT-3' R: 5'-GAATTGCCTTTGCAGACGC-3'	NM_194273	50
VEGFa	F: 5'-CATCATCCAGGAGTATCCCGA-3' R: 5'-CAGGACGGGATGTACGTGTG-3'	NM_131408	51
ZIP	F: 5'-CGTTTTTCGAGGTTTGGCTA-3' R: 5'-AACACCTTGGCATTCCGTCGT-3'	NM_001013540	50
EF1- $\alpha$	F: 5'-GTGCTGTGCTGATTGTGCT-3' R: 5'-TGTATGCGCTGACTTCCTTG-3'	NM_131263	201

F, forward; R, reverse.

initial denaturation at 95°C, 40 cycles of 95°C for 15 s, 60°C for 45 s, 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 *ef1 $\alpha$* , which did not change over the course of the experiment. Gene expression data were calculated using the  $2^{-\Delta\Delta ct}$  method (30). 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 Primer Express version 2.0 (Applied Biosystems, Foster City, CA).

**Statistical analysis.** Statistical analysis for Cu load, whole body cortisol, and qPCR validation was performed using Sigma Stat (SPSS, Chicago, IL). In particular, a one-way ANOVA and post hoc Tukey's test were used to test for pair-wise significance of waterborne Cu levels, Cu tissue load, whole body cortisol, NKA activity, and *atp1a1* gene expression. Additionally, least squares regression analysis was used for qPCR validation ( $P < 0.05$ ). qPCR data was transformed to  $\log_2$  values to match values obtained from the microarray experiment. All data have been expressed as a mean  $\pm$  SEM.

## RESULTS

Over the 21 day exposure period, we maintained a constant softwater environment (average hardness among treatments:  $8.9 \pm 0.2$  mg/l  $\text{CaCO}_3$ ) throughout all tanks, and only the Cu concentrations were significantly different between control,

Table 2. Concentrations of water ions, copper, pH, and total hardness for all experimental exposures

Treatment	Control	Cu, 8 $\mu\text{g/l}$	Cu, 15 $\mu\text{g/l}$
Na <sup>+</sup>	84.2 $\pm$ 4.5	80.1 $\pm$ 4.8	80.6 $\pm$ 6.4
Mg <sup>2+</sup>	26.7 $\pm$ 1.5	26.8 $\pm$ 1.4	26.6 $\pm$ 1.8
Ca <sup>2+</sup>	66.9 $\pm$ 4.7	62.8 $\pm$ 5.3	61.6 $\pm$ 5.6
Cu	1.8 $\pm$ 0.2 <sup>A</sup>	8.0 $\pm$ 0.4 <sup>B</sup>	14.4 $\pm$ 0.6 <sup>C</sup>
pH	7.01 $\pm$ 0.6	6.98 $\pm$ 0.7	6.96 $\pm$ 0.5
CaCO <sub>3</sub>	9.3 $\pm$ 0.3	8.9 $\pm$ 0.5	8.6 $\pm$ 0.5

Values are mean  $\pm$  SE. Values that do not share the same letters indicate significant difference ( $P < 0.05$ ,  $n = 21$ ). Cu, copper; CaCO<sub>3</sub>, total hardness.

low, and high Cu treatments (Table 2). There were no mortalities in the control and moderate exposure tanks; however, there was a 36% mortality rate in the high Cu exposure, although mortality occurred only within the first 4 days of the experiment. Tissue Cu load was consistent with previous experiments (9, 10), with significant Cu accumulation in the gills and liver in the high Cu exposure, yet no clear elevation under moderate Cu exposure (Fig. 1). There was also a distinct accumulation of Cu in the gut under both moderate and high Cu exposure (Fig. 1).

There were significant  $\sim$ 2.75- and 3.25-fold increases in whole body cortisol levels under moderate and high Cu exposure, respectively, indicative of a generalized stress response (Fig. 2). An enzyme known to be responsive to both Cu and elevated cortisol, NKA was assessed in the liver tissue for changes in both enzymatic activity and transcription of one of its primary subunits isoform 1a (*atp1a1*). We saw a distinct

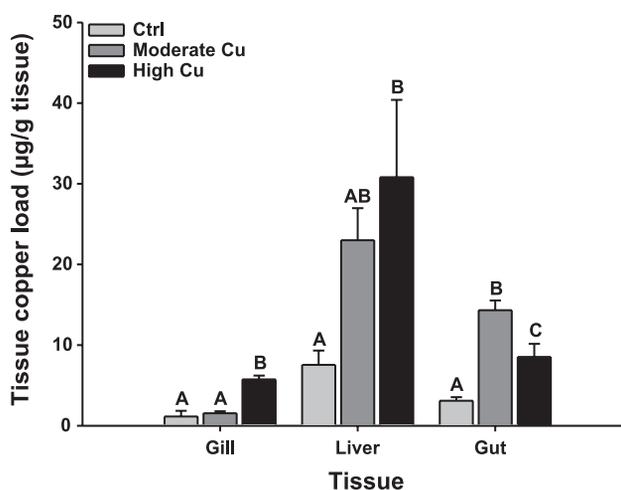


Fig. 1. Tissue copper (Cu) load in the gills, liver, and gut of softwater acclimated zebrafish exposed to control ( $1.8 \pm 0.2$   $\mu\text{g/l}$ ), moderate ( $8.0 \pm 0.4$   $\mu\text{g/l}$ ), and high ( $14.4 \pm 0.6$   $\mu\text{g/l}$ ) levels of waterborne Cu. Bars that do not share a like symbol are significantly different within a tissue group ( $P < 0.05$ ,  $n = 7$ ).

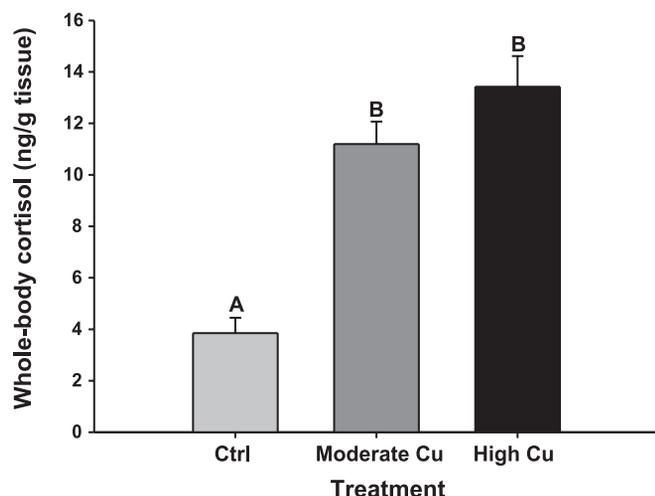


Fig. 2. Whole body cortisol levels of softwater acclimated zebrafish exposed to control ( $1.8 \pm 0.2 \mu\text{g/l}$ ), moderate ( $8.0 \pm 0.4 \mu\text{g/l}$ ), and high ( $14.4 \pm 0.6 \mu\text{g/l}$ ) levels of waterborne Cu. Bars that do not share the same letter are significantly different from each other within a specific tissue ( $P < 0.05$ ,  $n = 7$ ).

40–50% reduction of activity under both moderate and high Cu concentrations (Fig. 3). However, there were significantly elevated transcript expression levels ( $\sim 2.5$ -fold) under the same conditions (Fig. 3). The significant increase in *atp1a1* mRNA was also validated on the microarray (See Supplemental Table S1a and Table 2).<sup>1</sup>

Microarrays were validated for eight genes of interest (see MATERIALS AND METHODS) under the two exposure conditions by real-time qPCR, and there was a significant, positive correlation ( $R^2 = 0.787$ ,  $P < 0.001$ ) between the  $\log_2$ -transformed expression found on the arrays compared that of the qPCR measurements (Fig. 4).

After normalization and statistical analysis, there was a total of 573 genes that exhibited significant up or down changes in gene expression compared with the control arrays, representing

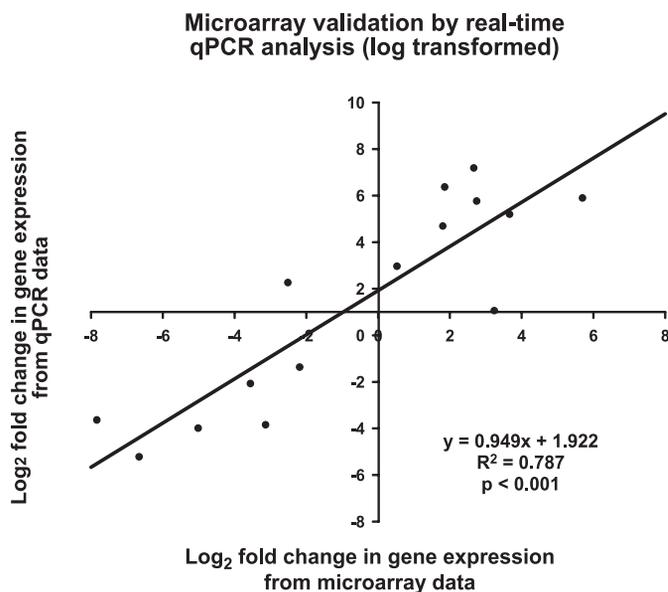


Fig. 4.  $\log_2$  transformed regression analysis in liver for the purpose of microarray validation using 8 selected genes [plus the housekeeping gene elongation factor 1 alpha (*ef1 $\alpha$* )] that had a fold change  $>1.5$  from 3 statistically distinct clusters: NKA isoform 1a (*atp1a1*), *ef1 $\alpha$* , estrogen receptor 1 (*esr1*), heat shock transcription factor 2 (*hsf2*), heat shock protein 60 (*hsp60*), heat shock protein 90a (*hsp90a*), metallothionein 2 (*mt2*), vascular endothelial growth factor (*vegf*), and zinc transporter (*zip*).

a 3.4% change in gene expression across the entire array. GO biological function analysis revealed predominant transcription regulation activity, with a strong significance related to negative transcription activity, and these ontological categories contained a number of transcription factors relating to cellular damage and carcinogenesis (i.e., zinc finger protein 281, Krüppel-like factor 12; Fig. 5A). Also interesting is the overrepresentation of genes found within the apoptosis category related to cellular damage (i.e., DAXX protein, p53, MAPKKK; Fig. 5A). GO molecular analysis further indicated a high degree of transcription regulation due to a significant overrepresentation of genes in the transcription factor category, indicating gene expression regulation (Fig. 5B). Also highlighted in the molecular category is the alteration in antioxidant activity, signified by changes in gene expression of glutathione transferase and glutathione peroxidase (Fig. 5B, Supplemental Table S1). Cluster analysis revealed three distinct clusters based on the expression profile across the treatments, containing 231, 201, and 141 significantly expressed genes (Fig. 6, A, B, C, respectively). A notable aspect of this cluster analysis is the opposing effects of treatment on expression profile, where there is little similarity in up- and downregulated genes when comparing moderate and high Cu treatments (Fig. 6; Supplemental Table S1, A–C). The clusters are essentially broken down into magnitude of opposing response, with few exceptions: *cluster 1* represents a strong increase in expression at a moderate Cu exposure, whereas there was a moderate decrease in expression under high Cu conditions (Fig. 6A). *Cluster 2* indicates a weak increase in expression at moderate levels, and a weak decrease in expression at high Cu levels (Fig. 6B). Finally, *cluster 3* depicts a strong decrease in expression at a moderate Cu exposure, and a weak decrease in expression at high Cu concentrations (Fig. 6C).

<sup>1</sup> The online version of this article contains supplemental material.

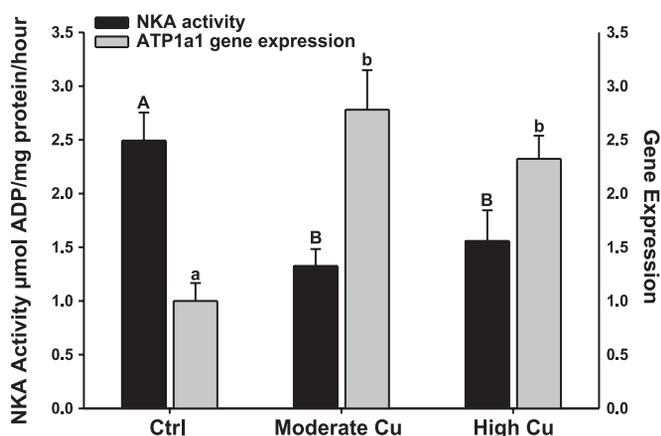


Fig. 3. Liver  $\text{Na}^+\text{-K}^+\text{-ATPase}$  (NKA) activity ( $\mu\text{mol ADP/mg protein/h}$ ; left-hand axis) and associated change in gene expression of *atp1a1* (normalized to *ef1* and relative to the control; right-hand axis) in softwater-acclimated zebrafish exposed to control ( $1.8 \pm 0.2 \mu\text{g/l}$ ), moderate ( $8.0 \pm 0.4 \mu\text{g/l}$ ), and high ( $14.4 \pm 0.6 \mu\text{g/l}$ ) levels of waterborne Cu. Bars that do not share the same letter are significantly different from each other ( $P < 0.05$ ,  $n = 6$ ).

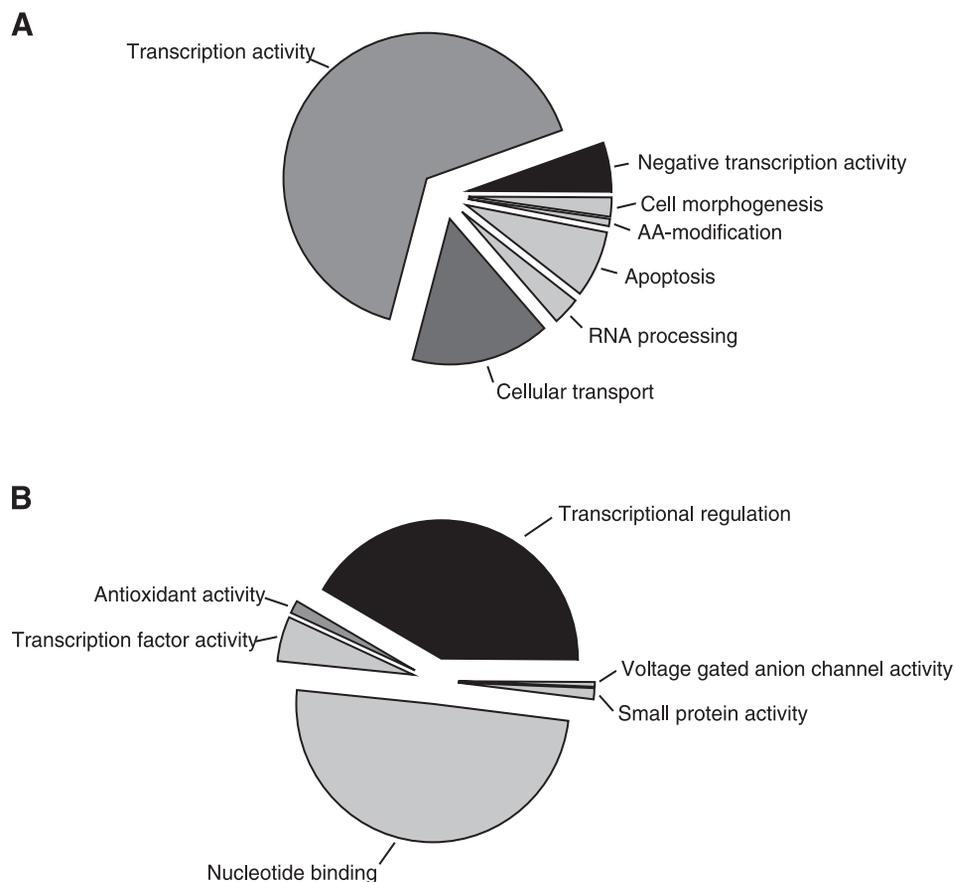


Fig. 5. GO Biological process (A) and molecular function (B) ontological analysis using DAVID. The graph displays terms that are significantly overrepresented ( $P < 0.05$ ) within GO molecular function. Color identifies the significance level of overrepresented categories: black  $P < 0.001$ , dark gray  $P < 0.01$ , light gray  $P < 0.05$ .

GRE analysis using NUBIscan v2.0 revealed that >44% of significantly expressed genes (up or down) contained at least one consensus GRE in the 2 kb promoter region directly upstream of the start codon (Supplemental Table S2). Additional MRE analysis (GCG version 11.1, Accelrys) indicated that 17% of significantly expressed genes contained at least one consensus motif (Supplemental Table S3). Further analysis isolated the shared and unique sequences: containing only GREs, only MREs, or containing both MRE and GRE motifs in the promoter region (Fig. 7, Supplemental Tables S2 and S3). A total of 172 (30%) genes contained only the GRE motif and could further be broken down into GO molecular process

categories of ribonucleotide binding (i.e., serum/glucocorticoid regulated kinase, MAPK4, protein kinase c;  $P < 0.01$ ), ATP Binding (ATPase1a1, HSP70, lysosomal  $H^+$ -ATPase;  $P < 0.01$ ), pyrophosphatase activity (i.e., ATP synthase lipid-binding protein;  $P < 0.05$ ). Further clusters of low significance ( $P < 0.1$ ) indicated some potential relationship to ion transport and binding (Fig. 7, Supplemental Table S3). There were 14 (out of 573 or 2.5%) genes that were unique in containing only an MRE motif, which were overrepresented in the category of metal binding (e.g., MT2,  $P < 0.05$ ) and nonsignificantly represented in the cellular transport (e.g., VEGFa,  $P < 0.1$ ) GO category (Fig. 7, Supplemental Table S3). Genes that shared

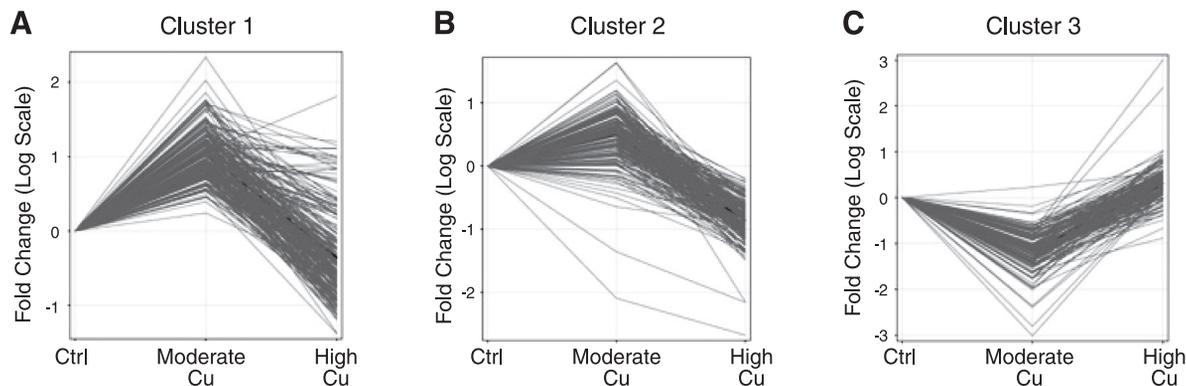


Fig. 6. Cluster analysis of liver genes that were statistically up- or downregulated >1.5-fold vs. control genes. Analysis revealed 3 distinct cluster patterns and indicated contradictory responses associated with varied levels of waterborne Cu exposure. Clusters contained 231 (A), 201 (B), and 141 (C) genes, and an associated identification of genes can be found in Supplemental Table S1.

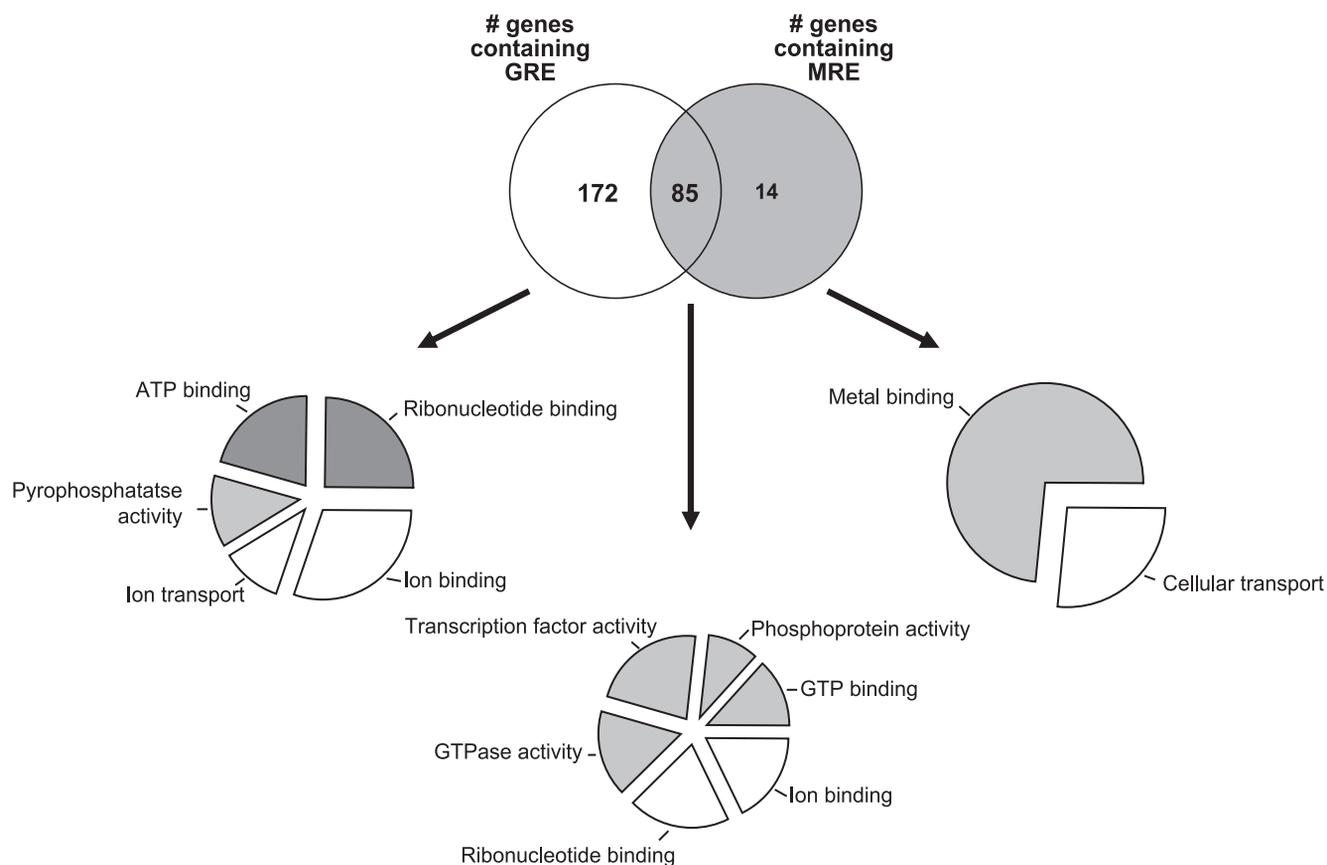


Fig. 7. Venn diagram of assessable genes that contained at least 1 consensus glucocorticoid response element (GRE) or metal response element (MRE), indicating the number of genes that contained the consensus promoter. Further details on specific genes can be found in Supplemental Table S3. Sequences were submitted to DAVID for ontological analysis of the molecular function category. Color identifies the levels of overrepresented category: dark gray  $P < 0.01$ , light gray  $P < 0.05$ , white  $P < 0.1$ .

both a GRE and MRE consensus motif totaled 85 (15%) and were overrepresented in the GO molecular categories of GTP binding (e.g., Rho family GTPase 3,  $P < 0.05$ ), phosphoprotein (e.g., protein tyrosine phosphatase 3,  $P < 0.05$ ), transcription activity (e.g., HOXB1,  $P < 0.05$ ), and GTPase activity ( $P < 0.05$ ; Fig. 7, Supplemental Tables S2 and S3). There were also categories of ribonucleotide- and ion-binding of low significance ( $P < 0.1$ ; Fig. 7, Supplemental Tables S2 and S3).

## DISCUSSION

The use of microarrays can highlight perturbations in previously unsuspected target pathways that are either beneficial or detrimental to a particular species faced with a toxicant. In this study, we have demonstrated a biphasic response associated with long-term (21 day) waterborne Cu exposure. There was principally a distinct upregulation of genes under moderate Cu exposure and predominantly a significant downregulation of genes under high Cu exposure (Fig. 5, Supplemental Table S1). A cursory glance at these results might erroneously suggest a direct role for Cu in modulating multiple pathways. However, Cu stimulates a systemic stress response, as indicated by increased whole body cortisol (Fig. 2). Although there were no differences in whole body cortisol levels between moderate and high Cu treatment groups (Fig. 2), moderate Cu exposure resulted in liver Cu burdens intermediate between control and high exposure (Fig. 1). Therefore, we can refine

our hypothesis that cortisol plays a significant role in changes in gene expression to one where Cu modulates cortisol-driven changes in gene expression depending on the exposure level. Indeed, in silico promoter region analysis revealed 44% of the significantly regulated genes contained at least one consensus GRE, while 30% of the genes contained only a GRE motif (i.e., no MRE, Supplemental Table S2). Furthermore, we found that 17% of regulated genes contained a consensus MRE motif, with 2.5% containing only an MRE motif (i.e., no GRE, Supplemental Table S3), indicating Cu may play a direct role in gene regulation. Overall, this study effectively identifies the gene expression response to waterborne Cu exposure in soft-water-acclimated zebrafish and highlights many of the complex homeostatic pathways involved.

**Waterborne Cu uptake.** Primary uptake pathways of trace metals are usually through the diet; however, in aquatic organisms Cu can also be taken up from the water (6, 17, 26). Because the liver is the primary Cu-sequestering organ (Fig. 1), we expected Cu-regulated gene expression to be highest in this tissue. As metals accumulate within cells above ambient levels, mechanisms of cellular protection are invoked, in particular metal-sequestering proteins, such as metallothioneins (MTs), are increased to reduce the amount of free Cu within a cell (21, 25). Isolated mouse hepatocytes demonstrated saturation kinetics associated with high external Cu, which indicates there is a maximum capacity inherent with increased Cu exposure (35).

Excess hepatic Cu in the moderate exposure group (where there was no significant elevation) was probably sequestered and excreted, as indicated by substantially elevated Cu levels within the gut, whereas excess Cu in the high treatment group (where there was a highly significant elevation) was probably not effectively sequestered and excreted, as indicated by the lower Cu burden in the gut (Fig. 1). Indeed, cellular damage associated with excessive free Cu ions may have impeded efficient Cu excretion. Previously, we reported extensive acute oxidative damage in the liver associated with this level of Cu exposure (9). Cu transport and excretion are regulated by a suite of accessory cofactors and two Cu-ATPases [ATP7a (Menkes gene); ATP7b (Wilson gene)] in humans (31), although to our knowledge, zebrafish appear to have only one Cu-ATPase (atp7a; Ref. 10). We speculate that with only one functional Cu transporter, efficiency in Cu excretion is lower in zebrafish and only a minor increase beyond a set Cu threshold may induce cellular damage and a negative transcriptional response, as discussed subsequently. Further experimentation into MT induction, Cu-ATPase function, and biliary Cu excretion in zebrafish is needed to test these ideas.

**Microarray validation.** Although phenotypic responses may not be identifiable, we know that genome-wide changes in expression are commonplace with environmental stressors (16, 24), and waterborne Cu exposure is no different (41). Congruent with current microarray experiments (i.e., Refs. 2, 41), validation by real-time qPCR was performed on eight identifiable genes of particular interest to metal exposure studies. This validation exercise indicated that gene expression results from the microarrays were in parallel with quantitative expression experiments (Fig. 4). The validation exercise also confirmed the biphasic response associated with the two distinct levels of waterborne Cu exposure. From our qPCR results, all but two genes (atp1a1 and mt2) had opposing responses in expression at the two Cu concentrations (Table 3, Supplemental Table S1). We have previously demonstrated that mt2 is a Cu-responsive isoform of MT in zebrafish (10), and it appears that independent of exposure level, the mt2 gene is upregulated by Cu. However, in mammalian studies, Cu induction of MTs is only detectable under high doses (37). Notably, the Zn uptake transporter ZIP1 (slc39a1) is downregulated under

moderate Cu exposure, which either decreases competitive effects or signifies a shared uptake pathway. Conversely, we see a distinct increase in ZIP1 expression under high Cu levels, which may reflect increased MT transcription (i.e., increased mt2 expression) via increased Zn levels and/or indicate Zn deficiency under excess Cu. This presents a potential interesting mode of interaction between two competitive ions and warrants further investigation. Similar to mt2, atp1a1, which is the catalytic subunit for the NKA enzyme (Table 3, Supplemental Table S1), showed an increase in gene expression under both levels of Cu exposure (Fig. 3). Cu is known to inhibit NKA enzymatic activity in the gills of both the juvenile tilapia and rainbow trout (15, 29, 63), and zebrafish liver tissue follows the same pattern (Fig. 3). Potentially, zebrafish are upregulating atp1a1 in effort to combat the inhibitory effects of excessive Cu accumulation on NKA activity, although further investigation is required.

Cu can also bind and activate estrogenic receptors and increase the risk of endocrine-related diseases (34). Under conditions of moderate Cu exposure, we found a significant upregulation of the *esr1* gene, whereas there was a significant downregulation in high Cu exposure (Table 3, Supplemental Table S1), indicative of endocrine disturbance, although further responses in this pathway were not investigated.

Cu-induced ROS may cause lipid, protein, and DNA damage (9, 14). Heat shock proteins are also known to be responsive to increased metal accumulation and ROS (65). Upon exposure to moderate Cu, we saw increased expression of hsp60 and its respective transcription factor hsf2, although transcription of another heat shock protein, hsp90a, decreased (Table 3, Supplemental Table S1). Distinctively, the opposite effects were seen under conditions of high Cu exposure (Table 3, Supplemental Table S1). Hsp90a has been implicated in the apoptosis pathway in humans (42, 64). Furthermore, both Cu and ROS have been shown to induce apoptosis (46, 54). Therefore, apoptosis may be occurring at an accelerated rate resulting in tissue damage and organ shut-down, explaining the significant mortality rate during the first few days of exposure in zebrafish exposed to high waterborne Cu. Increased expression of vegfa also suggests tissue damage under high Cu exposure, since Cu-induced oxidative damage has been shown to promote angiogenesis in human keratinocyte cell lines (53).

**Ontological analysis of microarrays.** Statistical analysis revealed that only 3.4% of the quantifiable genome exhibited changes in gene expression of >1.5-fold. After submission to DAVID for ontological analysis, we found that the predominant changes are related to transcriptional regulation, due to the significant overrepresentation in those GO categories (Fig. 5). Of particular note, is the significant ( $P < 0.001$ , Fig. 5A) overrepresentation in the negative regulation category, which can be defined as any process that prevents or reduces the frequency of reactions and pathways in a given cell/organism. Genes found within this category are often associated with apoptosis. To a lesser extent, we saw some significant changes in genes associated with ion regulation and homeostasis (i.e., atp1a1 and slc39a1; see above), including changes in membrane  $\text{Ca}^{2+}$ -ATPase transport, which may be linked competitive effects of  $\text{Ca}^{2+}$  uptake associated with excessive Cu (9). Overall, the predominant response of liver tissue to chronic waterborne Cu exposure in zebrafish seems to be an induction of transcription factors associated with increased oxidative

Table 3. Direction of change in expression of genes used for microarray validation grouped together according to similar patterns of expression

Gene Symbol	Gene Description	GeneBank ID	Expression Direction Moderate Cu	Expression Direction High Cu
ATP1a1	Na <sup>+</sup> -K <sup>+</sup> -ATPase1α1	AI105927.1	↑	↑
MT2	metallothionein 2	AW170968.1	↑	↑
ESR1	estrogen receptor 1	BF717829.1	↑	↓
HSF2	heat shock factor 2	CR926931.1	↑	↓
HSP60	heat shock protein 60	BI896507.1	↑	↓
HSP90a	heat shock protein 90	AI397223.1	↓	↑
VEGFa	vascular endothelial growth factor a	AW233384.1	↓	↑
ZIP1	zinc transporter 1	BM857883.1	↓	↑

Arrows indicate direction of significant expression >1.5-fold. The normalization gene elongation factor 1 alpha (EF1α) did not change significantly under any treatment regime.

stress pathways, backed by the overrepresented antioxidant category ( $P < 0.01$ , Fig. 5), in accord with our earlier findings of severe oxidative stress during acute waterborne Cu exposure (9). Here we see induction of several antioxidant related proteins (i.e., glutathione peroxidase 2, peroxiredoxin 5) that are known to aid in antioxidant defense (55, 61).

The biphasic response associated with moderate and high Cu exposures was particularly striking, as we see both the magnitude and direction of gene expression changing (Fig. 6, Supplemental Table S1). However, caution should be exercised as the magnitude of the gene expression response does not always confer an equal response in protein expression or activity, as seen in this and previous experiments (Fig. 3; Ref. 13).

**GRE and MRE analysis.** While this study provides evidence that waterborne Cu exposure can elicit a transcriptomic response in zebrafish, we also found that the exposure to Cu also induced a general stress response, as indicated by increased whole body cortisol levels (Fig. 2). Bury et al. (3) previously demonstrated that cortisol provides a protective effect at low doses against Cu-induced necrosis in cultured gill filaments, and our data suggest that Cu-induced cortisol release may play a similar role in vivo. This prompted an in silico analysis of GREs (Supplemental Table S2). We found 257 genes to contain at least one or more GREs in the promoter region, representing ~45% of significantly regulated genes (Fig. 7). We also know that Cu can induce a direct metal response through activation of MTF-1, which binds to MREs and initiates a transcriptional response (20, 51). This prompted an additional in silico analysis of MREs in the promoter region of significantly regulated genes, which showed 99 genes containing at least one consensus MRE motif, representing ~17% (Fig. 7). Venn analysis indicated an overlap of 85 genes that contained both MREs and GREs. As such, these candidate genes should be excluded from use as Cu-specific genetic endpoints.

With respect to genes that only contained GREs (~30%), we found categories essentially relating to transcriptional regulation and loosely relating to ion transport and binding (Fig. 7). With increased cortisol levels there are associated changes in ion homeostasis, a familiar phenomenon linked to altered environmental water chemistry (i.e., freshwater → seawater migration), additionally indicated by the GO molecular function categories of ion-binding and transport (Fig. 7; Refs. 7, 32). According to ontological evidence provided, gene expression as it relates to ion regulation under chronic Cu exposure may be modulated by cortisol, as opposed to direct stimulation from Cu. Conversely we see only a very short list of genes that contain only MREs in the promoter region. This suggests that the direct genetic response in response to chronic Cu exposure is very limited (Fig. 7, Supplemental Table S3). However, we did find that mt2 contains 2 MREs, which further identifies this gene as a genetic endpoint, although not specific to Cu, as other metals (i.e., Zn) may invoke increased MTF-1 binding to MREs as well (11, 22; Supplemental Table S3). Due to the relatively short list of genes found in this analysis, significant overrepresentation of GO categories is difficult to ascertain. However, it appears that genes containing MREs are associated with ion binding (e.g., mt2) and cellular transport, a broad descriptive category that includes genes such as vegf $\alpha$ . Certainly an in silico approach of this nature is prone to some false positives, and we cannot conclude that all the genes identified

are actually regulated by glucocorticoids or metal transcription factors. Rather, this analysis suggests that the gene transcriptional response after Cu exposure is associated more with a general stress response, as opposed to a direct metal response.

In summary, we have identified two potential genetic markers, atp1a1 (general stress response) and mt2 (metal stress response), of chronic Cu exposure under softwater conditions. Furthermore, we argue that genetic markers need to be coupled to measurements of functional components (enzyme activity/protein level) since mRNA expression level does not always coincide with a functional response (Fig. 3; Ref. 10). Moreover, we have validated in a tropical species that chronic Cu exposure primarily elicits a general stress response, which in turn can impact transcriptional regulation of genes thought to be mediated by solely by excessive metals. By determining both direct and indirect effects of contaminant exposure, one can elucidate specific, direct effects of a particular contaminant and highlight responsive genes as genetic endpoints. This study, along with many others, has taken the first initial steps toward identifying genetic endpoints of chronic metal exposure in teleosts.

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#### DISCLOSURES

No conflicts of interest are declared by the author(s).

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