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Water Chemistry Alters Gene Expression and Physiological End Points of Chronic Waterborne Copper Exposure in Zebrafish, *Danio rerio*

PAUL M. CRAIG,* CHRIS M. WOOD, AND GRANT B. MCCLELLAND

Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON, L8S 4K1, Canada

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This is the first study to implement a genomic approach to ascertain both transcriptional and functional end points of chronic Cu toxicity in fish associated with experimentally manipulated water chemistries. Over 21 d, zebrafish acclimated to softwater (Na^+ = 0.06 mM, Ca^{2+} = 0.08 mM, Mg^{2+} = 0.03 mM) were exposed to the following: soft-water (Ctrl); 12 μg L^{-1} Cu (Cu); 3.3 mM Na^+ (Na); 3.3 mM Na^+ + 12 μg L^{-1} Cu (Na + Cu); 3.3 mM Ca^{2+} (Ca); or 3.3 mM Ca^{2+} + 12 μg L^{-1} Cu (Ca + Cu). Although effective at reducing Cu load in all tissues, Na^+ in the presence of Cu did not decrease the degree of oxidative damage, particularly in the gill and gut. In contrast, Ca + Cu treatment decreased Cu accumulation in gill, but not liver or gut, with no reduction in oxidative damage. Transcriptional analysis of candidate genes (atp7a, ctr1, ECaC, esr1) showed principally a down regulation of transcripts with the Cu only treatment, while Ca + Cu treatment restored some of the genes to control levels. Conversely, the Na + Cu treatment had a strong, opposing affect when compared to that of Cu alone. Zebrafish Affymetrix GeneChips revealed significantly clustered patterns of expression. Changes in expression induced by Cu appeared to be opposite to the majority of the other treatments. Our data on the preventative or enhancing effects of Na^+ and Ca^{2+} both alone and in the presence of Cu, may, in the future, facilitate the incorporation of gene expression end points into a biotic ligand model predicting chronic Cu toxicity in this tropical model species of genomic importance.

Introduction

Water chemistry is the major factor influencing the toxic effects of contaminant metals in the natural environment. Water hardness, principally Ca^{2+} and Mg^{2+} concentrations, provides protection since these ions mitigate toxicity by competing with metal for uptake sites (*1*). The biotic ligand model (BLM *2, 3*), is a predictive model which takes into account the effects of protective ions and other aspects of water chemistry. This model is based on a fixed relationship between short-term gill metal accumulation and ultimate toxicity to an organism. However, the BLM is based on acute end points (metal accumulation and mortality) and does not take into account nonphenotypic changes related to increased metal contamination that can have potent effects on a chronic time scale, such as changes in gene expression. With advanced techniques in genomewide assessment (microarrays) now commercially available for many aquatic species (e.g., channel catfish, medaka, zebrafish, rainbow trout), incorporating gene expression end points into models of chronic toxicity for use in environmental protection standards is possible. Our primary objective was to use a microarray approach to examine the global gene expression response in zebrafish to chronic waterborne copper (Cu) exposure at environmentally realistic levels, under various water chemistries.

Although Cu is an essential micronutrient, excess Cu has a broad range of detrimental effects including oxidative damage (e.g., increased protein carbonyls), disrupted ion-regulation (e.g., competitive inhibition with Na^+K^+ATPase) and growth, and even endocrine disruption (*4–8*). These responses occur under relatively high levels of Cu exposure, yet chronic, environmentally realistic exposures may not necessarily elicit such pronounced effects. Measuring gene expression provides a useful tool to identify a transcriptomic response at lower, chronic metal exposures. We have taken two separate approaches to identify gene end points. We first used microarrays to determine global changes in transcription, where thousands of genes can be assessed at a given time point. The second approach was a candidate gene approach, as some genes are a priori expected to be affected by metals: copper transporter 1 (ctr-1 *5, 9*), Cu-ATPase (atp7a *5, 10, 11*), epithelial calcium channel (ECaC (*12*)), and estrogen receptor 1 (esr-1 *5, 13*).

The commercially available Affimetrix GeneChip Zebrafish Genome Array was used to ascertain the impact of chronic waterborne Cu exposure on the transcriptomic profile in zebrafish in liver tissue, as this is the primary site of Cu accumulation/detoxification and was expected to have the most significant changes in gene expression related to Cu exposure (*4, 5*). Furthermore, by altering concentrations of waterborne Ca^{2+} and Na^+ in a realistic context (freshwater values: Ca^{2+} range trace−6 mM; Na^+ range trace−3.6 M (*14*)), the protective effects of these cations were examined at both a functional (Cu load, enzymatic/oxidative damage) and transcriptional level. Manipulation of water chemistry allowed for future incorporation of gene expression end points into a BLM to predict chronic Cu toxicity in this tropical species.

Methods and Materials

Animals. Adult zebrafish (*Danio rerio*) of mixed sex were purchased from DAP International, Canada, and acclimated to soft-water (Table S1 of the Supporting Information) in an aerated 40 L aquarium as described previously (*15*). 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 = 420; 70/treatment) were weighed and placed in 8 L aerated tanks, with flow-through soft-water at 25 mL min^{-1}. Mariotte bottles were used to dose tanks with Cu (a concentrated Cu solution made from $CuSO_4$ dissolved in 0.05% HNO_3), Na (concentrated NaCl solution and 0.05% HNO_3), and Ca (concentrated $CaCl_2$ solution and 0.05% HNO_3) to six treatments of either control (Ctrl), Cu only (Cu), high Ca only (Ca), Ca + Cu (Ca + Cu), high Na only (Na), and Na + Cu (Na + Cu). (See Table S1 of the Supporting Information for additional water chemistry

* Corresponding author current address: Department of Biology, University of Ottawa, 550 Cumberland Street, Ottawa, ON, K1N 6N5, Canada; tel: (613) 562-5800 x6009; fax: (613) 562-5486; e-mail: pcraig@uottawa.ca.

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details). Fish were fed 2% body weight of commercial tropical fish food (Topfin, Phoenix, AZ; 8.3 ± 0.2 mg Cu kg^{-1} food), once per day. Tanks were monitored daily for mortality and cleaned of any accumulated food or waste. Water samples (10 mL) were taken from each tank, filtered (0.45 μm, Pall Corporation, East Hills, NY), added to 100 μL HNO_3, and kept at 4 C for analysis of Na^+, Ca^{2+}, Mg^{2+}, and Cu concentrations. Throughout the experiment, there were no mortalities. At the end of the exposure period, fish were quickly euthanized by an overdose of buffered anesthetic (MS-222, Sigma). Gill, liver, and gut were immediately frozen in liquid N_2 for further analysis of Cu burden, gene expression, and enzyme activity. Water and tissue ions and Cu levels were measured as described by Craig et al (*5*).

Protein Carbonyl and Enzyme Activities. Protein carbonyls in gill, liver, and gut were quantified using a commercial kit (Cayman Chemical Company, Ann Arbor, MI), following the work of Craig et al. (*4*). Catalase (CAT) activity was assayed at 28 C using a modified method based on the work of Claiborne (*16*) (see ref *4*). Tissue Na^+/K^+ATPase activity (NKA) was determined using the microassay method of McCormick (*17*).

Drinking Rates. Zebrafish drinking rates (n = 8/treatment) were measured following the protocol of Scott et al (*18*). Zebrafish were removed after 21 d of exposure and placed in static polyethylene chambers containing 100 mL of aereated water in three treatments (Ctrl, Na, and Ca). An 80 μCi portion of radiolabeled polyethylene glycol (^{3}H-PEG-4000, NEN Life Science Products, Boston, MA) was added, and 5 mL water samples were taken at 0, 1.5, and 3 h intervals. After exposure, zebrafish were terminally anaesthetized in MS-222, rinsed in clean ddH_2O, and the entire digestive tract was excised, weighed, and digested in 1 N HNO_3 at 65 C for 48 h. Drinking rates were expressed as the volume of water ingested based on quench-corrected counts from the digestive tract and water relative to body mass and time.

RNA Purification and Microarray Analysis. Total RNA was extracted from pooled liver tissue (two fish per pool; n = 4) following the work of Craig et al. (*5*). The integrity of RNA was assessed by capillary electrophoresis on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). A 5 μg portion of total RNA was used to prepare biotin labeled complementary RNA (cRNA) which was hybridized onto Affymetrix Zebrafish Genome arrays following standard protocols found in the Affymetrix Technical Manual (Affymetrix Santa Clara, CA). Raw fluorescence data (cel files) were converted to normalized expression indices using the RMA algorithm in Gene Spring v.10.0.1 (Agilent Technologies, Palo Alto CA, http://www.agilent.com). Differential expression analysis was via a one-way ANOVA with a Tukey post hoc test, and genes which demonstrated 2.0 fold or higher change in expression between any 2 treatments were selected for further analysis. K-means clustering algorithm was applied to genes with a p-value of 0.05 or less in the ANOVA analysis to gain understanding of the major changes in gene expression. The list of significantly expressed genes was submitted to the Database for Annotated, Visual, and Integrative Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp *19, 20*) for functional annotation clustering using the default settings, minus cellular component ontology. Functional annotation was ranked based on significant overrepresentation (p>0.05) and an elevated enrichment score, indicating a significant likelihood that genes fall within the listed annotation cluster. 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 (MGED) recommendations (Accession no. E-MEXP-2288).

Quantification of mRNA by Real-Time RT-PCR. First strand cDNA was synthesized from total RNA of gill, liver, and gut tissues (extracted as described above) using the methods described in the work of Craig et al (*5*), and expression data were calculated by the $2^{-\Delta\Delta ct}$ method, using EF1α as the housekeeping gene (*21*). A total of four pooled samples were run for each gene, allowing for the calculation of a mean ± SEM. Primers were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA), and target genes of interest (Table S2 of the Supporting Information) were verified by gel electrophoresis.

Statistical Analysis. Statistical analysis separate from microarray analysis was performed using Sigma Stat (SPSS Inc., Chicago, MI). One-way ANOVA and a posthoc Tukey test were used to test for pairwise significance for all data, with the exception of gene expression when related to control, where a Dunnett's test was used. Regression analysis was used for qPCR validation, (p < 0.05). All data are expressed as a mean ± SEM (N) where N = number of individuals (functional data) or pools (RT-PCR data).

Results and Discussion

Copper Accumulation. With Cu exposure, there were significant increases in Cu load in all tissues, compared to the Ctrl (Figure S1 of the Supporting Information). Elevated waterborne Na^+ and Ca^{2+} both reduced Cu accumulation in the gills to Ctrl levels (0.66 ± 0.11 μg g^{-1} tissue), but Ca^{2+} appeared to enhance Cu load in both the liver (28.24 ± 5.18 μg g^{-1} tissue) and gut (63.27 ± 10.9 μg g^{-1} tissue). Primary absorption of Cu is thought to occur in the gut from the diet (*22, 23, 26, 28*), but Cu is also known to be taken up from the external medium via the gills. Na^+ and Ca^{2+} compete with Cu for binding sites on the gill (*2, 3, 24, 25, 27*). Less is known about protection in the liver by waterborne cations. In fish, the liver is the primary site of accumulation of excess Cu, which is then excreted into the gut in the bile (*4, 5, 29, 30*). In mammals (*31*), treatment with a combined high Ca^{2+} and high Cu diet provided substantial protection from liver Cu accumulation and also reduced Cu-related liver damage. Moreover, pre-exposure to elevated waterborne Ca^+ reduced Cu toxicity in tilapia (*32*). Interestingly, our Ca + Cu treatment did not protect against hepatic Cu accumulation or protein carbonyls (Figures S1 and S3 of the Supporting Information), but rather promoted Cu and Ca^{2+} (16.4 ± 2.4 mmol kg^{-1}) accumulation in the liver.

Shared uptake pathways in the gills between Na^+ and Cu are well-established (*24, 27*) and further supported in this study by the reduced Cu load with Na + Cu treatment in the liver as well as in the gill. However, despite an apparent inhibition of Cu uptake by high external Na^+, protein carbonyls increased in both the gut and gill of Na + Cu treated zebrafish (Figure S3 of the Supporting Information). Previously, high NaCl has been shown to induce oxidative stress in both rat renal medullary cell cultures (*33*) and isolated perfused rat livers (*34*). Possibly, the combination of high external Na and Cu leads to increased oxidative damage. However, on an acute time frame, Craig et al (*4*) found no increase in oxidative damage in the gills or liver under combined conditions of increased Na^+ and Cu and, likewise, no increased carbonyls in the gills or liver associated with Na^+ exposure alone, an indication that prolonged Na + Cu exposure may be more detrimental.

A fascinating aspect of our data are the large increases in Cu load in the gut (63.3 ± 10.9 μg g^{-1} tissue) and liver (28.2 ± 5.2 μg g^{-1} tissue) in the Ca + Cu treatment. Fish in marine environments drink copious amounts of seawater to avoid dehydration, and associated with this are high pH and HCO_3^- concentrations in the intestinal fluids due to the apical Cl^-/HCO_3^- exchange which precipitates Ca and Mg carbonates (*35–37*). The waterborne Ca^{2+} concentration appears to play a key role in regulating this response (*37*). We found that the Ca treatment significantly increased the drinking rate (3.26

TABLE 1. Quantitative Expression Levels Assessed by qPCR of ctr-1, esr1, ECaC, and atp7a from the Liver, Gill, and Gut of Acclimated Zebrafish Exposed to Control (soft-water), Cu (12 μg/L), Ca (3.3 mM), Ca + Cu (3.3 mM Ca + 12 μg/L Cu), Na (3.3 mM), and Na + Cu (3.3 mM Na + 12 μg/L Cu)[a,b,c]

			treatment					
gene	tissue		Ctrl	Cu	Ca	Ca + Cu	Na	Na + Cu
ctr-1	liver	fold change	1.01 ± 0.10[a]	0.35 ± 0.08[ab]	1.09 ± 0.03[a]	0.51 ± 0.06[a]	0.92 ± 0.22[a]	2.46 ± 0.16[c]
		direction	–	↓	–	–	–	↑
	gill	fold change	1.12 ± 0.34	1.07 ± 0.14	1.29 ± 0.11	0.57 ± 0.07	1.29 ± 0.13	1.98 ± 0.52
		direction	–	–	–	–	–	–
	gut	fold change	1.08 ± 0.24[a]	0.27 ± 0.02[b]	0.65 ± 0.05[ab]	0.28 ± 0.01[b]	0.46 ± 0.01[b]	0.71 ± 0.03[ab]
		direction	–	↓	–	↓	↓	–
esr-1	liver	fold change	1.02 ± 0.13[a]	0.02 ± 0.01[b]	0.09 ± 0.01[ab]	1.58 ± 0.19[abc]	0.22 ± 0.09[ab]	9.03 ± 0.44[d]
		direction	–	↓	↓	–	↓	↑
	gill	fold change	1.14 ± 0.39[a]	0.21 ± 0.01[b]	0.26 ± 0.03[b]	0.15 ± 0.02[b]	0.43 ± 0.02[ab]	0.59 ± 0.06[ab]
		direction	–	↓	↓	↓	–	–
	gut	fold change	1.17 ± 0.40[a]	0.02 ± 0.01[b]	0.20 ± 0.10[b]	0.39 ± 0.08[ab]	0.18 ± 0.02[b]	2.21 ± 0.26[c]
		direction	–	↓	↓	–	↓	↑
ECaC	liver	fold change	1.16 ± 0.37	1.18 ± 0.88	0.31 ± 0.09	0.57 ± 0.17	1.40 ± 0.67	0.86 ± 0.39
		direction	–	–	–	–	–	–
	gill	fold change	1.11 ± 0.33[a]	5.26 ± 0.41[ab]	1.88 ± 0.26[a]	0.24 ± 0.04[a]	3.66 ± 0.68[a]	13.86 ± 4.99[b]
		direction	–	↑	–	–	–	↑
	gut	fold change	1.05 ± 0.19[ab]	1.86 ± 0.47[ab]	0.315 ± 0.11[ab]	0.32 ± 0.06[a]	2.04 ± 0.69[ab]	0.45 ± 0.07[a]
		direction	–	–	–	–	–	–
atp7a	liver	fold change	1.29 ± 0.58[a]	4.88 ± 1.14[b]	2.38 ± 0.48[ab]	0.42 ± 0.03[a]	1.79 ± 0.24[a]	1.00 ± 0.14[a]
		direction	–	↑	–	–	–	–
	gill	fold change	1.06 ± 0.20[a]	0.65 ± 0.04[ab]	0.91 ± 0.04[ab]	0.35 ± 0.04[b]	0.84 ± 0.06[ab]	0.69 ± 0.24[ab]
		direction	–	–	–	↓	–	–
	gut	fold change	1.07 ± 0.22[a]	0.37 ± 0.04[b]	1.09 ± 0.13[a]	0.41 ± 0.01b	1.19 ± 0.16[a]	0.85 ± 0.02[a]
		direction	–	↓	–	↓	–	–

[a,b,c] Means ± 1 SEM (n = 4). Fold change values that do not share a like symbol are significantly different from each other ($p < 0.05$). Directional arrows indicate a significant fold change compared to that of the control (Dunnett's test, $p < 0.05$; fold change > 2).

± 0.64 mL kg^{-1} h^{-1}) nearly 2-fold compared to the control with no significant increase associated with the Na treatment (1.66 ± 0.18 vs 2.18 ± 0.35 mL kg^{-1} h^{-1}, respectively). Although the fish were exposed to a Ca^{2+} level equivalent of only $^1/_3$ seawater, Ca^{2+} appears to have initiated an osmoregulatory response, as there were additional significant increases in NKA activity (Figure S5 of the Supporting Information) associated with both Ca only and Ca + Cu treatments in the gill (4.70 ± 0.39 and 3.27 ± 0.74 mol ADP mg $protein^{-1}$ h^{-1}, respectively) and with Ca + Cu treament in the gut (1.39 ± 0.12 mol ADP mg $protein^{-1}$ h^{-1}). Also, waterborne Ca^{2+} may trigger increased drinking of waterborne Cu, and, in part, explain elevated gut and liver Cu accumulation. Waterborne Cu speciation in freshwater is either free Cu^{2+} or Cu bound to organic/inorganic compounds (*38*), yet with elevated pH in the intestinal lumen, a precipitate of Cu carbonate may form and serve as a detoxification mechanism in the gut. Nevertheless, we did not see any precipitate while harvesting the intestinal tissues, although the gall bladders of zebrafish exposed to Cu were a distinct blue-green color similar to that of $CuCO_3$. Although speculative, this definitely warrants further investigation, as this may be a key aspect of protection against divalent metal contaminants in marine environments.

Enzyme Activities. Cu competes with Na^+ for entry pathways at the apical side of gill ionocytes, and ultimately inhibits basolateral NKA activity (Figure S4 of the Supporting Information *24, 27*). Not surprising was a rescue of gill NKA activity with increased ambient Na^+. Increased NKA activity is routinely associated with freshwater/seawater migration in euryhaline teleosts (*39–43*). High Ca^{2+} has been shown to stimulate gill NKA activity in *Cyprinodon variegatus* but to decrease its activity in *Mysidopsis bahia,* two euryhaline species commonly used for toxicological studies (*44*). In the present study, there was a parallel decrease across all treatments in liver NKA and catalase activities (Figures S4 and S5). Increased reactive oxygen species (ROS) can have a negative impact on both catalase (*45*) and NKA (*46*); indications of increased ROS (elevated protein carbonyls) were seen with most treatments (Figure S3 of the Supporting Information).

Gene Expression and Microarrays. The candidate gene approach allowed us to both validate microarray data and extend gene expression measures to tissues not used in the microarray experiments. Quantitative PCR analysis was clear in validating our microarray experiment ($R^2 = 0.776$; $p < 0.001$) and demonstrated a similar pattern of expression related to other tissues (Table 1). The microarray approach revealed a high degree of inherent physiological variability indicated by principal component analysis (Figure S6 of the Supporting Information), perhaps a result of using a commercially purchased, heterogeneous-wildtype zebrafish population. However, this is a more natural depiction of gene response to environmental disruption at the population level in the wild. Our microarray results from liver tissue revealed four distinct clustered patterns of expression, and predominantly indicated that Cu alone had an opposing effect when compared to the other treatment groups. Chiefly, Cu alone had a distinct impact on the transcriptional and translational machineries as determined by functional annotation (Figure 1), a finding further supported in a previous zebrafish study using a different source of microarrays and two Cu exposure conditions (*15*).

Cluster 1 indicates that Cu had an up-regulatory effect, while the other treatments resulted in a down regulatory effect on transcript expression (Figure 1A). Here, we see over-representation in three separate categories, but the greatest number of genes were involved in active transmembrane transport. This is not surprising since Cu is known to interrupt ion homeostasis (*2, 3, 24, 27, 47*). Also not surprising was a stimulation of exogenous chemical response genes, such as glutathione peroxidase, a known antioxidant scavenger related to increased ROS (*48, 49*). The second cluster revealed a unique pattern associated with Cu, Ca^{2+}, and Na^+ having a negative impact on gene expression, whereas Ca + Cu and

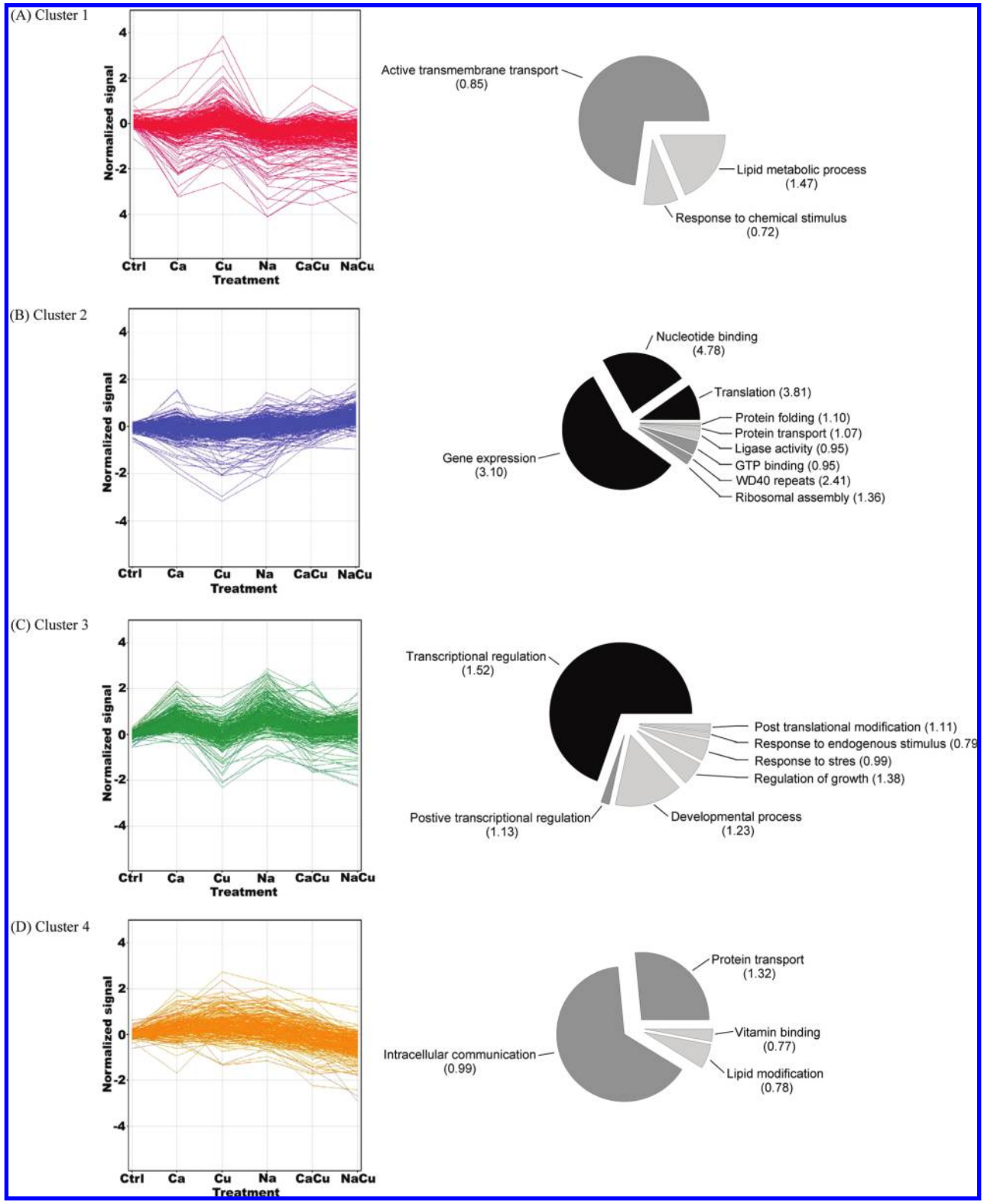

FIGURE 1. Pictorial cluster analysis of microarray results from liver in zebrafish exposed to ctrl (softwater), Cu (12 μg/L), Ca (3.3 mM), Ca + Cu (3.3 mM Ca + 12 μg/L Cu), Na (3.3 mM), and Na + Cu (3.3 mM Na + 12 μg/L Cu) and DAVID functional annotation clustering results ($p < 0.05$). Four distinct cluster patterns were identified. Normalized signals are represented in Log 2 format, and signal intensities greater than 1 were used for functional annotation. Numerical values in brackets = enrichment scores, where the higher the score, the greater likelihood that selected genes from a given cluster fall within the annotation indicated.

Na + Cu in combination minimized this effect, potentially a protective transcriptional response (Figure 1B). Functional ontological analysis revealed a significant impact on initiation of transcription, translation, and protein modification and transport (Figure 1B). This could indicate reduced energetics associated with Cu, where nonessential processes, such as performance (*50*), growth (*8*), and reproduction (*51*) are reduced in favor of higher, essential functions. Further revealed are two key genes associated with Cu exposure, esr-1 and ctr-1, which will be further discussed (Table S3B of the Supporting Information). Cluster 3 had similar functional characteristics as cluster 2, although the pattern

of expression was altered, where Cu had negative impacts on transcription, with an attenuated effect in the Na + Cu and Ca + Cu treatments (Figure 1C). Cluster 4 had a weak pattern of expression and contained the lowest significance related to functional, ontological annotation (Figure 1D). Predominant increases in transcripts were associated with Cu, Ca, and Na treatments, whereas Ca + Cu and Na + Cu treatments induced a negative response (Figure 1D). A feature of this cluster is changes in HIF1α expression, a transcription factor known to be induced by increased ROS in zebrafish (*52*). Overall, the microarray results reveal that the patterns of expression were more important than specific genes involved; global transcriptional regulation is a complex response.

A more viable approach to gene end point assessment is a directed analysis of a handful of known responsive genes under our exposure regime. Copper uptake and transport are primarily regulated by ctr-1 and atp7a. Altered levels of external Cu and ion concentrations were associated with changes in transcript levels (*4, 5, 9*). Within the liver, we found a significant decrease in ctr-1 and an increase in atp7a expression under the Cu only treatment, which validated our microarray experiment, although atp7a is not found on the Affymetrix GeneChip (Table 1). These changes may signify an attempt to reduce Cu load within the liver by reducing uptake by ctr-1 and increasing expulsion by atp7A. Craig et al. (*5*) found a similar pattern of atp7a expression in the liver of zebrafish chronically exposed to 8 μg L^{-1} Cu suggesting this as a compensatory response. However, there were no changes in transcription of either ctr-1 or atp7a in gills associated with the Cu only treatment, although atp7a did decrease in the Ca + Cu exposure (Table 1). Ca + Cu treatment did reduce ctr-1 expression compared to Cu only exposure in the gill (Table 1), coinciding with reduced Cu load in the gills. The gut exhibited a similar response with a down-regulation of transcript expression for both ctr-1 and atp7a, and this again may be a response to reduce further absorption of Cu from the gut, as there was a substantial increase in Cu load in the gut associated with both Cu and Ca + Cu treatments.

The microarray analysis revealed that Ca^{2+}, both alone and in combination with Cu played a significant role in altering gene expression, when compared to treatments with Na^+ (Figure 1, Table S4 of the Supporting Information). Tissue Ca^{2+} uptake occurs through ECaC in the apical membrane, then basolaterally through Ca^{2+}-ATPase or Na^+/Ca^{2+} exchangers (*53, 54*). There was no response of ECaC expression in any treatment in the liver or gut, but a considerable increase in ECaC transcript expression with Cu and Na + Cu treatments in the gill (Table 1). Previously, Craig et al. (*55*) showed that acclimation to a Ca^{2+}-deficient environment induces protein and gene expression of gill ECaC, an important factor to consider in this experiment as ambient Ca^{2+} concentrations were low in the Cu and Na + Cu exposure groups. However, low ambient Ca^{2+} levels were also present for the Ctrl and Na exposure groups and there was no significant change in gill ECaC expression (Table 1). This empirical competition between Cu and Ca^{2+} is backed by novel gene expression evidence as only the Cu treatments associated with low ambient Ca^{2+} increased gill ECaC expression (Table 1).

Our final approach examined the potential endocrine disruptive capacity of Cu exposure in zebrafish. In mammalian studies, it has long be known that Cu, in a dose-dependent manner, reduces both estrogen and progesterone concentrations (*56*). Furthermore, estrogen receptors are known metal-binding proteins, and contain zinc binding sites which promote binding to estrogen response elements. Divalent metals have been shown to interact with these zinc binding sites (*57, 58*). Furthermore, in mammalian cell lines, exposure to Cu decreased the concentration of estrogen receptor proteins and mRNA by 40−60% (*59*). Likewise, there was a substantial decrease in transcript expression under Cu or Ca only conditions in all tissues (Table 1). Furthermore, esr-1 expression decreased in gill tissue with Ca + Cu exposure, and liver and gut demonstrated a decrease associated with Na treatment (Table 1). Conversely, a large increase in esr-1 expression was associated with Na + Cu treatment, yet the mechanistic reason is unclear (Table 1). Taken as a whole, Cu seems to play a significant role in estrogen receptor disruption, which in turn can reduce the sensitivity to estradiol and impact reproductive capacity in fish (*60*). Further investigations in this area are warranted, as these are not reproductively active tissues.

Further analysis was directed toward establishing gene expression end points for possible future incorporation into a chronic BLM for the zebrafish. Implications for development of chronic BLMs using the current approach are discussed in more detail in the Supporting Information. This study recognizes the importance of examining multiple water chemistries to elucidate the transcriptional and physiological effects of Cu in zebrafish. Traditional approaches to studying Cu toxicity are mostly associated with measurements of mortality, and environmental standards are based on these predictions. However, chronic, low Cu doses, sometimes lacking direct and readily visible effects, can show distinct transcriptional patterns eventually associated with decreased growth, impaired ion homeostasis, and endocrine disruption. Although the protective effects of ions, such as Ca^{2+} and Na^+ are functionally well-documented (*2, 3, 25, 27*), the protection that cations provide at the level of the genome is poorly understood. Our microarray data demonstrates that there is some degree of protection given by Na^+ and Ca^{2+} against Cu at the level of the transcriptome but that these seemingly benign ions may also have substantial transcriptional effects by themselves. Since environmental conditions have complex effects on global gene expression patterns, further study is needed to truly understand any protective effects they may have.

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Supporting Information Available

Supporting information, figures, and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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