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Identifying Health Impacts of Exposure to Copper Using Transcriptomics and Metabolomics in a Fish Model

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Copper (Cu) is a micronutrient essential for the biochemical functioning of numerous processes in vertebrates but is also often present in the aquatic environment at concentrations able to cause adverse health effects in aquatic organisms. This study investigated the signaling pathways mediating the effects of exposure to Cu using a toxicogenomic approach in a fish model, the stickleback (*Gasterosteus aculeatus*). Freshwater-acclimated male fish were exposed via the water to Cu, including at environmentally relevant concentrations (3.2−128 μg of Cu/L for 4 days), and the biological responses explored through analyses of the hepatic transcriptome and metabolome and phenotypic end points, including assessment of DNA damage in blood cells. The Cu exposures resulted in DNA strand breaks in blood cells at all exposure concentrations and alterations in hepatic gene expression and metabolite concentrations in a concentration-dependent manner (from 10 μg of Cu/L). Genes associated with the cholesterol biosynthesis pathway were significantly over-represented and consistently down-regulated (at 128 μg of Cu/L), similar to that occurring in a mouse model for Wilson's disease. Additionally, inductions in metallothionein and catalase were also observed. The concentrations of NAD^+ and lactate increased significantly with the Cu exposure, consistent with a shift toward anaerobic metabolism, and these aligned closely with changes observed in gene expression. The pathways of Cu toxicity identified in our study support the conserved mechanisms of Cu toxicity from lower vertebrates to mammals, provide novel insights into the deleterious effects of Cu in fish, and further demonstrate the utility of fish as environmental sentinels for chemical impacts on both environmental and human health.

Introduction

Water bodies receive numerous contaminants from both natural and anthropogenic sources, and there are often health implications for exposed aquatic organisms. Collectively, fish are valuable sentinel organisms for the protection of environmental (and human) health from contaminants in the aquatic environment, and they share many similar metabolic and physiological features with other vertebrates. Furthermore, fish are especially vulnerable to contaminants discharged into the environment as they are often exposed to pollutants of concern continuously and throughout their lives.

Chemicals of concern in the aquatic environment are diverse and include both organic (e.g., pharmaceuticals, detergents, plastics, hydrocarbons, and pesticides) and inorganic (e.g., heavy metals, including Cu, cadmium, zinc, and iron) contaminants. Cu is particularly interesting as an environmental contaminant, because it is both a micronutrient essential to life but also is toxic to most organisms at concentrations found commonly in the aquatic environment. Cu concentrations vary widely in freshwater systems (0.2−30 μg/L) and are particularly high in water bodies associated with mining activities (100−200000 μg/L) (*1*). In rivers in urbanized areas, typical concentrations of dissolved Cu are in the low microgram range, but there are areas where much higher concentrations have been recorded: for example, up to 70 μg/L in Chesapeake Bay (*2*), over 70 μg/L in Watarase Basin, Japan (*3*), and 124 μg/L in the catchment of the River Lee, United Kingdom (*4*). These concentrations are higher than those reported to cause adverse effects to fish and used to define the water quality criteria for Cu (the concentrations calculated to cause no effects in aquatic species by the U.S. EPA are within the low microgram per liter range; http://www.epa.gov/waterscience/criteria/copper/).

Cu is essential as a catalytic and structural cofactor for multiple enzymes involved in energy production, iron acquisition, oxygen transport, cellular metabolism, peptide hormone maturation, blood clotting, and signal transduction, among others. Thus, organisms have evolved highly regulated mechanisms of uptake, transport, storage, and excretion for this metal to maintain homeostasis in an environment in which the availability of trace nutrients fluctuates (reviewed in ref *5*). The mechanism of storage for Cu involves the up-regulation of metallothioneins (MTs), proteins that are able to sequester and store large quantities of Cu (*6*). In turn, MTs (both transcripts and proteins) have become well-established biomarkers for Cu and other metal exposures as they are readily induced in a concentration-dependent manner (*7, 8*). Excess Cu leads to complex toxicological effects including oxidative stress (*1, 9*), DNA damage (*10, 11*), and lipid peroxidation (*10*). In humans, Cu toxicity is particularly well studied as a condition known as Wilson's disease can occur. In this disease, a mutation in the gene encoding for a Cu transporter that is essential for Cu excretion from hepatocytes (ATPase, Cu^{2+}-transporting β-polypeptide; ATP7B) leads to accumulation of Cu in the liver and the subsequent biochemical effects described above (reviewed in ref *12*). In fish, Cu is taken up via the diet (as in mammals), but it is also transported from the water across the gill epithelium. The high volume of water passing through the gill epithelium renders fish especially susceptible to Cu toxicity, and the lethal concentration causing 50% mortalities for waterborne

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exposures varies from 210 μg of Cu/L for trout (*Oncorhynchus mykiss*) to 661 μg of Cu/L for common carp (*Cyprinus carpio*) and 1398 μg of Cu/L for gibel carp (*Carassius auratus gibelio*) (*13*). This vulnerability of fish to Cu exposure and toxicity highlights the need to better understand the risks posed by this metal to fish populations.

Systems biology approaches in toxicology offer an unprecedented potential to unravel the complex effect pathways of chemical contaminants and significantly enhance our understanding of their effects on the health of organisms. In concert with phenotypic measures of chemical exposure and health effects, the application of "omics" techniques has provided the opportunity to distinguish alterations associated with adverse health effects from those involved in normal acclimation to changing conditions. Such information is also of value in the identification of appropriate biomarkers of exposure, able to act as early warning systems for the protection of environmental and human health. Toxicogenomic data sets on the effects of metals on aquatic organisms are starting to emerge (*14–17*), but integrative assessments of the changes encoding for adverse health effects are still relatively unexplored. The present study was designed to generate a mechanistic understanding of the effects of Cu exposure and thus potential health implications of such exposures on a widely used and geographically dispersed model fish species, the stickleback. Fish were exposed to five concentrations of Cu, covering those likely to be found in freshwater systems globally, and hepatic transcriptome and metabolome changes as well as phenotypic end points indicative of health effects were measured. Data were interrogated to search for the molecular effect pathways of Cu across the range of exposure concentrations tested and to identify the molecular pathways signaling for adverse health effects.

Experimental Section

Chemicals. All chemicals and reagents were obtained from Sigma-Aldrich (United Kingdom) unless stated otherwise.

Fish Exposure to Copper. Mature male stickleback were exposed to five concentrations of Cu (3.2, 10, 32, 64, and 128 μg/L) in duplicate tanks for a period of 4 days. A water control treatment was also included. At the end of the exposure regime, fish were sacrificed humanely, the blood was collected for determination of DNA damage, the livers were dissected, frozen in liquid nitrogen, and processed for determination of transcriptomic and metabolomic profiles, and the gonads were dissected to confirm the sex of each individual. Water samples were collected throughout the exposure, and the total dissolved Cu was determined by differential pulse anodic stripping voltametry at a hanging mercury drop electrode (DPASV-HMDE). A detailed description of the fish origin and husbandry, exposure procedures and sampling, and chemical analysis of the water is given in the Supporting Information.

Health Effect Assessments. A number of morphometric parameters were determined to assess the general health and stage of sexual development, including the gonadosomatic index (GSI, gonad wt/body wt $\times$ 100), the hepatosomatic index (HSI, liver wt/body wt $\times$ 100), and the condition factor (body wt/(length)^3 $\times$ 100). DNA strand breaks were measured in blood cells (principally red blood cells) from individual stickleback using the comet assay (*18*) as an indication of DNA damage. Comparisons between groups for the morphometric and physiological measurements were performed using one-way analysis of variance (ANOVA). When data did not meet the requirements of normality or equal variance, comparisons were performed using ANOVA on ranks. Differences between groups were considered to be statistically significant when $p < 0.05$.

Transcriptomic and Metabolomic Analyses. Measurements of transcriptomic and metabolomic profiles were conducted on stickleback livers, using a specific cDNA microarray and one-dimensional ^{1}H NMR spectroscopy, according to the methods described previously (see ref *19* and references within). Data analyses were performed using significance analysis of microarrays (SAM) under the R programming environment, and the statistical tools available within GeneSpring GX 7.3 (Agilent), as previously described (see ref *19* and references within). A full description of the methodologies and statistical approaches employed is given in the Supporting Information. The transcriptomic data presented in this paper are available under the accession numbers E-MAXD-42 and E-MAXD-43 in the ArrayExpress database at the European Molecular Biology Laboratory, European Bioinformatics Institute, EMBL-EBI (http://www.ebi.ac.uk/microarray-as/ae/).

Validation of the transcriptomic profiles was conducted using real-time quantitative polymerase chain reaction (RT-QPCR) analyses with five target genes, including metallothionein (induced by Cu exposure), and four genes belonging to the cholesterol biosynthesis pathway that were down-regulated as a result of the Cu exposure (24-dehydrocholesterol reductase, hydroxymethylglutaryl-CoA reductase, hydroxymethylglutaryl-CoA synthase, and isopentenyl-diphosphate δ isomerase). Primer sequences, PCR product sizes, and annealing temperatures are summarized in Table S1 in the Supporting Information. RT-QPCR analyses were conducted on independent biological samples, exposed to the same chemical treatment as those used for the transcriptomics and metabolomics experiments (see the Supporting Information for the full methodology).

Results

Water Chemistry. Chemical analysis of the water for concentrations of Cu showed that the exposure concentrations were between 70% and 88% of the nominal concentrations for all treatments throughout the study (Figure S1, Supporting Information).

Morphometric and Health Effect Measurements. Throughout this study, fish remained in good condition with no mortalities attributed to treatment. The mean body weight, length, and condition factor of the fish were 1.11 $\pm$ 0.03 g, 4.72 $\pm$ 0.04 cm, and 1.03 $\pm$ 0.01, respectively, and there were no significant differences between treatment groups. The mean GSI and HSI were 0.50 $\pm$ 0.02 and 4.13 $\pm$ 0.10, respectively, with no statistically significant differences between treatments. All Cu treatments induced an increase of DNA strand breaks in blood cells, which occurred in a concentration-dependent manner ($p = 0.012$, $R^2 = 0.80$, Figure 1). The percentage tail DNA of the cells (indicating DNA strand breakage) ranged in severity from 17.38 $\pm$ 1.23% for the control fish to 37.23 $\pm$ 1.78% for fish exposed to the highest concentration tested (128 μg of Cu/L).

Concentration-Related Effects of Cu on the Hepatic Transcriptome. Pooled RNA samples from eight individuals within each treatment group were used in an initial study to identify the Cu treatments that resulted in significant gene responses and to select treatments for subsequent analyses of individual transcriptomes. The global trends in the data set were investigated using principal component analysis (PCA) based on the list of consistently expressed genes. On the basis of the first three components, the gene expression profiles for each treatment pool defined a curve closely associated with concentration, from 0 to 128 μg of Cu/L (Figure S2A, Supporting Information). In addition, we searched for genes whose expression was correlated with the concentration of Cu in the water, and this approach identified 568 genes (Figure S2B and Table S2A in the Supporting Information). On the basis of these data, we

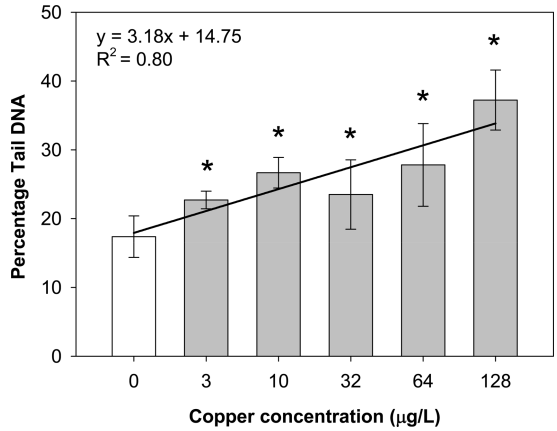

FIGURE 1. DNA damage (comet assay, percentage tail DNA) in blood cells of male stickleback exposed to a water control and 3.2, 10, 32, 64, and 100 μg of Cu/L for 4 days. Each column represents the mean ± standard deviation. Asterisks indicate treatments that were significantly different from the water control ($p < 0.05$, Mann−Whitney *U* test).

prioritized analyses of the hepatic transcriptomes of individual fish exposed to concentrations of 10, 32, and 128 μg of Cu/L. Analysis of the gene expression profiles based on individual fish identified 311 genes ($p < 0.05$) for which their expression correlated with the Cu exposure concentration, and there was a strong overlap between this gene list and that identified in the pooled experiment ($p = 1.11E{-}55$). SAM identified 102 features corresponding to 32 unique genes differentially expressed between treatment groups (false discovery rate (FDR) < 0.1, Table S3 in the Supporting Information). PCA based on these genes showed that individuals exposed to the same treatment were grouped together, in particular for the exposure to 128 μg of Cu/L (Figure 2A). Quality threshold (QT) clustering of the differentially expressed genes identified both monotonic and nonmonotonic responses for the regulated genes (Figure S3, Supporting Information). Analysis of over-representation of gene ontologies (GOs) among the nonredundant genes regulated by Cu showed a bias toward specific ontologies (Table S4, Supporting Information). The most significantly over-represented GO terms were related to steroid biosynthesis and metabolism, including cholesterol biosynthesis and metabolism (FDR < 0.0001). Within these processes, genes for several enzymes that are part of the cholesterol biosynthesis pathway were the most affected (strongly down-regulated), and in fish exposed to 128 μg of Cu/L there was an up to 6-fold down-regulation for some of these genes (Table S3). These results were validated by RT-QPCR performed on independent biological replicates (Figure S4, Supporting Information), confirming the trends in gene expression observed for all cases.

Other GO terms over-represented among the genes regulated by Cu included response to chemical stimulus and ion homeostasis. The gene encoding for metallothionein, a classic biomarker of metal exposure, was significantly (but modestly) up-regulated. The gene expression pattern for metallothionein was verified by RT-QPCR and, similarly, confirmed a concentration-dependent response curve for this gene (Figure S4, Supporting Information). The gene betaine homocysteine methyltransferase, encoding the cytosolic enzyme catalyzing the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively, was also significantly induced following exposure to Cu. In addition, genes involved in response to oxidative stress (e.g., catalase), genes involved in cell cycle regulation (e.g., cyclin G1), and genes encoding components of the cell

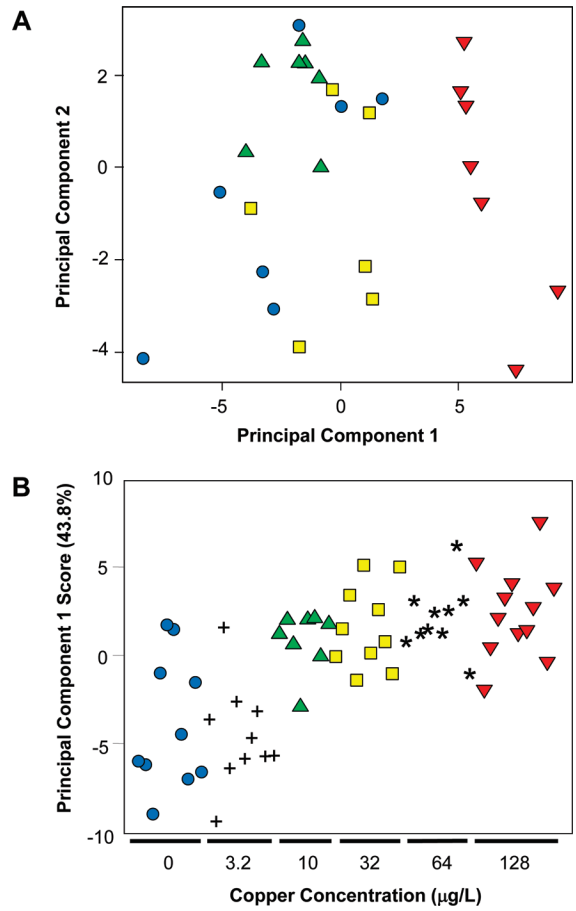

FIGURE 2. (A) Scores plot from a principal component analysis of the individual hepatic gene expression profiles following exposure to Cu (based on all genes showing statistical differences in expression between the treatments (SAM with FDR < 0.1): blue circles, control; green triangles, 10 μg of Cu/L; yellow squares, 32 μg of Cu/L; red triangles, 128 μg of Cu/L. (B) PC1 scores versus sample number plot from a principal components analysis of the 60 filtered NMR spectra of individual stickleback livers, which each contain 424 bins (SAM with FDR < 0.1), following treatment with water control and 3.2, 10, 32, 64, or 128 μg of Cu/L. To highlight the concentration-dependent effect of Cu on the metabolome, the sample numbers (1−60) are presented in terms of their corresponding Cu concentrations.

structure (e.g., β-actin) were all up-regulated as a result of exposure to Cu (at 128 μg/L).

Concentration-Related Effects of Cu on the Hepatic Metabolome. A number of identified metabolites were found to be significantly altered in the liver in response to the Cu exposure (as determined by SAM, FDR < 0.01), and these are listed in Table S5 in the Supporting Information. The fold changes in metabolite concentrations were small (all less than 2-fold), but statistically significant; e.g., lactate increased by only 24% from the water control to the highest dose of 128 μg/L Cu ($p = 0.006$). The changes in the relative concentrations of these metabolites are shown in Figure S5 in the Supporting Information and clearly illustrate a concentration-dependent effect and an apparent onset of metabolic alteration at around 32 μg/L Cu. Using a less stringent FDR of <0.1, a total of 424 NMR bins were found to be significantly different across the exposure groups. PCA of all 60 NMR spectra, using just these 424 bins, yielded the scores plot shown in Figure 2B. A clear Cu concentration-dependent relationship was evident along the principal component 1 (PC1) axis, which takes into account changes in multiple metabolites. Considering this multimarker profile,

the onset of metabolic alteration occurs at 10 μg/L Cu (one-way ANOVA of PC1 scores, $p = 6.01 \times 10^{-9}$, with the water control group significantly different from the 10, 32, 64, and 128 μg of Cu/L treatment groups using a Tukey family error rate of 5%).

Discussion

Pathways of Cu Toxicity. In this study, the exposure concentrations of Cu were chosen to represent those likely to occur in the aquatic environment and below those expected to induce overt toxic effects or mortality. No effects were seen on the mortality, condition factor, HSI, GSI, or swimming and feeding behavior, indicating that the exposures were indeed below the threshold for overt toxicity. However, a concentration-related increase in DNA damage was seen in blood cells, supporting previous work (*10, 20*) where it has been shown that Cu causes oxidative stress and associated DNA damage in rainbow trout gill cells and bacteriophage λ DNA, respectively. This finding potentially has implications for the longer term health of the individuals, when considering that the exposures undertaken were both short-term and environmentally relevant, and many fish (and other aquatic animals) will be exposed to Cu at these concentrations throughout their lives. The likelihood of adverse health effects, however, will depend on many factors, including the balance of activity between DNA damage and DNA repair.

Some of the gene responses in stickleback following exposure to Cu followed hormetic concentration−response curves (see the QT cluster analysis of the genes regulated by Cu, Figure S3 in the Supporting Information), with induction of transcripts at low concentrations and their inhibition at high concentrations (and vice versa). This type of response has been observed in many ecotoxicological studies (reviewed in ref *21*, including a recent study that employed toxicogenomics to investigate Cu toxicity in the earthworm, *Lumbricus rubellus* (*22*). This is especially relevant here as Cu is an essential metal, required for the normal physiology of organisms, and therefore, exposure to very low concentrations may have beneficial outcomes for the organism and/or may lead to acclimation, whereas relatively high concentrations will lead to toxic effects. However, the increased DNA damage observed in blood cells indicates that adverse effects may be occurring even at the lowest concentration tested (3.2 μg of Cu/L).

The mechanisms of the toxicological responses as determined by the transcriptomic analysis in fish exposed to Cu (most notably at 128 μg of Cu/L) were in line with those previously documented in the literature, including increased toxic stress, probably, in part, via the generation of reactive oxygen species (ROS). This was implied by the induction of catalase in the liver, a gene responsive to oxidative stress and inducible by short-term exposure to Cu (*1*). ROS were also likely to have contributed to the increased DNA damage observed. It is well documented that ROS can contribute to the process of cell death through apoptosis and necrosis (reviewed in ref *23*), and similar effects of Cu in other organisms have been extensively documented (*24–26*). Other mechanistic pathways for Cu toxicity observed included a putative impairment of oxidative phosphorylation in the mitochondria, which would potentially result in depletion of energy reserves. Specifically, there was a concentration-dependent increase in the expression of genes for several enzymes involved in glycolysis (including glyceraldehyde 3-phosphate dehydrogenase ($p = 0.0103$), fructose bisphosphate aldolase A ($p = 0.0000445$), and fructose 1,6-bisphosphatase 1 ($p = 0.00256$)) and a concentration-dependent decrease in the expression of genes for enzymes constituting the electron transport chain (including cytochrome *c* oxidase subunit 1 ($p = 0.00229$) and cytochrome *c* oxidase subunit VIa ($p = 0.0471$), p values for the correlation between gene expression and Cu concentration in the experiment performed on individuals). The metabolomics data supported these findings, with both lactate and NAD^+ up-regulated in response to increasing Cu concentrations. Lactate results from the process of glycolysis, and its increase signals for an increase in anaerobic respiration. NAD^+ is involved in multiple biological processes and is a key regulator of metabolism. Alterations in the concentration of these molecules signal for a disruption of the energy production pathways. Together, these results are consistent with the reported toxicological effect of Cu on oxidative phosphorylation (*1, 22, 27*) and with previous studies where Cu exposure in wild fish resulted in an impairment of their aerobic capacity (*28*).

In our study, metallothionein gene expression showed a modest, but consistent increase at threshold concentrations of 32 μg of Cu/L, congruent with increased cellular capacity for storage and detoxification of Cu (reviewed in ref *29*). For other metals, metallothionein is induced to a much greater extent, suggesting that the value of this protein as a biomarker for Cu exposure is less important than that for other nonessential metals, for example, cadmium. Similar findings have been reported elsewhere for both vertebrates and invertebrates (*15, 16, 27*). The expression of Cu-metabolism MURR1 domain containing protein 1 was also significantly increased as a result of the Cu exposure. This gene has been implicated in the regulation of Cu homeostasis and is likely to be involved in the detoxification of this metal in the liver through its interactions with the Cu transporter ATP7B (*30*).

The expression of betaine homocysteine methyltransferase showed a consistent concentration-dependent up-regulation following exposure to Cu. This is a cytosolic enzyme that catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively, a reaction that is also required for the irreversible oxidation of choline (*31*). This enzyme requires zinc for its functioning (*32*) and has been shown to be down-regulated in Cu-deficient rats (*33*), but its involvement in the toxicological response to Cu has not been previously documented. It has, however, been established that betaine homocysteine methyltransferase is irreversibly oxidized by H_2O_2, leading to the loss of zinc and loss of enzymatic activity (*34*). The effect observed in the stickleback could be occurring as a result of the oxidative conditions resulting from the exposure to Cu, and in turn this is likely to have led to an induction of the transcription of the gene encoding this enzyme. Interestingly, the decrease in choline as a result of the Cu exposure is possibly linked with the loss of activity of this enzyme. These findings warrant further study on potential epigenetic effects of Cu exposure since choline deficiency has been associated with changes in DNA methylation and hepatic carcinogenesis in rodents (*35*) and with hepatocellular adenomas in fish (*36*).

The exposure of stickleback to Cu resulted in the alteration of the expression of genes involved in protein biosynthesis and protein degradation, including many ribosomal proteins, constituents of the ubiquitin and proteasome pathways, and proteases. In parallel, there was a significant dose-dependent increase in asparagine and glutamine, indicating that exposure to Cu impacted aspects of protein metabolism. Similar results were observed as a response to Cu in fathead minnow embryos, where an induction of genes associated with the ribosome and proteasome was observed following exposure to Cu (*37*). The functional significance of the regulation of protein biosynthesis and turnover as a result of Cu exposure may be linked with the need to remove and replace proteins where damage has occurred as a result of oxidative conditions, as previously suggested for cadmium (*16*).

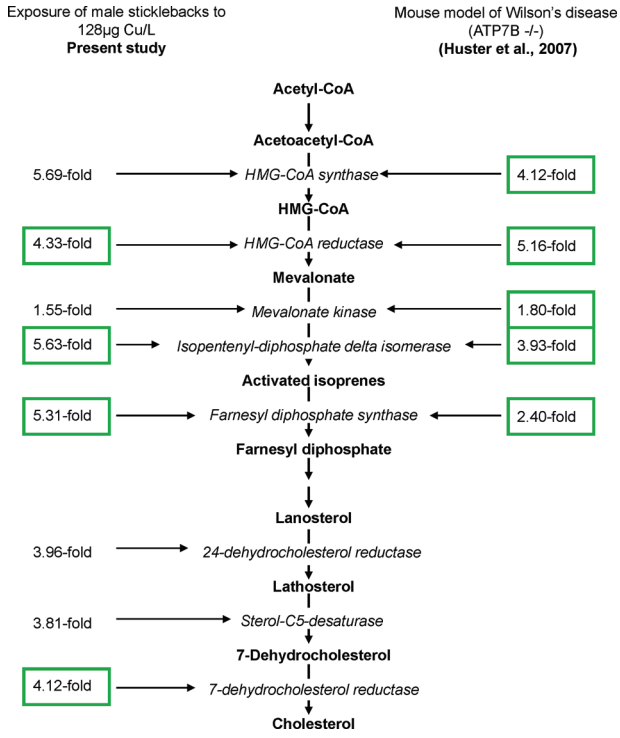

FIGURE 3. Comparative analysis of the effects of Cu toxicity on the cholesterol biosynthesis pathway. Down-regulation of the expression of multiple genes encoding for enzymes involved in the biosynthesis of cholesterol in the present study and in a study employing a mouse model for Wilson's disease, lacking the Cu transporter ATP7B (*38*). Expression data represent the average fold down-regulation of the differentially expressed reporters for each enzyme. Data highlighted by gray boxes were statistically significant (SAM, FDR < 0.1).

Down-Regulation of Cholesterol Biosynthesis. The most pronounced hepatic changes in gene expression observed following 4 days of exposure to Cu (128 μg of Cu/L) occurred for genes encoding enzymes that constitute the cholesterol biosynthesis pathway (Figure 3). There was a significant down-regulation of many of these genes, including hydroxymethylglutaryl-CoA reductase (4.33-fold down-regulated, catalyzing the commitment step in cholesterol biosynthesis), 7-dehydrocholesterol reductase (4.12-fold down-regulated, encoding the penultimate enzyme of mammalian sterol biosynthesis that converts 7-dehydrocholesterol to cholesterol), and isopentenyl-diphosphate δ isomerase (5.63-fold down-regulated, involved in intermediate steps for the synthesis of cholesterol). The reproducibility of these findings was verified by RT-QPCR analysis of the gene expression profiles of 24-dehydrocholesterol reductase, isopentenyl-diphosphate δ isomerase, hydroxymethylglutaryl-CoA synthase, and hydroxymethylglutaryl-CoA reductase performed on independent biological replicates exposed to the same treatment conditions (Figure S4, Supporting Information). Strikingly, the gene expression responses in this study were similar to those observed in a recent study on Wilson's disease conducted using mice (*38*) (Figure 3). In this disease, the lack of a functional ATP7B results in hepatocytes being unable to excrete Cu, leading to accumulation of Cu in the liver, followed by toxicity and death. Huster et al. employed mice that lack functional ATP7B transporters and investigated the early toxicological process accompanying this disease when Cu was accumulating in the liver and before overt toxicity occurred (*38*). The similarity in the inhibition of the cholesterol biosynthesis pathway between those occurring for Wilson's disease in the mouse and in the stickleback for our study, as a result of Cu exposure, is remarkable and suggests that this pathway is a target for the early stages of Cu toxicity in both mammals and fish. The liver is the primary organ for cholesterol production in vertebrates, and reduction in the expression of several genes involved in cholesterol biosynthesis has potentially major implications for health status. Cholesterol is required for membrane stability, formation of billiary acids, and biosynthesis of steroid hormones, illustrating the range of potential health impacts following Cu exposure. The metabolomics analyses conducted within this study were focused on polar metabolites and therefore could not provide additional insight into the consequences of down-regulation of genes involved in cholesterol biosynthesis. Dietary Cu supplementation has been previously reported to lower tissue and serum levels of cholesterol in a number of vertebrates, including chickens (*39, 40*), rats (*41*), and humans (*42*). For fish, the literature is not clear and studies have reported increased cholesterol in the blood (*43*), decreased cholesterol in the liver and gonads (*44*), or no effects on the concentrations of cholesterol (*45*). The exact mechanisms by which Cu exerts its effects on cholesterol levels are unclear, but appear to involve the down-regulation of genes involved in the cholesterol biosynthesis pathway (*40*). Our data are in agreement with this and suggest that early stages of Cu toxicity involve a consistent and very significant down-regulation of the genes constituting this pathway across vertebrate species. Further studies are required to elucidate the exact regulatory mechanism by which Cu causes these coordinated responses and the implications for the health of exposed organisms.

In summary, we report the impacts of Cu exposure on the health of a model fish species, the stickleback. The effects observed include those that were expected, including induction of DNA damage and changes in the expression of specific genes and metabolites, and we also showed that effects occurred at environmentally relevant concentrations. The most striking effect observed at the level of the transcriptome was a very significant down-regulation of the expression of genes encoding for enzymes which mediate the biosynthesis of cholesterol. This finding aligns closely with that reported for a mouse model of Wilson's disease, and to our knowledge, it is the first time that a consistent and pronounced effect on this pathway has been observed as a result of Cu exposure in fish. Gene expression and metabolomic changes aligned closely for specific pathways and suggested that Cu causes a switch from aerobic to anaerobic respiration, as has been reported previously for other species. This data set, therefore, illustrates the power of toxicogenomics to uncover novel biological targets of chemical toxicity that were unlikely to be identified by the classic toxicological approaches, provides novel insights into the impacts of Cu in fish, and further supports the use of fish as environmental sentinels and models for information on potential contaminant effects on human health.

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Supporting Information Available

Supplemental Experimental Section, (Figure S1) measured concentrations of Cu in the tank water, (Figure S2) principal component analysis of the hepatic gene expression profiles and cluster diagram of the genes showing statistical association with the treatment concentration, (Figure S3) QT cluster diagram of genes differentially expressed between Cu treatments, (Figure S4) expression profiles of hepatic genes in sticklebacks exposed to Cu as determined by microarray analysis and by RT-QPCR on independent biological samples, (Figure S5) relative concentrations of identified metabolites that changed significantly across treatment groups, (Table S1) target genes, primers, and assay details for RT-QPCR analysis, (Table S2) genes significantly correlated with the concentration of Cu in the analysis conducted on (A) pooled samples and (B) individual samples, (Table S3) differentially expressed genes between treatments for the microarray experiment conducted on individual fish and the fold change for each treatment compared to controls, (Table S4) gene ontology analysis of the genes differentially expressed between treatment groups on the microarray experiment conducted on individual fish, and (Table S5) identified metabolites showing significant changes in concentration in stickleback liver extracts following exposure to Cu. This information is available free of charge via the Internet at http://pubs.acs.org.

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