Daphnia magna Ecotoxicogenomics Provides Mechanistic Insights into Metal Toxicity

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Toxicogenomics has provided innovative approaches to chemical screening, risk assessment, and predictive toxicology. If applied to ecotoxicology, genomics tools could greatly enhance the ability to understand the modes of toxicity in environmentally relevant organisms. *Daphnia magna*, a small aquatic crustacean, is considered a "keystone" species in ecological food webs and is an indicator species for toxicant exposure. Our objective was to demonstrate the potential utility of gene expression profiling in ecotoxicology by identifying novel biomarkers and uncovering potential modes of action in *D. magna*. Using a custom *D. magna* cDNA microarray, we identified distinct expression profiles in response to sublethal copper, cadmium, and zinc exposures and discovered specific biomarkers of exposure including two probable metallothioneins, and a ferritin mRNA with a functional IRE. The gene expression patterns support known mechanisms of metal toxicity and reveal novel modes of action including zinc inhibition of chitinase activity. By integrating gene expression profiling into an environmentally important organism, this study provides experimental support for the utility of ecotoxicogenomics.

Introduction

From evolutionary biology to the medical sciences, almost every field of biology has benefited from the genomics

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revolution. In particular, toxicology has thrived by integrating genomics into toxicological studies. In 1999, Nuwaysir et al. described how microarray technologies could transform toxicology, presenting the possibilities of a new field called toxicogenomics (*1*). Since then, a proliferation of studies has substantiated these promises. Gene expression profiling has been used to identify and confirm mechanisms of action of different toxicants (*2*), discern the effects of chemical mixtures (*3*), and identify novel biomarkers of exposure including exposure to copper (*4*) and other metals (*5*). Perhaps the greatest potential for microarrays in toxicology is the ability to classify chemicals based on their gene expression profiles, thus revealing characteristics about their mode of action and toxicity to organisms. Several studies have provided validation for such a classification system (*6*), and databases such as Chemical Effects in Biological Systems (CEBS) are presently being developed to store large amounts of gene expression data to make this a reality (*7*).

The accomplishments of toxicogenomics, if applied to ecotoxicology, could facilitate the basic tasks of monitoring contaminant levels, identifying the chemicals responsible for toxicity in impaired waters, and helping protect ecosystems and human health by encouraging the design of less harmful chemicals (*8*). A few studies have shown how genomic technologies could be employed in ecotoxicology and have been reviewed recently (*9*). In particular, a *Daphnia magna* cDNA microarray enriched in transcripts related to reproduction was used to evaluate the effects of the fungicide, proiconazole, on gene expression (*10*). This study successfully illustrated the usefulness of custom-made microarrays for studying gene expression in a nontraditional organism.

In the present study, we continue to build on the proof-of-principle studies completed in ecotoxicogenomics through the development of a cDNA microarray for *D. magna* (water flea), a standard test organism for freshwater ecotoxicity studies (*11*). We identified distinct gene expression profiles for three metal exposures: copper, cadmium, and zinc at sublethal concentrations. Moreover, we have discovered previously unknown *D. magna* genes encoding two putative metallothioneins, and homologues to glutathione-*S*-transferase, chitinase, and ferritin. Our genomic approach allowed us to uncover unknown modes of toxicity, including a downregulation of genes related to exoskeleton maintenance during zinc exposure. Further investigation confirmed that Zn causes a decrease in chitinase activity, linking the gene expression changes to a physiological response. This study illustrates the power of genomics to go beyond a focus on individual genes by establishing expression profiles of toxicant exposure and revealing biological pathways that may contribute to the toxicity of these three metals.

Materials and Methods

Maintenance of *D. magna* Cultures. Genetically homogeneous *D. magna*, purchased from Aquatic Research Organisms (Hampton, NH), were cultured in COMBO modified for water hardness (the constituents of COMBO media are described in ref *12*) and maintained at 23.5 °C in a Percival environmental chamber according to standard protocols (*11, 13*). Following exposure studies, pH, dissolved oxygen, water hardness, and alkalinity were measured, recorded, and reported in Table S2 (Supporting Information).

Acute and Chronic Toxicity Assays. Acute and chronic toxicity assays were conducted using protocols similar to the USEPA Whole Effluent Toxicity (WET) protocol (*11*) and USEPA chronic toxicity WET protocol (*13*). First instar *D. magna* were placed in 25 mL of media containing varying

concentrations of copper sulfate (Fisher Scientific, Hampton, NH), cadmium sulfate (Fisher Scientific), or zinc chloride (Sigma-Aldrich, St. Louis, MO). Eight concentrations were tested for each contaminant and a zero concentration control. End points measured in these toxicity tests included lethality for the acute test and reproduction for the chronic test. The LC_{50}, EC_{50}, and NOEC were determined using the statistical procedures outlined by the USEPA (*11*).

Printing to Microarray. A total of 5000 randomly selected cDNA clones from the *Daphnia* Genome Consortium (DGC) library (generous gift from D. Bauer at University of New Hampshire, NH, and J. Colbourne at Indiana University, IN) were PCR amplified from the pDNR-LIB vector using the following primers: forward, AGTCGACGGTACCGGACATA, and reverse, GCCAAACGAATGGTCTAGAAA. PCR products were purified by ethanol precipitation and resuspended in distilled water. Clones were checked by electrophoresis on a 1.2% agarose gel to ensure uniform PCR products. cDNA clones were printed onto lysine-coated glass slides by the UCB Nutritional Sciences and Toxicology genomics facility.

Chemical Exposures. Chemical exposures were performed using ~40 adult (16−18 day old) *D. magna* placed in 2 L of COMBO media for 24 h. For microarray hybridizations, we exposed *D. magna* to a sublethal concentration of 1/10th the LC_{50} value determined in the acute toxicity bioassay (see Table S1, Supporting Information) for each metal. A zero concentration control was performed alongside the metal exposures. For the chitinase enzyme assay, *D. magna* were exposed to the 1/10 LC_{50} for each metal. Additional concentrations of Zn including 100, 200, and 1000 μg/L were used to provide a dose response of chitinase activity. For the microarray experiments, three biological replicates were performed for each metal exposure on separate dates. A fourth replicate exposure was performed for *real-time* PCR confirmation, and three separate exposures were done for the chitinase enzyme assays. Following each 24-h exposure, *D. magna* were collected and RNA was extracted for microarray hybridizations and real-time PCR or crude protein was isolated for chitinase activity studies.

RNA Isolation. *D. magna* were harvested by gently removing each daphnid from the culture and immediately grinding them in liquid nitrogen using a pestle and mortar. RNA was isolated using Trizol according to standard methods (Invitrogen, Carlsbad, CA).

Microarray Hybridization. Before proceeding to reverse transcription, RNA from both the unexposed and exposed *D. magna* was split into two pools, to provide two replicate hybridizations for each metal exposure. cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) from total RNA in the presence of aminoallyl-labeled dUTP. Fluorescence labeling proceeded by incubating the aminoallyl-labeled cDNA with Cy5 or Cy3 fluorescent dyes (Amersham Biosciences, Piscataway, NJ). The dyes were switched for each technical replicate so that the control cDNA was labeled with Cy3 in one hybridization and Cy5 in the other. The labeled cDNA pools from the unexposed and exposed *D. magna* were mixed and hybridized to the *Daphnia* microarray (protocols available at http://cmgm.stanford.edu/ pbrown/mguuide). Because three exposures were performed for each metal, and RNA from each exposure was hybridized to two different microarrays, there were six hybridizations for each metal. Scanning and quantification was performed using an arrayWoRx Biochip Reader (Applied Precision, Issaquah, WA) and GenePix software version 3.01 (Axon Instruments, Union City, CA). Detailed information about the experimental design, raw signal intensity values, and other MAIME compliant data are available at the Gene Expression Omnibus (GEO) (located at http://www.ncbi.nlm.nih.gov/ geo) with the accession number GSE4759.

Identification of Candidate Differentially Expressed Genes. The statistical methods used to normalize the data and identify differentially expressed genes are described in detail in Loguinov et al. (*14*). Briefly, technical replicates were normalized to remove possible nonlinearity, if any, and checked for homogeneity using box plots. As an alternative to between-slide normalization, we applied an approach based on sequential single-slide data analysis and utilized the α-outlier-generating model and outlier regions approach to identify differentially expressed cDNAs. Our algorithms are implemented as software written in S-plus language (R version of the software is available in Louginov et al. (*14*)). The software used microarray data from single-slide experiments as an input and generated tables with candidate differentially expressed cDNAs, different types of the corresponding ratios, unadjusted *p*-values, adjusted *p*-values, and *q*-values. We applied an average false positive cutoff of 1 to identify candidates for differential gene expression. cDNAs differentially expressed in both technical replicates, and in two of the three, biological replicates were chosen as candidate differentially expressed cDNAs.

Sequencing of Differentially Expressed cDNAs. The plasmids containing the cDNAs determined to be differentially expressed were isolated from bacterial clones using the R.E.A.L. Prep 96 Plasmid Kit (Qiagen, Valencia, CA). The clones were sequenced in one direction using the following primer: AGTCGACGGTACCGGACATA.

Identification of Protein Homologues and Prediction of Protein Function. Translated BLAST searches (tblastx) were performed to determine the closest protein homologue to the sequenced cDNA clones (http://greengene.uml.edu/ Batch.html). cDNAs without homology to known proteins and cDNAs whose closest protein homologue was uncharacterized were further analyzed for possible protein function. cDNAs were translated using Expasy translation tool (http://us.expasy.org/tools/dna.html), and all possible protein fragments were characterized using PredictProtein (http://www.embl-heidelberg.de/predictprotein/predictprotein.html).

Real-Time PCR. To confirm the differential expression, several genes were chosen for quantitative PCR analysis. RNA isolated following 1/10 LC_{50} metal exposures was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). Primer sequences are available in Table S3 (Supporting Information). PCR amplification was performed using a SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA) and the following program: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. PCR products were quantified in real time using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). cDNA from unexposed *D. magna* was used to create standard curves for each primer set. PCR reactions were done in triplicate and compared to the standard curve to determine fold induction relative to the unexposed samples. The 18S rRNA expression levels were used to normalize for cDNA content.

RNA Oligomer Preparation. To confirm the functionality of the *D. magna* IRE, a 40-nucleotide RNA oligomer was designed based on the 5′ region of the *D. magna* ferritin mRNA sequence (AJ292556): UCUGUUUUGCUUCGCCAGU-GUGUGAACAAGCAGUUUCUAC. The 40-nucleotide oligomer was purchased from HHMI/Keck Biotech at Yale University, deprotected and desalted according to the manufacturer's guidelines. The RNA was further purified by PAGE on a 15%, 8 M urea gel. The 40-nt RNA band was detected by UV shadowing, excised, and the RNA eluted from the gel by incubating the gel fragment in water overnight. The RNA was concentrated by ethanol precipitation. For mobility shift assay experiments, the RNA was ^{32}P radiolabeled with T4 Kinase (New England Biolabs, Beverly, MA) and purified by G-50 spin column.
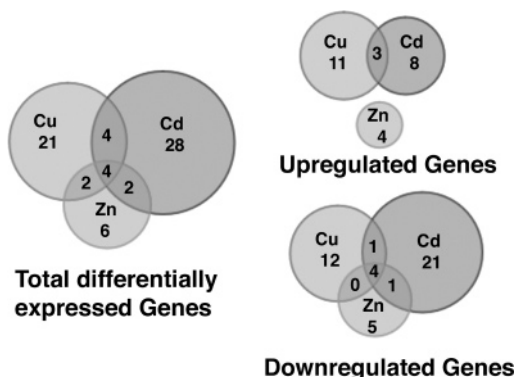
FIGURE 1. Copper, cadmium, and zinc causing the differential expression of a unique set of cDNAs. The number of differentially expressed genes determined by microarray analysis for each metal are shown including the genes wthat overlap in expression by more than one metal.

Electromobility Shift Assay. A 0.72-pmol aliquot of RNA 40-mer oligo was denatured at 90 °C for 5 min and allowed to reanneal by slow cooling to room temperature in 40 mM HEPES pH 7.2 with 100 mM KCl. The reannealed RNA was incubated for 30 min with the indicated amount of recombinant rabbit IRP1 (generously provided by William Walden, University of Chicago, IL) in 4% glycerol, 2% β-mercaptoethanol, and 4 mM $MgCl_2$. The RNA−protein complex was separated from unbound RNA on a nondenaturing 4% (19:1) polyacrylamide gel with 0.5× TBE buffer at 5V/cm for 1.5 h, and the band intensities were quantified by Phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). The percent complex was determined by dividing the band intensity of the RNA−protein complex by the total radioactivity in the same lane.

Protein Isolation. *D. magna* were harvested as described for RNA isolation and protein was isolated using the method of Zou and Bonvillian (*15*). Total protein content was determined using the BioRad protein assay based on the Bradford method (*16*).

Chitinase Assay. To determine if the downregulation of the exoskeleton genes resulted in a decrease in chitinase enzyme activity, a chitinase assay was performed. Chitinase activity was assayed according to the method of Zou and Bonvillain (*15*) using 0.2 mg/mL protein extract and the substrate 4-nitrophenyl *N,N′*-diacetyl-β-D-chitobioside (Sigma-Aldrich). Each protein extract was assayed three times and 0.007 unit/mL chitinase from *Streptomyces griseus* (Sigma-Aldrich) was used as a positive control.

Results and Discussion

Identification of Gene Expression Profiles and Candidate Biomarkers. Because we were interested in linking the acute gene expression responses to an adverse chronic outcome, we exposed *D. magna* to the $1/10$ LC_{50} for the microarray studies (see Table S2). This concentration is below levels that cause acute lethal toxicity, which may produce a nonspecific stress response, but within the range that causes chronic effects to reproduction. (See Table S2. The $1/10$ LC_{50} is greater than the chronic EC_{50} for Cd and Zn and close to the chronic NOEC for Cu.) After a 24-h waterborne exposure to the $1/10$ LC_{50} of Cu, Cd, or Zn, RNA from *D. magna* was extracted and hybridized to the 5000-element cDNA microarray. Differentially expressed clones were sequenced, and preliminary analysis of these cDNAs reveals that each metal had a unique expression profile as shown in Figure 1. A few genes are differentially expressed in response to more than one metal; however, the majority is specific for each metal exposure. We characterized the cDNAs using translated BLAST searches and PredictProtein, and organized the

TABLE 1. Predicted Function of Differentially Expressed Genes after Exposure to Copper, Cadmium, or Zinc at the 1/10 LC50[1]

Exp. level Cu Cd Zn	Acc #	Predicted Protein Class
METAL BINDING AND TRANSPORT		
	AJ292556	Ferritin
	DV437799	Metallothionein
	DV437826	Metallothionein
	DV437852	Ferritin
	DV437849	Ferritin subunit
	DV437835	Heavy metal binding protein, Collagen α chain
DIGESTION AND NUTRIENT ABSORPTION		
	DV437797	Cellulase
	DV437796	Endo-β-1,4-glucanase precursor
	DV437795	Endo-β-1,4-mannanase
	DV437794	α- amylase
	DV437819	Cubilin
	DV437815	Preamylase precursor
EXOSKELETON RELATED PROTEINS		
	DV437807	Chitin binding and metabolism
	DV437809	Chitinase
	DV437800	Chitin binding and metabolism
	DV437850	Chitin binding and metabolism
	DV437857	Chitinase
	DV437858	Chitinase precursor
	DV437856	Cuticle protein
CELL SIGNALING		
	DV437806	Inositol monophosphatase
	DV437805	Leucine-rich protein phosphatase
	DV437828	Protein kinase
	DV437832	Ras-related protein
IMMUNE FUNCTION		
	DV437831	Interferon γ-inducible protein
	DV437813	Lectin-like protein
	DV437823	β-1,3-glucan binding protein, Glycoside hydrolase 16
	DV437848	Cerebellin precursor-like protein
OXIDATIVE STRESS RESPONSE		
	DV437830	Glutathione-S-transferase
	DV437833	Glutathione-S-transferase
	DV437829	Peroxiredoxin V protein
MONOOYGENASES		
	DV437798	Monooxygenase
	DV437827	Dopamine β-hydroxylase
	DV437820	Dopamine β-hydroxylase
	DV437836	Copper type II, ascorbate-dependent monooxygenase
PROTEASES		
	DV437812	Aminopeptidase
	DV437825	Trypsin precursor
	DV437854	Carboxypeptidase A1 precursor
	DV437853	Trypsin
	DV437803	Chymotrypsin BII precursor
	DV437822	Zinc metallopeptidase
	DV437841	Serine collagenase precursor
	DV437834	Chymotrypsin precursor
	DV437842	Serine protease
	DV437843	Transmembrane serine protease
	DV437839	Transmembrane serine protease
	DV437838	Trypsin
	DV437840	Trypsin
	DV437846	Trypsin, cytoskeleton related
SULFOTRANSFERASES		
	DV437811	Sulfotransferase
	DV437801	Retinol dehydratase
	DV437802	Sulfotransferase, retinol dehydratase
DEVELOPMENTALLY RELATED PROTEINS		
	DV437824	posterior end mark
	DV437847	CUB and sushi domain containing protein
	AB114859	Vitellogenin SOD
	DV437844	Anionic trypsin II precursor, nude1
OTHER FUNCTIONS		
	DV437810	Mitochondrial import inner membrane translocase
	DV437817	Fatty acid binding protein
	DV437837	GM2 activator protein
	DV437845	NADH dehydrogenase
	DV437804	Fatty acid binding protein

[1]Red represents upregulated cDNAs and green represents downregulated cDNAs. ■ Log_2 Ratio: 0.32-0.50, ■ Log_2 Ratio: 0.51-0.80, ■ Log_2 Ratio: 0.81-1.35, ■ Log_2 Ratio: -0.38- -0.50, ■ Log_2 Ratio: -0.51- -0.80, ■ Log_2 Ratio: -0.81- -1.33. "Exp. Level" refers to the expression level of the genes relative to the control. "Acc#" refers to GenBank accession numbers for the *D. magna* cDNAs.

predicted proteins by function (Table 1). (Table S4, Supporting Information, provides the complete list of protein homologues for each differentially expressed cDNA.)

We selected candidate biomarkers of exposure and confirmed their differential expression using real-time PCR (RT-PCR) (Table S5, Supporting Information). For many genes, the RT-PCR results agreed with the microarray results. For others, including ferritin (AJ292556), and the inositol monophosphatase (DV437806), RT-PCR detected differential

expression when the microarray results did not. Further replication would be needed to confirm the differential expression of the genes, which disagreed with the microarray results. The inositol monophosphatase (DV437806) still remains a promising biomarker of exposure to Cu because the differential expression in response to the other metals was in the opposite direction.

Of particular interest to ecotoxicology is the identification of two putative metallothionein (MT) cDNAs, DV437826 and DV437799, which were induced following metal exposure (Figure S1, Supporting Information). MTs are low molecular weight proteins involved in the transport and detoxification of heavy metals and have been used as biomarkers of exposure to metals for many years (17). Despite the interest in using *D. magna* and other daphnids in ecotoxicology, no MT genes have been cloned from these organisms. The translated sequences for DV437826 and DV437799 identified in this study create short peptides with an abundance of cysteine residues, many of them found in the characteristic C−X−C motif of MT proteins, although it appears we only identified a partial sequence for DV437799. They share other properties with MTs including no aromatic residues, no histidine residues, and a low molecular weight (18) and were also identified as probable MTs by the computer algorithm ProDom (19). However, they are distinct from other crustacean MTs (Figure S1A). Only a few amino acids outside of the cysteine residues are conserved between the *D. magna* and other crustacean MTs and the *D. magna* MTs do not cluster with other crustacean MTs (Figure S1B).

Another metal binding protein and potential biomarker of metal exposure is a previously identified *D. magna* ferritin gene, AJ292556 (NCBI accession number). It is similar to the cytosolic heavy-chain ferritin characterized in *Daphnia pulex*. Microarray analyses identified this ferritin as upregulated by copper and cadmium exposure (Table 1 and Table S4), and RT-PCR analyses revealed that zinc also caused its induction (Table S5). Numerous ferritins characterized in insects are involved in the storage and scavenging of iron and are transcriptionally and translationally upregulated by Fe (20). Ferritin is also regulated by oxidative stress (21), and ferritin mRNA induction by Cd was shown in *Xenopus leavis* (22). The transcriptional induction of ferritin therefore, may be an indirect effect, mediated by oxidative stress caused by these metals.

Prior to this study, only the partial sequence of the *D. magna* ferritin was known. When we investigated the 5′ untranslated region of the EST sequence for this gene, we found an iron-response element (IRE) (Figure 2). Translational control of ferritins in many species has been shown to be the result of iron-response protein (IRP)/IRE interactions. IRPs bind to the hairpin structure created by the IRE, which rests upstream of the ferritin start codon, and inhibit translation of the mRNA. Iron decreases IRP binding, enabling translation of the ferritin protein. Other factors including nitric oxide, hydrogen peroxide, and oxygen have been shown to affect IRP binding (23). IREs have been characterized in multiple species including the crustacean, *Pacifastacus leniusculus* (or crayfish) (24). Figure 2A reveals the similarity in nucleotide sequence between the *D. magna* IRE, the *P. leniusculus* IRE, and the consensus sequence of insect ferritin IREs. Figure 2B shows the predicted secondary structure of the *D. magna* IRE, which forms the typical hairpin loop to which an IRP can bind. To determine if the *D. magna* IRE is able to bind IRP1, we performed an electromobility shift assay with recombinant rabbit IRP1. As shown in Figure 2C, IRP1 binds to the *D. magna* IRE and the extent of complex formation is dependent on the protein concentration, confirming the functionality of the *D. magna* IRE. Two partial *D. magna* cDNAs obtained from the *Daphnia* genome
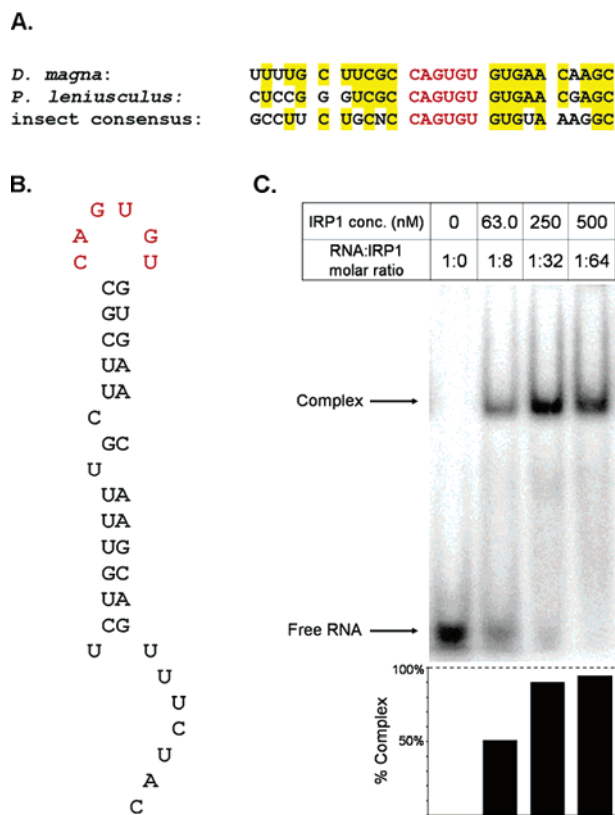

FIGURE 2. 5′ Untranslated region (UTR) of the *D. magna* ferritin mRNA containing a functional IRE. (A) Sequence of the *D. magna* IRE is similar to IREs found in the crayfish, *P. leniusculus,* and the consensus IRE sequence of insect ferritins (20). (B) The secondary structure of the *D. magna* IRE as predicted by Mfold (38). (C) Rabbit IRP1 gel shift experiment. 5′-^{32}P-labeled *D. magna* IRE 40-nt 7.8 nM was incubated with increasing amounts of IRP1. The free RNA was separated from the complex by 4% (19:1) acrylamide gel electrophoresis. The bar graph represents the percentage of RNA−protein complex in each lane measured by densitometric analysis, revealing full binding at the RNA−protein ratio of 1:32. The gel shown is a representation of three experiments with two different protein preparations. The specificity of the rabbit IRP1 for the IRE structure has been shown previously (39).

database wFleaBase (http://wfleabase.org/), WFes0007968 and WFes0007967, have high homology to the crayfish IRP1. The functionality of the *D. magna* IRE and the presence of IRP1 homologues in *D. magna* provide strong evidence that *D. magna* ferritin is under the translational control of iron through IRP1.

Prediction of Toxic Modes of Action. By inspecting the expression data for patterns that could predict modes of action and biological pathways affected by metal exposure, we identified four possible mechanisms: (1) the effect of metals on digestion, (2) possible oxidative stress caused by Cd, (3) immune suppression induced by Cu, and (4) Zn's influence on chitinase activity.

The only genes downregulated by all three metals are all involved in digestion (Table 1 and Table S4). We verified that all three metals caused a repression of the α-amylase homologue by RT-PCR (Table S5). Studies have shown a slowing of digestion and a suppression of feeding rates after exposure to Cd or Zn (25, 26). These observations were accompanied by a decrease in digestive enzyme activity after Cd exposure, which could not be replicated in vitro, implying that the observed decrease in enzymatic activity was caused by a decrease in the expression of the enzymes (25). An investigation of the chronic effects of metals to *D. magna* revealed that Cd and Zn caused several effects to the digestive
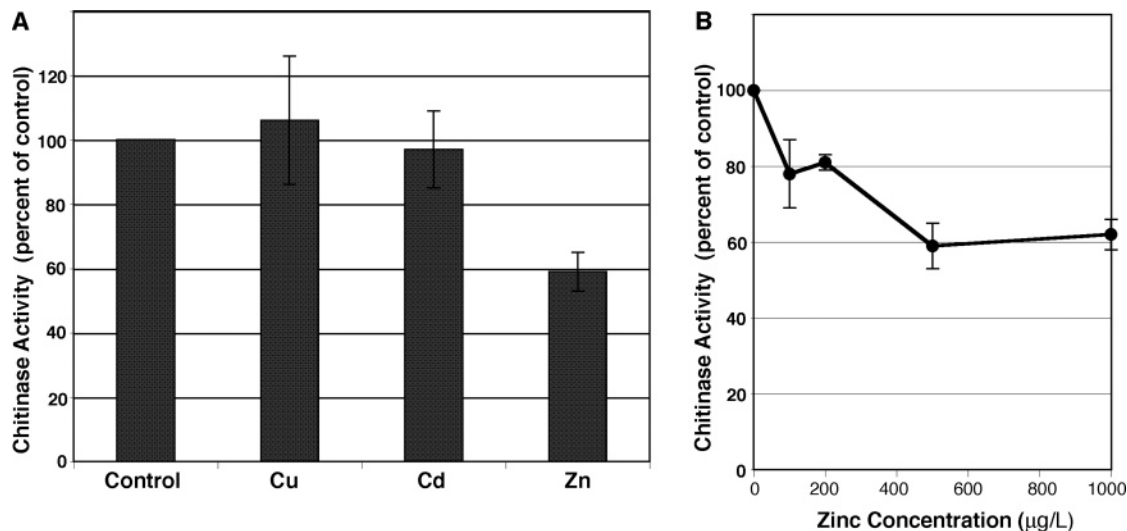
FIGURE 3. Decrease in chitinase activity caused by zinc that is dose dependent. The total chitinase activity was measured following 24-h exposure to Cu, Cd, or Zn, normalized by total protein content, and divided by the chitinase activity in the nonexposed control to determine the percent activity relative to the control. (A). *D. magna* were exposed to the 1/10 LC_{50} for each metal: Cu (6 μg/L), Cd (18 μg/L), and Zn (500 μg/L). (B). A dose response was performed from 100 μg/L Zn to the NOEC of 1000 μg/L Zn.

organ including shrinking and paralysis (*27*). These effects suggest an overall dysfunction of the *D. magna* digestive system that would likely cause a decrease in expression of digestive enzymes.

Three genes potentially involved in the oxidative stress response are upregulated by Cd exposure. These genes include DV437830 and DV437833 (homologues to glutathione-*S*-transferases, GST) and DV437829 (a homolog to peroxiredoxin V) (see Tables 1 and S4). We confirmed the upregulation of DV437830 by RT-PCR (Table S5). GST protects cells from oxidative damage and has been shown to be transcriptionally regulated by oxidative stress (*28*). Consistent with our results, a recent study showed that GST activity was increased in *D. magna* by Cd exposure (*29*).

Peroxiredoxins, or thiol peroxidases, also play a protective role against reactive oxygen species. The peroxiredoxin genes identified in insects have peroxidase activity in the presence of DTT and protect cells from oxidative stress and Cd-induced cytotoxicity in tissue culture (*30*). The *D. magna* gene DV437829 is similar to peroxiredoxins found in yeast and insects and contains a conserved cysteine residue found in the catalytic site of peroxiredoxins (Figure S2).

Two genes possibly involved in immune response, DV437823 and DV437821, were downregulated by copper exposure (Table 1), and the downregulation of DV437823 was confirmed by RT-PCR (Table S5). The proteins encoded by these genes show similarities to β-1,3-glucan binding proteins (LGBP) and lectins, proteins involved in the arthropod innate immune response. They are responsible for the recognition of an infection and are the first actors in a cascade of events leading to the activation of the pro-phenoloxidase system and a complement-like system found in invertebrates (*31*). While infection causes an increased expression of these proteins (*32*), one would expect that a decrease in expression levels would lead to a suppression of the immune response. Indeed, studies have shown an impairment of immune factors and a higher susceptibility to infection in crustaceans and mollusks after exposure to sublethal concentrations of copper (*33, 34*). It seems reasonable that the decrease in expression of β-1,3-glucan binding proteins and lectin observed in the copper exposed *D. magna* could be responsible for the immune system suppression seen in copper-exposed organisms. Further confirmation of this linkage is needed to fully understand how copper affects the invertebrate immune system.

Two genes (DV437857 and DV437858) downregulated by zinc code for homologues to chitinase proteins that contain the conserved chitinase signature sequence: YD/GFDGLDL/MDWEYP (*35*). The only other gene shown to be uniquely downregulated by zinc by the microarray analysis is DV437856 (Table S5), whose translated sequence contains repeats of AAPA, a motif commonly found in cuticle proteins (*36*). To further confirm the influence of zinc on chitin metabolism, we investigated how zinc affects chitinase activity. As shown in Figure 3A, at the 1/10 LC_{50} Zn causes a significant decrease in chitinase activity compared to the control and this effect was not seen from exposure to Cu or Cd. The decrease in chitinase activity is dose-dependent with 100 μg/L causing a 20% decrease in activity (see Figure 3B) and also correlates with chronic effects to reproduction, which are first evident at concentrations above 100 μg/L (Table S1). At 500 μg/L, we see a 40% decrease, which appears to be highest level of suppression before reaching acutely toxic concentrations above the NOEC of 1000 μg/L.

The downregulation of the chitinase gene and subsequent decrease in enzyme activity suggests that zinc is interfering with exoskeleton maintenance and molting. Molting is a highly controlled process in arthropods regulated by hormones including 20-hydroxyecdysone (*35*). Toxicants including PCBs, endosulfan, and DES inhibit molting in *D. magna* possibly by interfering with endocrine processes (*37*). Chitinase activity has also been shown to be hormone-regulated, and certain xenobiotics can inhibit chitinase activity (*15*); however, to our knowledge, no studies have shown a metal to inhibit chitinase activity. Because daphnids must shed their exoskeleton in order to release a new brood of neonates, zinc may be interfering with reproduction. We have shown that the decrease in chitinase activity correlates with chronic effects on reproduction. Whether these effects are caused by a decrease in chitinase activity is an interesting question for future studies.

We have shown that three metals, toxicants of the same chemical class, have distinct expression profiles in a classic ecotoxicology test organism, *D. magna*. These expression patterns are robust, confirmed by an independent method, RT-PCR, and correlate well with existing knowledge about the toxicity of metals to crustaceans. This study also illustrates the power of genomics to view effects to biological processes by toxicants and has shown how a whole system analysis can aid in the identification of modes of action for environmental

toxicants. The differentially expressed genes identified can be used as biomarkers of exposure and may be used in field studies to detect metals in water. Applying genomic tools such as cDNA microarrays to ecotoxicology will require further validation and faces obstacles ahead, but this study has provided evidence for the feasibility of ecotoxicogenomics.

Supporting Information Available

Toxicity data (Table S1), details on water parameters (Table S2) and RT-PCR primers (Table S3), a complete list of differentially expressed genes by each metal (Table S4), and real-time PCR results (Table S5). Sequence alignments of *D. magna* metallothionein (Figure S1) and peroxiredoxin (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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