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LINKING MOLECULAR BIOMARKERS WITH HIGHER LEVEL CONDITION INDICATORS TO IDENTIFY EFFECTS OF COPPER EXPOSURES ON THE ENDANGERED DELTA SMELT (*HYPOMESUS TRANSPACIFICUS*)

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Abstract—The delta smelt (*Hypomesus transpacificus*) is an endangered pelagic fish species endemic to the Sacramento–San Joaquin estuary (CA, USA), and considered an indicator of ecosystem health. Copper is a contaminant of concern in Californian waterways that may affect the development and survival of this endangered species. The experimental combination of molecular biomarkers with higher level effects may allow for interpretation of responses in a functional context that can be used to predict detrimental outcomes caused by exposure. A delta smelt microarray was developed and applied to screen for candidate molecular biomarkers that may be used in monitoring programs. Functional classifications of microarray responses were used along with quantitative polymerase chain reaction determining effects upon neuromuscular, digestive, and immune responses in Cu-exposed delta smelt. Differences in sensitivity were measured between juveniles and larvae (median lethal concentration = 25.2 and 80.4 μg/L Cu^{2+}, respectively). Swimming velocity declined with higher exposure concentrations in a dose-dependent manner ($r = -0.911$, $p < 0.05$), though was not statistically significant to controls. Genes encoding for aspartoacylase, hemopexin, α-actin, and calcium regulation proteins were significantly affected by exposure and were functionally interpreted with measured swimming responses. Effects on digestion were measured by upregulation of chitinase and downregulation of amylase, whereas downregulation of tumor necrosis factor indicated a probable compromised immune system. Results from this study, and many others, support the use of functionally characterized molecular biomarkers to assess effects of contaminants in field scenarios. We thus propose that to attribute environmental relevance to molecular biomarkers, research should concentrate on their application in field studies with the aim of assisting monitoring programs. Environ. Toxicol. Chem. © 2010 SETAC

Keywords—*Hypomesus transpacificus* Delta smelt Microarray Biomarker Copper

INTRODUCTION

The application and significance of biomarkers in an environmental context has been criticized due to the lack of linkage between biomarker response in individual organisms and effects [1], such as reproductive fitness or population decline, at higher levels of organization (e.g., populations and ecosystems). The experimental combination of biomarkers with indicators of population or ecosystem condition, allows for the evaluation of effects upon an individual and subsequent extrapolation of endpoints such as population effects. Recent studies have hypothesized, identified, and demonstrated links between gene expression and higher levels of organization [2–4]. The success behind the use of biomarkers as early and sensitive warning tools thus lies in interpreting biomarker responses in individuals in the context of affected cellular pathways, integrated with extensive life history knowledge of the species in question. This requirement is especially true when assessing effects on non-model species, or organisms living in ecological systems where sensitivity to stressors could greatly differ from model organisms.

In fish, not only swimming behavior, but maintenance of optimal swimming performance is of particular importance for optimal fitness. A number of life history variables are dependent on swimming ability, including respiration, feeding, predator–prey interactions, and social interactions such as courtship and spawning, which are fundamental to survival, growth, and reproduction, the most important traits in evolutionary success. Contaminant exposures that predominantly affect neuromuscular structure and activity may translate to swimming impairments; however, other maintenance aspects, such as immune system and acquisition of adequate nutrients, may also play a role. Furthermore, exposure may affect olfactory senses and related behavioral responses, such as contaminant avoidance and homing, that will further affect individuals' chances of survival and reproduction, and as such have direct effects on population dynamics. Contaminant avoidance is, in itself, generally seen as a beneficial response; however, should the avoidance coincide with homing and identification of limited spawning sites, it would impinge on reproduction and consequently population dynamics [5].

The delta smelt (*Hypomesus transpacificus*) is an endangered pelagic fish species endemic to the Sacramento–San Joaquin estuary, California (USA), whose abundance has dramatically declined since the 1980 s, and more precipitously in recent years [6]. A number of complex factors, such as freshwater export and habitat destruction, have been attributed to the decline of delta smelt in its native environment, with

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contaminants being another key issue due to intense agriculture, urban runoff, and other anthropogenic activities [2]. A recent steep decline has prompted considerable efforts to understand the causative factors of reduced population size [6], especially because several other pelagic species have shown similar population trends.

Delta smelt are restricted to the Sacramento–San Joaquin estuary, spawning in late winter and early spring, at limited freshwater sites both known and speculated, based on sediment type and other physicochemical properties such as slope, vegetation, depth, temperature, and salinity [7]. A major site of delta smelt spawning is in the Lower Sacramento River, from which hatched larvae are transported with the flow to brackish waters downstream, where they mature, during a short-lived one-year cycle [8]. This spawning site is located within agricultural areas and downstream from the Sacramento municipal wastewater treatment plant. Thus, emerging larvae are potentially exposed to contaminant mixtures, coinciding with pesticide applications that mix with urban effluent contaminants.

Copper is a contaminant of concern in Californian waterways. It is not only common in urban storm water runoff, and transport off old mining sites, but it is regularly used as a pesticide and fungicide in agricultural areas [2]. Seasonally fluctuating dissolved Cu concentrations in the Sacramento River have been reported approximating $2\ \mu g/L^{-1}$, however, concentrations in tributaries (e.g., Arcade Creek) have been measured above 6 μg/L [9] and have been reported to exceed 500 μg/L in rice field effluents following application [10].

Copper, although essential for multiple cellular proteins, can be toxic to many aquatic organisms, including fish. The mode of action of Cu in several fish species has been reported to involve inhibition of Na^+ channels in gill epithelium, although other mechanisms are likely to be important as well [11]. Knowledge of the effects of Cu on model organisms is extensive, making this an ideal contaminant to utilize in this biomarker assessment proof-of-concept study, where our aim is to link molecular responses with higher level condition indicators—in this study, swimming performance. In the present study, we describe the effects of Cu on delta smelt at sensitive larval and juvenile developmental stages, the development of molecular biomarkers, and their link with swimming performance. The need for inclusion of molecular biomarkers in monitoring programs is emphasized to understand mechanisms by which contaminants exert effects upon endangered organisms.

MATERIALS AND METHODS

Fish exposures and swimming assessments

Delta smelt were obtained from the Fish Conservation and Culture Laboratory (FCCL), University of California (UC) Davis (CA, USA) and maintained for a minimum of 24 h in experimental conditions prior to test initiation. Two separate tests were conducted with juveniles and larvae, and used to assess gene expression through microarray and quantitative polymerase chain reaction (q-PCR) applications, respectively. Swimming behavior of larval fish was investigated and compared to q-PCR responses as detailed below. All experiments and use of test organisms were approved by the UC Davis Institutional Animal Care and Use Committee (Animal Use Protocol for Animal Care and Use 13361). This institution is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare, assurance number A3433-01.

Exposures used for microarray analysis. Juvenile delta smelt (90-d-old) were exposed for 7 d to 5, 10, 25, and 50 μg/L total Cu (nominal), sourced from $CuCl_2$–dihydrate (American Chemical Society reagent purity ≥99.0%; Sigma-Aldrich). Controls were maintained in diluted well-water; salinity <1 ppt, adjusted to a specific conductance of $450\ \mu S/cm^{-1}$, with deionized water, and a pH of 8.45, using hydrochloric acid. Total water hardness, expressed as $CaCO_3$ was 92 mg/L, and temperature was maintained at 21°C ± 1. Juveniles were acclimated to control water for 24 h prior to test initiation. Replicate experimental treatments ($n = 4$) were initiated with 10 juveniles in 7 L of water at 20°C. Fish were fed twice daily with <48-h-old *Artemia franciscana* (Argent Chemical Laboratories). The light:dark cycle was 16 h:8 h. Approximately 80% of the water in each replicate was renewed at test initiation and on days 2, 4, and 6. At test end, surviving fish were euthanized with MS-222 (tricaine methanesulfonate; Sigma), and snap-frozen in liquid nitrogen and stored at −80°C for subsequent analyses. Comprehensive Environmental Toxicity Information System (CETIS) by Tidepool Scientific Software was used to calculate nominal lethal concentrations. Surviving juveniles from 50.0 μg/L Cu^{2+} were assessed against controls, utilizing the developed microarrays (see below). Due to mortality at this concentration, three of the four replicates were used, and four surviving fish from each replicate were pooled for this assessment.

Exposures used for q-PCR and swimming analyses. Larval delta smelt (47-d-old) were exposed for 4 days to 27, 53, 106, 213 μg/L total Cu (nominal), sourced from $CuCl_2$–dihydrate (American Chemical Society reagent purity ≥99.0%; Sigma-Aldrich). Controls were maintained in hatchery water with specific conductance of $930\ \mu S/cm^{-1}$ using Instant Ocean (Aquarium Systems), salinity <1 ppt, and pH of 7.9. Total water hardness, expressed as $CaCO_3$ was 100 mg/L, and temperature was maintained at 17°C ± 1. Larvae were acclimated to control water for 24 h prior to test initiation. Antibiotics (Maracyn and Maracyn-2, Virbac Animal Health) were added during the acclimation period at concentrations of $5.3\ mg/L^{-1}$ Maracyn (erythromycin) and $0.26\ mg/L^{-1}$ Maracyn-2 (minocycline), to eliminate any gram-positive and gram-negative bacteria, respectively. Fish were fed twice daily with *A. franciscana* (<48-h-old). The light:dark cycle was 16 h:8 h. Approximately 80% of the water in each replicate was renewed at test initiation and on the second exposure day. At test end, a subset of fish were used for swimming assessments, and remaining fish were snap-frozen and stored at −80°C for subsequent biomarker analyses. The CETIS software was used to calculate nominal lethal concentrations.

Swimming assessments were performed at test takedown. Fish were placed in rectangular tanks (12 × 6 × 9 cm) containing control water, and allowed to acclimate for 5 min. Three-minute video imaging, recorded in MPEG-2 format, was performed at 30 frames per second using a black and white Panasonic CCTV camera (12VDC) connected to a laptop computer via a USB framegrabber (Model WinTV-HVR 950). Video analysis was carried out using Ethovision XT (Ver 6.1.326, Noldus Information Technology). Average velocity was determined for each fish by analyzing a total of 72 s per video test. One-way analysis of variance (ANOVA), with Dunnett's multiple comparison post hoc tests was used to compare swimming data of exposed treatments to controls. The ANOVA assumptions were verified using F_{max}. Prism 4.0

(Graphpad Software) was used to perform swimming data ANOVA and dose–response correlation analyses.

Experimental physicochemistry

For all exposure tests, water temperature, pH, and DO were measured daily. Water hardness was measured at test initiation, and total ammonia concentrations were measured prior to each water renewal and at test termination. Dissolved Cu analyses were carried out by the Department of Fish and Game—Water Pollution Laboratory (Rancho Cordova, CA, USA).

Microarray application

Development of the delta smelt microarray was described in Connon et al. [4]; briefly, we have constructed a cDNA microarray with 8,448 expressed sequence tags which were pin-printed in duplicate onto epoxysilane-coated glass slides.

Total RNA was extracted from whole, individual organisms, using Trizol Reagent (Invitrogen) as per manufacturer's guidelines. Fifteen micrograms of total RNA were used for cDNA synthesis, spiked with *Arabidopsis thaliana* control RNA (SpotReport™ genes: Chlorophyll A-B binding protein [CAB], rubisco activase [RCA], ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [RBCL], and lipid transfer protein 4 [Ltp4]; Stratagene) and labeled with Alexa fluor dyes, using SuperScript™ Plus Indirect cDNA Labeling System (Invitrogen). Microarray assessments were carried out using three replicate treatments. Each experimental sample or control was combined with a reference pool cDNA prior to hybridization using an automated Tecan HS4800 hybridization station. Slides were scanned using a GenePix 4000B scanner (Axon Instruments). Microarray data are available for download through the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov) accession number GSE23238.

Normalization and analytical methods are described in Connon et al. [4] and Loguinov et al. [12]. In brief, print tip normalization was carried out within slides, and sequential single-slide data analysis was carried out as an alternative to between-slide normalization. An α-outlier-generating model was used to identify differentially expressed genes by applying the following decision rule for multiple-slide data analysis: A given gene was selected as a candidate if it was consistently detected as up- or downregulated in three of three replicates (raw p value $= 0.0625$ using exact binomial test and considering outcomes as Bernoulli trials). The approach did not use scale estimation for statistical inference and, due to limited replication, between-slide normalizations were not performed [12]. A higher than usual cut-off point of 0.0625, due to normal microarray normalization stringencies, was used because the purpose of this investigation was to identify genes that could be assessed as probable q-PCR–based molecular biomarkers for future monitoring programs (see below).

Sequencing was performed at the College of Agriculture and Environmental Sciences' Genomic Facility (UC Davis). Using the Basic Local Alignment Search Tool, translated nucleotide (BLASTx) searches were performed on specific fragments that responded significantly to the exposure treatments. Only genes that were differentially expressed following exposure were sequenced. Sequences were annotated according to homologies to protein database searches using translated nucleotide sequences and direct nucleotide queries (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were only annotated if found to have a BLASTx match with an expectation value smaller than 1×10^{-5} and a score above 50.

Functional classifications

Differentially expressed genes were classified according to the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/kegg2.html) and gene ontology (http://www.uniprot.org/uniprot), and information gathered from literature, into functional groups. Classification was carried out based on gene expression changes in control and exposed organisms, regardless of whether these were up- or downregulated. Specific genes of interest were selected for further investigation using q-PCR (see below).

Biomarker development

Genes were selected according to level of expression significance, knowledge base from literature, and functional classification. Primer and probes for q-PCR analyses were designed using Roche Universal Probe Library Assay Design Center (http://www.roche-applied-science.com). Designed primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com), and TaqMan® probes were supplied by Roche. Sequences for all genes assessed by q-PCR analyses have been submitted to GenBank (http://www.ncbi.nlm.nih.gov). Primers and probes for investigated biomarkers are detailed in Table 1.

Quantitative PCR. Complementary cDNA was synthesized using 1.0 μg total RNA, with random primers and SuperScript® III reverse transcriptase (Invitrogen), and diluted to a total of 120 μl with nuclease-free water to generate sufficient template for q-PCR analysis. TaqMan Universal PCR Mastermix (Applied Biosystems) was used in q-PCR amplifications in a reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 μl of cDNA sample in a final volume of 12 μl. The samples were placed in 384 well plates and cDNA was amplified in an automated fluorometer (ABI PRISM® 7900 Sequence Detection System; Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescence of samples was measured every 7 s, and signals were considered positive if fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle, CT). The SDS 2.2.1 software (Applied Biosystems) was used to quantify transcription.

The geNorm algorithm [13] was used to estimate the variability of the reference genes, and to determine an optimal normalization gene. Quantitative PCR data was analyzed using the relative quantification 2(-Delta Delta CT) method [14]. Expression was calculated relative to β-actin determined by geNorm as the least variable gene in this study. Quantitative PCR data were not normally distributed, therefore, significant differences in gene expression, relative to the unexposed controls, were assessed using two-tailed Mann–Whitney U test, single comparison alpha $= 0.05$, with Bonferroni's correction experiment-wide alpha $= 0.15$, treating each gene as a separate experiment. Prism 4.0 (Graphpad Software) was used to perform this analysis along with correlation tests with swimming performance.

RESULTS AND DISCUSSION

Experimental water physicochemistry

Water physicochemical parameters for all tests remained stable throughout the exposures (Table 2), except for a lower ammonia concentration in the 50.0 μg/L Cu^{2+} 7-d test used for microarray analysis, which was attributed to a lower number of remaining fish due to high mortality. Discrepancy in ammonia

Table 1. Molecular biomarkers: Primer and probe sequences used for quantitative-polymerase chain reaction (PCR) analyses of gene expression in *Hypomesus transpacificus*

Gene	GenBank accession No.		q-PCR primer sequences	Roche probe No.
Myozenin	FJ711583	F	ccaatgtcgtgctggtacacc	106
		R	ctgccagacattgatgtagcca	
Creatine kinase	FJ711584	F	cgatcggcgttggagatg	163
		R	gccaagttcaacgagattctgg	
Sarcoendoplasmic reticulum Ca ATPase	GU564437	F	catgatcattgggggagca	148
		R	tgctgtgatgacaacgaggac	
α-Actin	GU564441	F	cctgcctcgtcgtactcctg	11
		R	catcctggcttccctgtcc	
m-Calpain	GU564439	F	ccctccgacatgggaagagt	30
		R	accaactatgccttgcccaa	
Aspartoacylase	FJ711577	F	ggaggcacacatgggaatg	109
		R	cttcctctgaatctctgttccattatc	
Hemopexin	FJ711579	F	catgcactacgaggacgacaag	143
		R	tggtagtagctgaacaccttgctg	
Chitinase	GU564440	F	tgtgatcaagttcctccgtcagt	147
		R	ccggggtattcccagtcaat	
α-Amylase	GU564441	F	gatcaccatgttcttgatctgacg	99
		R	ccatcaatcctgaccaaacctg	
Transforming growth factor-β	GU564442	F	caacggcatagtgcatgtgg	76
		R	gaatgtgtgcacgttgttggt	
Tumor necrosis factor—decoy receptor	GU564443	F	ctttttccgctgttccatgttc	2
		R	gttaccagcatacgcagtgtcc	
β-Actin[a]	GU564444	F	tgccacaggactccatacc	12
		R	catcggcaacgagaggtt	

[a] Reference gene.

concentrations between exposure sets are attributed to development stage, age, and size differences between juvenile and larval delta smelt. Toxicity of ammonia is influenced by pH, temperature, and salinity, and is known to affect ion transport and membrane permeability, especially important in gills. The present study did not investigate interactions of ammonia with Cu. Water hardness is known to influence Cu toxicity; the California Toxic Rules Criteria and U.S. Environmental Protection Agency National Ambient Water Quality Criteria (NAWQC) for protection of aquatic life (http://www.epa.gov/waterscience) set 9.0 μg/L Cu^{2+} at 100 mg/L $CaCO_3$, thus the concentration chosen for microarray assessments was over four times higher than this criteria. Concentrations presented throughout the remaining sections represent measured dissolved Cu.

Fish exposures and swimming assessments

Exposures used for microarray analyses. Juvenile delta smelt (90-d-old) were highly sensitive to Cu exposure (Table 3), resulting in an estimated 7-d median lethal concentration ($LC50_{7d}$) of 17.8 μg/L Cu^{2+}. In test controls, there was no mortality. Microarray assessments were carried out at 42 μg/L Cu^{2+}; a higher than ambient concentration was chosen to ensure that Cu-specific responses were elicited in exposed juveniles towards biomarker development.

Exposures used for q-PCR and swimming video-analyses. Exposure of 47-d-old larval delta smelt to Cu for 4 d resulted in an $LC50_{96h} = 80.4\ \mu g/L^{-1}$ Cu^{2+} (Table 3). Differences in Cu sensitivity between juvenile and larval exposures were attributed not only to age and size, but also to temperature and conductivity,

Table 2. Physicochemical parameters of control and test waters from 4- and 7-d copper exposures[a]

Treatment	Measured Cu^{2+} (μg/L)	Temp (°C) ±1	pH	EC (μS/cm)	DO (mg/L)	NH4/NH3 (mg/L)	NH_3 (mg/L)	Hardness ($CaCO_3$ mg/L)
Exposures used for microarray analysis (90-d-old juvenile, 7-d exposure)								
Control water	NA	21	8.40	431	8.8	0.28	0.025	92.0
5 μg/L Cu^{2+}	NA	21	8.49	456	8.7	0.24	0.026	NA
10 μg/L Cu^{2+}	NA	21	8.48	461	9.0	0.23	0.024	NA
25 μg/L Cu^{2+}	NA	21	8.46	455	8.8	0.37	0.037	NA
50 μg/L Cu^{2+}	42.0	21	8.39	457	8.9	0.14	0.012	NA
Experiment mean		21	8.44	452	8.84	0.25	0.025	
Exposures used for quantitative polymerase chain reaction and swimming ability analyses (47-d- old larvae, 4-d exposure)								
Control water	1.6	17	7.86	931	9.1	0.025	0.000	100.0
27.0 μg/L Cu^{2+}	24.0	17	7.84	926	9.3	0.037	0.001	N/A
53.0 μg/L Cu^{2+}	52.4	17	7.89	927	9.4	0.057	0.001	N/A
106.0 μg/L Cu^{2+}	105.0	17	7.93	931	9.4	0.047	0.001	N/A
213.0 μg/L Cu^{2+}	213.0	17	7.89	931	9.5	0.033	0.001	N/A
Experiment mean		17	7.88	929	9.34	0.039	0.001	

[a] EC = electric conductance; DO = dissolved oxygen; NA = data not available.

Table 3. Acute toxicity data from copper-exposed 90-d-old juvenile and 47-d-old larval *Hypomesus transpacificus* (μg/L dissolved copper)[a]

	Endpoint		Juvenile exposure		Larval exposure	
	96-h	95% CI	7-d	95% CI	96-h	95% CI
Control survival	100%	—	100%	—	93%	—
NOEC	8.4	—	8.4	—	53.0	—
LOEC	21.0	—	21.0	—	106.0	—
LC10	9.6	4.2–11.4	9.0	4.2–10.3	9.3	27.0–77.8
LC25	13.4	10.6–18.0	11.7	9.7–13.0	44.8	27.0–83.1
LC50	25.2	16.4–35.4	17.8	14.4–22.4	80.4	48.7–227.2

[a] NOEC = no-observed-effect concentration; LOEC = lowest-observed-effect concentration; LC10, LC25, and LC50 = estimated lethal concentrations percentages; CI = confidence interval; — = data not available.

which are known to affect metal uptake and toxicity [15]. In test controls, there was 93% survival. Due to high mortality resulting at the highest Cu concentration, surviving fish numbers were not sufficient for use in q-PCR tests and thus were discarded from further analysis.

Video-analysis of larval swimming performance (Fig. 1) has indicated an overall effect of Cu exposure correlating with declining velocity in a dose-dependent manner ($r = -0.911$, $p < 0.05$). However, swimming velocity was not statistically different from controls ($p = 0.439$).

Comparative toxicological data. Differences in the above-reported toxicity between juvenile and larval delta smelt (Table 3) are due to exposure duration discrepancies and developmental stage, but variations in experimental conditions, such as differences in water conductivity, pH, and temperature, also contributed to the higher Cu toxicity to juveniles. Furthermore, the larval exposure tests were carried out using antibiotic treatments. Though juvenile delta smelt appear more sensitive to Cu exposure than do larvae, acute toxicity results are thus inconclusive; however, genomic responses have been analyzed successfully in a comparative manner (see below).

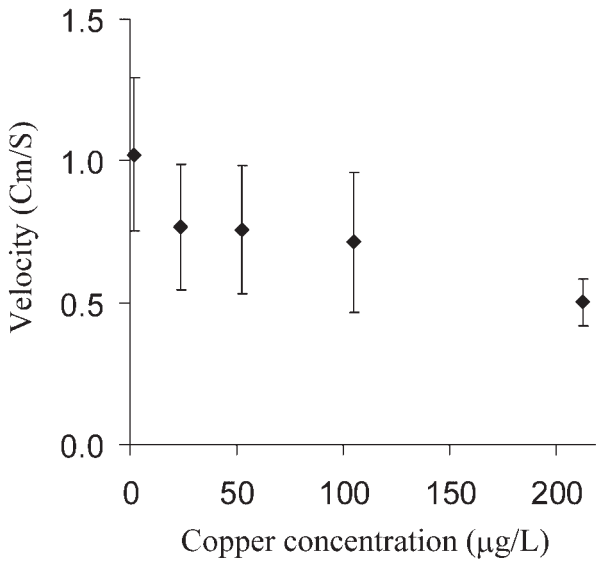

Fig. 1. Swimming performance of 47-d-old larval delta smelt exposed for 96 h to 0, 27, 53, 106, and 213 μg/L total copper (nominal).

Microarray responses

Differentially expressed genes resulting from exposure to 42.0 μg/L Cu^{2+} are presented in Table 4. A functional classification based on KEGG and gene ontology of up- and down-regulated genes responding to Cu exposure are presented in Figure 2.

Copper exposure impacted on neuromuscular activity, affecting muscle integrity and contraction activity (e.g., creatine kinase, myozenin, sarcoendoplasmic reticulum calcium ATPase [SER Ca], titin a), neurological effects resulting in Ca and phosphate signaling (e.g., m-calpain, cyclophilin-a), and nerve maintenance (hemopexin and aspartoacylase [ASPA]). Copper is reported to inhibit iron storage through interaction with peroxidases causing oxidative stress which leads to disruption in Ca homeostasis [16]. Digestion was also affected by Cu exposure, including genes encoding a number of proteins involved in glycolysis, cholesterol efflux, lipid transport, chymotrypsin activity, and proteolysis (e.g., amylase-3, gastric chitinase). Other responses indicate compromised immunity (e.g., tumor necrosis factor [TNF], transforming growth factor-beta [TGF-β]), and cellular homeostasis and tumor malignancy (e.g., vitronectin); changes in expression of these proteins have been implicated in a variety of diseases.

Gene classification from KEGG orthology analyses also indicate effects on expression of genes encoding proteins involved in the peroxisome proliferator-activated receptor pathway, receptors that function as transcription factors regulating gene expression, playing an essential role in the regulation of cellular differentiation, development, and metabolism of carbohydrates, lipids, and proteins, and tumorgenesis. This pathway integrates the majority of genes classified into digestion and metabolism.

Molecular biomarker responses

Genes were selected according to level of expression significance and to represent the identified functional classifications. Thus, genes involved in muscular, neurological, digestive, and immune system functional groups were further investigated, using q-PCR, and assessed as probable biomarkers of copper exposure in *H. transpacificus*.

Results confirm microarray identified functional responses. Quantitative PCR verified copper elicited responses in neuromuscular, digestive, and immune system functions (Fig. 3a–d), with significant differences in expression of muscle α-actin, ASPA, hemopexin, chitinase, and TNF ($p < 0.05$). Remaining assessed genes displayed dose–response relationships, and/or differences in expression trends, but were not statistically significant compared to controls. Although not statistically significant in their expression level, responses from these genes directly facilitate the interpretation of functionally affected systems and are thus interpreted with biological significance.

Muscular structure and activity. Muscular structure and integrity appears to have been affected by Cu exposure (Fig. 3a), as indicated by effects on contractile muscle systems, α-actin and myozenin. Skeletal α-actin, was significantly down-regulated at all concentrations, correlating with swimming performance ($r = 0.957$). Alpha actin is reported to induce expression of a number of other myogenic genes essential for muscle formation [17], thus it may serve as holistic muscle integrity and functioning effect biomarker. Myozenin is a Z-line, α-actinin- and γ-filamin-binding protein expressed predominantly in skeletal muscle, and has been suggested as

Table 4. Annotation, gene ontology (GO), and regulation of gene expression, in juvenile *Hypomesus transpacificus* exposed to copper (42.0 μg/L Cu^{2+})[a,b]

Gene most similar to	Species match	Accession No.	E-value	Score	GO	Fold-change
Pancreatic protein with two somatomedin B domains	*Paralichthys olivaceus*	BAA88246	2.00E-95	352	GO:0005179	7.54
Cell division cycle 14 homolog A	*Danio rerio*	CAP09233	3.00E-19	99	GO:0004725	5.76
Corticotropin-lipotropin A precursor	*Oncorhynchus mykiss*	Q04617	7.00E-63	244	GO:0005179	5.20
Elastase 2-like protein	*Sparus aurata*	AAT45251	2.00E-89	332	GO:0006508	5.08
Actin alpha 2, skeletal muscle[a]	*Pagrus major*	BAF80060	1.00E-94	384	GO:0003774	4.88
Phosphoglucose isomerase-2	*Plecoglossus altivelis*	BAF91566	1.00E-120	435	GO:0006096	4.86
Apolipoprotein A-I-2 precursor	*Oncorhynchus mykiss*	O57524	4.00E-71	271	GO:0033344	4.81
Pepsinogen A form IIa	*Pseudopleuronectes americanus*	AAD56283	1.00E-105	384	GO:0004194	4.65
Arachidonate 12-lipoxygenase	*Danio rerio*	NP_955912	4.00E-23	112	GO:0004052	4.42
Chitinase1	*Paralichthys olivaceus*	BAD15059	1.00E-127	458	GO:0004568	4.25
Lipoxygenase 12R (Predicted: similar to)	*Ornithorhynchus anatinus*	XP_001518171	8.00E-06	55	GO:0016165	4.17
Apolipoprotein Eb	*Danio rerio*	NP_571173	2.00E-38	162	GO:0033344	4.16
SPARC: secreted protein, acidic, rich in cysteine	*Danio rerio*	AAT01213	2.00E-31	139	GO:0006816	4.14
Pepsin A2	*Trematomus bernacchii*	CAD80096	2.00E-88	253	GO:0004194	4.05
Apolipoprotein A-I-1 precursor (Apo-AI-1)	*Oncorhynchus mykiss*	O57523	8.00E-76	286	GO:0033344	3.99
Chymotrypsinogen 2-like protein	*Sparus aurata*	AAT45254	1.00E-20	101	GO:0004263	3.93
Myozenin 1[a]	*Danio rerio*	NP_991241	2.00E-25	119	GO:0030346	3.91
NADH dehydrogenase subunit 5	*Osmerus mordax*	ABI35911	1.00E-107	390	GO:0008137	3.88
Astacin-like metallo-protease	*Oryzias latipes*	NP_001098207	2.00E-83	311	GO:0008533	3.87
Hect domain and RLD 4 (PREDICTED: similar to)	*Danio rerio*	XP_685685	7.00E-76	286	GO:0006512	3.76
Actin, alpha 2, smooth muscle, aorta	*Danio rerio*	AAH75896	1.00E-107	391	GO:0003774	3.75
Chitinase[a]	*Oncorhynchus mykiss*	CAD59687	9.00E-68	260	GO:0004568	3.75
F-type lectin	*Morone saxatilis*	ABB29997	1.00E-46	188	GO:0016467	3.73
Pgk1(phosphoglycerate kinase 1) protein	*Danio rerio*	AAH65888	9.00E-84	313	GO:0006096	3.67
Aldolase a, fructose-bisphosphate	*Danio rerio*	NP_919358	1.00E-124	447	GO:0006096	3.47
NADH dehydrogenase subunit 5	*Osmerus mordax*	ABI35911	5.00E-94	308	GO:0008137	3.47
Pepsinogen C (progastricsin)	*Salvelinus fontinalis*	AAG35646	1.00E-107	390	GO:0004194	3.41
Amylase-3 protein	*Tetraodon nigroviridis*	CAC87127	3.00E-54	213	GO:0004556	3.36
Simple type II keratin K8b (S2)	*Oncorhynchus mykiss*	CAA63300	3.00E-74	281	GO:0005882	3.28
Glutamate dehydrogenase 1	*Danio rerio*	NP_955839	1.00E-107	392	GO:0004352	3.24
α-Amylase[a]	*Pseudopleuronectes americanus*	AAF65827	1.00E-144	513	GO:0004556	3.06
Pepsinogen	*Paralichthys olivaceus*	BAC87742	3.00E-77	291	GO:0004194	3.04
NADH dehydrogenase subunit 6	*Salangichthys microdon*	NP_795843	1.00E-107	392	GO:0008137	3.03
Hemopexin[a]	*Danio rerio*	NP_001104617	1.00E-59	233	GO:0046872	3.02
Gamma2-synuclein	*Takifugu rubripes*	NP_001029019	2.00E-41	172	GO:0030424	2.93
Actin, alpha, cardiac muscle 1-like	*Danio rerio*	NP_001001409	1.00E-141	503	GO:0003774	2.92
Cardiac muscle ATP synthase,alpha 1,	*Danio rerio*	NP_001070823	7.00E-62	240	GO:0015662	2.91
Selenoprotein P, 1a	*Danio rerio*	NP_840082	1.00E-53	213	GO:0001887	2.86
Intestinal fatty acid-binding protein	*Danio rerio*	AAF00925	3.00E-56	221	GO:0008289	2.82
L-arginine:glycine amidinotransferase	*Danio rerio*	NP_955825	5.00E-83	310	GO:0016740	2.76
Apolipoprotein A-IV	*Danio rerio*	AAH93239	1.00E-73	279	GO:0006869	2.72
Peptidylprolyl isomerase A (cyclophilin)	*Danio rerio*	AAQ91263	2.00E-74	282	GO:0003755	2.66
Histone methyltransferase SmyD1b	*Danio rerio*	ABC54714	1.00E-108	394	GO:0030239	2.62
Sarcoendoplasmic reticulum calcium ATPase	*Silurus lanzhouensis*	ABG90496	8.00E-79	297	GO:0006937	2.47
1-Acylglycerol-3-phosphate O-acyltransferase 3	*Danio rerio*	NP_998590	4.00E-68	261	GO:0003841	2.36
Chitin-binding peritrophin-A domain	*Danio rerio*	AAH45331	4.00E-69	264	GO:0016490	2.34
Apolipoprotein A-I	*Danio rerio*	NP_571203	1.00E-81	306	GO:0033344	2.28
Calpain 1 protein	*Danio rerio*	AAH91999	2.00E-68	262	GO:0005509	2.27
Apolipoprotein B	*Salmo salar*	CAA57449	3.00E-24	115	GO:0030301	2.23
Sarcoendoplasmic reticulum calcium ATPase[a]	*Makaira nigricans*	AAB08097	1.00E-83	313	GO:0006937	2.22
Muscle creatine kinase[a]	*Danio rerio*	CAM16434	1.00E-112	406	GO:0004111	2.21
Transmembrane protein 38A	*Danio rerio*	NP_957194	8.00E-81	303	GO:0005267	2.21
Tripartite motif-containing 45	*Xenopus tropicalis*	NP_001011026	3.00E-27	125	GO:0046872	2.20
Titin a	*Danio rerio*	ABG48500	3.00E-88	328	GO:0031432	2.19
C1q-like protein	*Dissostichus mawsoni*	ABN45966	3.00E-38	162	GO:0006817	2.17
Apolipoprotein CII	*Oncorhynchus mykiss*	AAG11410	1.00E-19	100	GO:0006869	2.02
Guanine nucleotide-binding protein (G protein), beta 1	*Danio rerio*	NP_997774	1.00E-117	424	GO:0003924	1.88
Alpha tubulin (protein LOC573122)	*Danio rerio*	NP_001098596	1.00E-120	434	GO:0007018	1.86
DAZAP2-like protein (deleted in azoospermia-associated)	*Takifugu rubripes*	NP_001072102	5.00E-59	230	GO:0030154	1.86
Aacyl-CoA synthetase long-chain family member 5	*Tetraodon nigroviridis*	CAG06540	1.00E-102	375	GO:0004467	1.83
Carboxypeptidase H	*Paralichthys olivaceus*	AAO92752	1.00E-82	309	GO:0004183	1.82
Apolipoprotein	*Tetraodon nigroviridis*	CAG03661	1.00E-38	78	GO:0030301	1.80
Transforming growth factor, beta-induced[a]	*Danio rerio*	NP_878282	3.00E-21	105	GO:0008083	1.59

Table 4. (*Continued*)

Gene most similar to	Species match	Accession No.	E-value	Score	GO	Fold-change
Neurotransmitter transporter, glycine, member 9 (SLC6A9)	*Danio rerio*	CAM14205	1.00E-100	367	GO:0006836	0.65
Calcium-binding protein 39	*Danio rerio*	NP_998666	1.00E-76	290	GO:0019855	0.63
Cytochrome P450, family 46, subfamily A, polypeptide 1	*Danio rerio*	NP_001018358	2.00E-65	252	GO:0004497	0.63
E3 ubiquitin-protein ligase MARCH2	*Danio rerio*	Q1LVZ2	2.00E-87	325	GO:0006512	0.60
Calcitonin receptor-like receptor	*Oncorhynchus gorbuscha*	CAD48406	5.00E-56	221	GO:0004948	0.59
Dopachrome tautomerase	*Salmo salar*	ABD73808	1.00E-85	318	GO:0016491	0.56
Tetraspanin 7b	*Danio rerio*	NP_001005581	1.00E-110	400	GO:0022857	0.54
Isocitrate dehydrogenase 3 (NAD+) gamma	*Danio rerio*	NP_001017713	2.00E-14	83	GO:0016616	0.53
Cofilin 2 (muscle)	*Danio rerio*	NP_991263	5.00E-84	314	GO:0003779	0.50
m-Calpain[a]	*Oncorhynchus mykiss*	BAD77825	1.00E-108	396	GO:0005509	0.50
Zona pellucida protein X	*Sparus aurata*	AAY21008	1.00E-68	263	GO:0032190	0.50
Suppressor of ypt1	*Danrio rerio*	NP_878281	1.00E-122	442	GO:0016192	0.47
Thioredoxin-like 1	*Danio rerio*	NP_957432	1.00E-107	391	GO:0045454	0.44
Lactase-phlorizin hydrolase (Predicted: similar to)	*Danio rerio*	XP_001336765	1.00E-110	401	GO:0005975	0.43
Potassium channel tetramerization domain-containing 5	*Danio rerio*	NP_996932	2.00E-76	288	GO:0005249	0.39
Zinc finger protein 503	*Danio rerio*	NP_942137	3.00E-63	245	GO:0003676	0.39
Ornithine decarboxylase	*Paralichthys olivaceus*	AAO92750	9.00E-67	256	GO:0006596	0.35
Proteasome subunit alpha type 7	*Danio rerio*	NP_998331	1.00E-112	409	GO:0030163	0.35
Proteasome (prosome, macropain) 26S subunit, ATPase, 4	*Danio rerio*	AAI53480	1.00E-109	396	GO:0030163	0.34
TNF (tumor necrosis factor) decoy receptor[a]	*Oncorhynchus mykiss*	AAK91758	5.00E-67	257	GO:0004872	0.26
APEX nuclease (apurinic/apyrimidinic endonuclease) 2	*Xenopus tropicalis*	NP_001006804	6.00E-25	118	GO:0006281	0.22

[a] Genes selected for biomarker development.
[b] NAD = nicotinamide adenine dinucleotide; NADH = reduced form of NAD.

a biomarker for muscular dystrophy and other neuromuscular disorders [18].

Muscle activity was also affected by Cu exposure, altering Ca^{2+} homeostasis, denoted by SER Ca ATPase and creatine kinase-altered expression. Sarcoendoplasmic reticulum Ca ATPase is a muscle calcium ATPase pump responsible for the transfer of calcium from the cytoplasm into the SER after muscle activity [19]. It was downregulated by Cu exposure in a dose-dependent manner ($r = -0.976$) and correlated with resulting swimming performance ($r = 0.965$). This is suggestive of a potential biomarker of muscular activity, indicative of mobility impairments, and likely apoptotic responses. Downregulation of SER Ca ATPase mRNA signifies a decrease in enzyme synthesis, likely causing a decrease in Ca^{2+} in the SER lumen. Disruption of Ca^{2+} homeostasis within the SER has been postulated as an early warning sign of apoptosis, thus inhibition of SER Ca ATPase could lead to cell death [20].

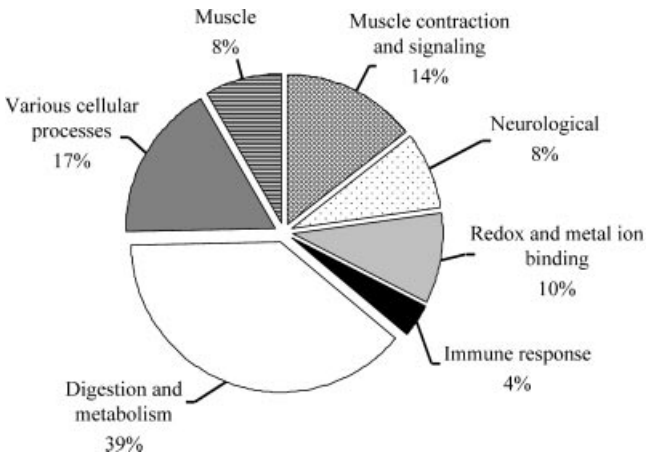

Fig. 2. Microarray responses: Systematic analysis of Kyoto Encyclopedia for Genes and Genomes (KEGG) orthology and gene ontology-based functional classification of delta smelt genes significantly differing in juveniles exposed to copper (42.0 μg/L Cu^{2+}) for 7 d.

A further biomarker assessed to measure muscular effects of Cu exposure was creatine kinase. Protein concentrations are used as a diagnosis of diseases like cardiac infarction and skeletal muscle necrosis [21]. It is specifically bound to sarcoendoplasmic reticulum, and regulates Ca uptake and ATP/ADP ratios [22], thus is directly linked with SER Ca ATPase and involved in muscle activity. Though not statistically significant, creatine kinase expression was also reduced by increasing Cu concentration, in a similar manner displayed by SER Ca and α-actin, suggesting a decline in Ca regulation and overall energetic activity.

Neurological activity. Copper exposure is known to affect the nervous system through the formation of reactive radicals [16], an effect that was sustained in this study (Fig. 3b), through expression of hemopexin, which was significantly upregulated at lower concentrations. Hemopexin is synthesized by Schwann cells following nerve injury, has been reported in the peripheral nervous system, and is specifically regulated during repair [23]. The measured downregulation at higher concentrations could imply inhibition of repair mechanisms. Wallerian degeneration generally occurs following axonal injury and is critical for its repair. This is characterized by axonal and myelin degeneration, thus ASPA, a gene identified in a previous study [4], was investigated because it is expressed in myelin sheaths and involved in their maintenance. It was chosen to further assess neurological damage, because of its functional proximity to hemopexin, however, ASPA did not respond significantly to Cu exposure but displayed an increase at lower concentrations,

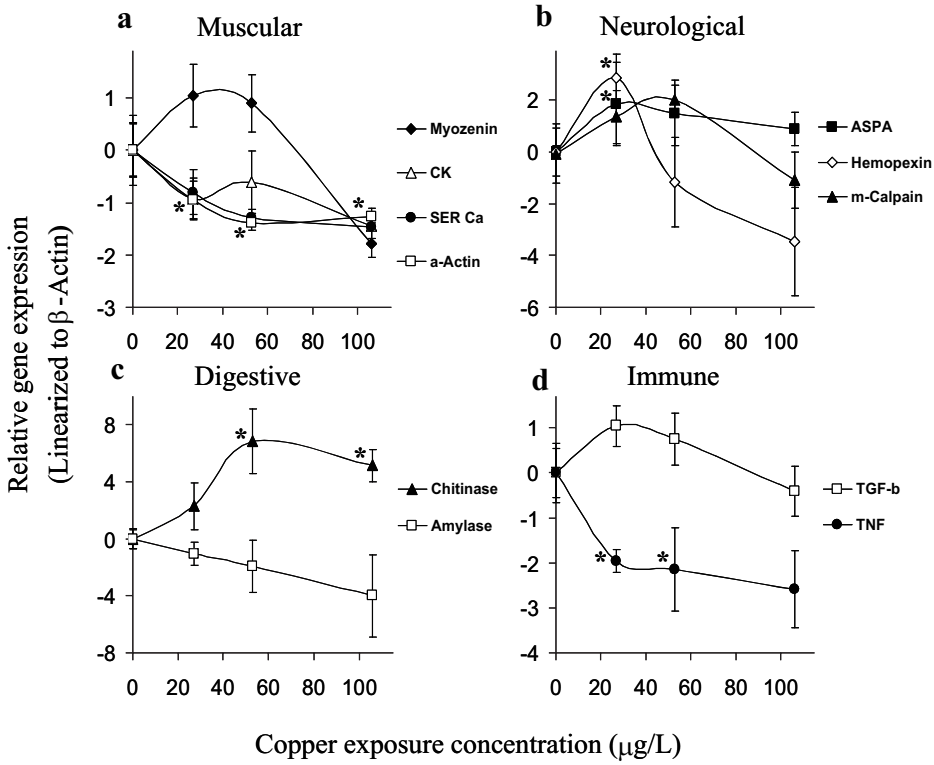

Fig. 3. (**a–d**) Quantitative polymerase chain reaction expression assessments of selected delta smelt genes responding to copper-exposed larvae. CK = creatine kinase; SER Ca = sarcoendoplasmic reticulum Ca ATPase; ASPA = aspartoacylase; TGF = transforming growth factor; TNF = tumor necrosis factor. The asterisk (*) indicates tests' significant differences; $p < 0.05$ at $n = 9, 9, 8$, and 5 for concentrations 1.6, 24.0, 52.4, and 105.0, respectively. (Data points have been connected to aid visualization.)

supporting measured differences in hemopexin transcription. Both hemopexin and ASPA expression were elevated at lower concentrations and reduced at higher concentrations.

Effects of Cu exposure on Ca availability were further corroborated by changes in m-calpain expression (Fig. 3b). m-Calpain is a Ca-dependent cysteine protease, known to co-localize with a calcium-sensing receptor, where calcium not only activates the m-calpain enzyme, but also causes it to undergo autolysis through subunit dissociation [24]. The physiological roles of calpains are still poorly understood; however, they have been shown to control cell fusions in myoblasts, playing an important role during myogenesis and thus muscle regeneration [25]. Interestingly, activation of m-calpain in the peripheral nervous system has been also reported to be involved in Wallerian degeneration, with increased expression being initiated following nerve injury [26], which was also indicated by changes in ASPA expression levels (see above). Thus, m-calpain is a potential biomarker of neuromuscular activity and, as such, clustered into both muscular and neurological responses. However, due to high variability in control subjects, and the low number of replicates available in this study, changes in expression were not significantly different in this test.

Digestion. Copper exposure resulted in significant responses in transcription of genes involved in delta smelt digestion (Fig. 3c). Alpha-amylase is a digestive enzyme that hydrolyzes starch into maltose [27]. It was downregulated, significantly correlating ($r = 0.978$) with increasing Cu concentration. Downregulation of α-amylase transcription has previously been associated with Cu exposure [28]. Chitinase, an enzyme required in the digestion of chitin structures in the exoskeletons of crustaceans and many insects, was significantly upregulated on exposure to Cu. Kurokawa et al. [29] have demonstrated that fish express chitinase in their guts, thus hypothesized to be involved in arthropod digestion, and also in the defense against Gram-positive bacteria and fungal pathogens.

Immune responses. Microarray analysis also identified effects upon the immune system that were confirmed through q-PCR (Fig. 3d). Tumor necrosis factor, a proinflammatory cytokine, was significantly downregulated by copper exposure, indicating a compromised immune system. Produced in many cell types, TNF plays an important role in immunity and inflammation, and in the control of cell proliferation, differentiation, and apoptosis; its downregulation has been implicated with various diseases in humans, such as Crohn's, arthritis, multiple sclerosis, and Alzheimer's [30]. Balkwill et al. [30] indicate that TNF knock-out causes autoreactive T cells' regulation, resulting epitope spreading, thus leading to a state of disease. Transforming growth factor-β is an anti-inflammatory cytokine expressed functionally during development, and in tissue maintenance and homeostasis, regulating proliferation and differentiation, cell survival, and apoptosis [31]. Upregulation of TGF-β has been linked with neurodegenerative diseases and ischemic injuries [32]; interestingly, it is reported to induce muscle α-actin expression [33], which was downregulated by Cu exposure. Transforming growth factor-β displayed an upregulation at lower levels of Cu exposure, in similar trends observed in ASPA and hemopexin, further suggesting immune responses resulting from probable neurological or signaling impairments.

Molecular biomarkers in monitoring programs. Molecular biomarkers have, for many years, successfully been used in human medicine as diagnostic tools, for example, the assessment of prostate specific antigen gene expression as an indicator

of prostate cancer [34], and are increasingly being used in the pharmaceutical arena to assess the mode of action, safety, efficiency, and targeted effects of novel and developed drugs [35]. Research in ecotoxicology has been concentrating on attributing or defining an ecological relevance to responses measured using biomarkers, through measuring responses at the individual level and attempting to extrapolate results to population responses. This work has been the subject of extensive critical discussion that has successfully strengthened biomarker research, but has concurrently discouraged widespread application in field studies. However, a limited number of researchers have successfully applied molecular biomarkers in field studies, identifying contaminant stress-induced responses [36] and metal-contamination effects [37] in various field-collected aquatic organisms.

In most aquatic organisms, and particularly in fish, a number of life-cycle characteristics are dependent on swimming behavior, from respiration to reproduction, thus swimming performance in fish is likely the single most significant environmentally relevant parameter that can be measured because it is indicative of effects upon behavior. Thus, effects upon individual fish, however minimal, will reduce reproductive success of a population. Neuromuscular alterations will likely have significant effects on swimming performance. In this study, we utilize a suite of molecular biomarkers, designed specifically to address neuromuscular disturbances, in an attempt to indicate, allow interpretation of, and correlate stressor modes of action that may impinge on swimming ability.

The use of biomarkers to specifically address key health parameters have extensively been investigated, through proteomics, a global genomics approach, or with the application of molecular probes, such as neuromuscular activity, digestion, and immune responses, as in this study, or with the addition of developmental assessments, such as links with endocrine responses, growth, and sexual development. In the search for biomarkers of effect and exposure, it is traditional to ascertain as useful only those whose functional responses correlate with exposure concentration. However, hormetic or biphasic dose responses appear to be indicative of changes in homeostasis [38], and as such should be treated as an invaluable technique to identify concentrations at which organisms can no longer compensate adequately to exposure. It is our contention that biphasic responses can therefore be more informative than dose-responsive biomarkers, which are solely indicative of exposures, without identifying lowest concentrations at which detrimental effects may occur. Thus, a suite of biomarkers, both biphasic and dose-respondent, should be utilized in conjunction to elucidate effects upon an organism. Previously, we have demonstrated the ability to link neurological effects from pyrethroid exposures in the delta smelt, with changes in gene expression that correlated with swimming behavior [4]. To attribute environmental relevance to molecular biomarkers, research should concentrate on their application in field studies with the aim of developing monitoring programs. Only through the inclusion of these techniques in monitoring programs, can biomarkers truly be evaluated.

Molecular biomarkers in the delta smelt. Delta smelt are highly sensitive to handling and extremely difficult to work with under laboratory conditions, and these studies are not necessarily informative of what the organisms are exposed to in their habitat. Thus, reliable biomarkers for determining the health status and exposure history of delta smelt in field-based studies are essential. Molecular biomarkers such as those identified in this study, could therefore be coupled to, and carried out in collaboration with, the Californian Department of Fish and Game annual townet surveys and monitoring programs (http://www.dfg.ca.gov/delta), thus creating an informative database of genomic responses indicative of delta smelt health status, aiding the process of toxicity identification and evaluation through the identification of contaminant-specific responses within complex chemical mixtures. Baseline gene expression, for comparative purposes, could be generated from assessing hatchery-raised larval, juvenile, and adult delta smelt, alongside temporal, site-specific variations in townet-surveyed wild fish.

Recommendations for field-based monitoring studies. A number of delta smelt genes, identified in this and previous studies [4], have been demonstrated as specific and informative biomarkers to warrant their application in field studies. Utilizing the molecular biomarkers identified this far, we are able to identify and further understand neuromuscular effects resulting from copper and pesticide exposures. Combining gene expression changes in aspartoacylase and hemopexin, for example, has proven successful in the identification of neurological insults, suggesting degradation of axon myelin sheaths and nerve repair [4]. Biphasic changes in expression seemingly differentiate homeostatic responses, allowing repair mechanisms, from concentrations that are chronically detrimental, as implied by aspartoacylase and hemopexin responses in this study. Effects of Cu on muscular activity and integrity can also be ascertained through the resulting downregulation of SER Ca ATPase and α-actin, respectively. Furthermore, the health status of an organism can be assessed in terms of digestion capacity and immune system functioning. Additional to the q-PCR–based biomarkers already described, a number of prospective genes have been identified with the microarray application and will be isolated from future studies, to expand genomic information towards a suite of functionally classified biomarkers to be included in monitoring programs, and identify classes of contaminants present in the delta smelt habitat range that may be responsible for toxicity. Thus, the presently assessed suite of biomarkers could be applied to field studies, comprising site-specific collected water-sample exposures within laboratory conditions, as well as upon wild specimens caught during townet surveys.

Linking molecular responses to swimming performance. The short-term Cu exposure (4-d) in this study resulted in an overall decrease in swimming velocity in larval delta smelt with increasing concentration. Though not statistically significant to controls, the reduction in swimming activity could possibly be explained by alterations measured by all neuromuscular molecular biomarkers, because these indicate the mechanisms of action of Cu upon the delta smelt. SER Ca ATPase was a particularly informative gene, because the measured downregulation supports indications of interference of Cu with Ca^{2+} homeostasis, neurological signaling, and muscle activity. The strong downregulation of α-actin, at all concentrations, sustains reported effects of Cu on contractile muscle proteins [28], further supported by increases in m-calpain expression, involved in muscle regeneration [25].

Food consumption was not measured in this test; thus it is not clear from this study whether the measured digestive changes are directly due to Cu exposure acting upon specific enzymes, or whether this is a result of altered swimming performance impinging on prey capture and ingestion. Under unexposed conditions, elevated levels of chitinase would likely signify higher levels of ingestion. It is unlikely that Cu-exposed fish had a higher consumption level; thus our contention is that Cu directly acts upon digestion, either impeding the proteolytic process through synthesis inhibition, enzyme degradation, or mRNA expression inhibition, as indicated through amylase

expression. Further responses to Cu exposure indicate a compromised immune system, with specific links to the central nervous system, as suggested by the measured upregulation of TGF-β, reported to be linked to neurodegenerative diseases [32]; its involvement in the induction of contractile muscle protein [33] further supports neuromuscular damage.

CONCLUSIONS

Copper concentrations used in this study, though high, are not uncommon in Californian surface waters [39], especially the lowest concentration investigated. Results from this study are indicative of short-term exposure responses. Bioaccumulation properties of heavy metals are well researched [40], and we extrapolate that longer-term exposures to lower levels of Cu are likely to have detrimental effects on swimming performance and alter the overall chances of delta smelt survival in the wild. Indisputably, the primary reason for the decline in number of pelagic organisms in the Sacramento–San Joaquin Delta is directly related to water exports [6]. However, organisms that manage to survive this habitat destruction are exposed to elevated concentrations of contaminant resulting from industrial, agricultural, and urban pollution, and lower water flows combined with a lesser dilution rate. Management systems to monitor the extent of change resulting from anthropogenic loads are essential, and this study enhances the argument for the use of a suite of molecular biomarkers as a successful approach towards identifying effects and causal factors of species decline.

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