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Sublethal responses to ammonia exposure in the endangered delta smelt; *Hypomesus transpacificus* (Fam. Osmeridae)

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ABSTRACT

The delta smelt (*Hypomesus transpacificus*) is an endangered pelagic fish species endemic to the Sacramento-San Joaquin Estuary in Northern California, which acts as an indicator of ecosystem health in its habitat range. Interrogative tools are required to successfully monitor effects of contaminants upon the delta smelt, and to research potential causes of population decline in this species. We used microarray technology to investigate genome-wide effects in fish exposed to ammonia; one of multiple contaminants arising from wastewater treatment plants and agricultural runoff. A 4-day exposure of 57-day old juveniles resulted in a total ammonium (NH_4^+–N) median lethal concentration (LC50) of 13 mg/L, and a corresponding un-ionized ammonia (NH_3) LC50 of 147 μg/L. Using the previously designed delta smelt microarray we assessed altered gene transcription in juveniles exposed to 10 mg/L NH_4^+–N from this 4-day exposure. Over half of the responding genes were associated with membrane integrity and function, however, neurological and muscular function was also affected. Amongst the notable pathways affected by ammonium exposure, directly associated with cellular membranes, are energy metabolism through oxidative phosphorylation, cellular responses to environmental stimuli, highlighted through signal transduction and molecular interactions, cellular processes encompassing transport and catabolism, along with cell motility, development, communication and cell death. To assess these impacts further, key genes were selected as potential biomarkers and investigated using quantitative PCR analysis on fish exposed to 2.5, 5, 10, 20 and 40 mg/L NH_4^+–N. Quantitative PCR results indicate biphasic responses, pivoting around the estimated no-observed effect concentration (NOEC; 5.0 mg/L NH_4^+–N) and below. Genes significantly affected by ammonia exposure include *claudin-10*, *Keratin-15*, *Septin-3*, *Transmembrane protein 4*, *superfamily 4* (membrane), *Tropomyosin*, *Myosin light chain*, *Calmodulin* (muscular), *Tubulin cofactor beta* (neurological), *Sirtuin-6* (development), and *Rhesus associated type C glycoprotein 1* (gill- and skin-specific ammonium transporter). The quantitation of the ammonium transporter may highlight the capacity of delta smelt to contend with elevated levels of ammonia, the peak response of which may be indicative of short-term thresholds of tolerance. Our study supports the notion that exposure to ammonia results in cell membrane destabilization, potentially affecting membrane permeability, enhancing uptake and thus synergistic effects of multiple-contaminant exposure.

1. Introduction

Contaminants and their potential deleterious effects to fish in the Sacramento-San Joaquin Estuary in Northern California are of particular interest due to negative long-term population trends and an observed step decline of several pelagic fish species (Bryant and Souza, 2004; Hieb et al., 2005; Feyrer et al., 2007; Sommer et al., 2007). This trend, known as the pelagic organism decline, has been the focus of an increasing number of investigations over the past decade (Sommer et al., 2007; Brown et al., 2009; Connon et al., 2009, 2011). Delta smelt (*Hypomesus transpacificus*), an endemic species to the Sacramento-San Joaquin Estuary, is one of the pelagic species of concern and has been listed as endangered under both the USA Federal and Californian State Endangered Species Acts. The role that contaminants may play in the decline of this important species, and other pelagic organisms, is in need of further investigation.

Ammonia and ammonium originating from municipal wastewater treatment plants, agricultural activity and numerous other sources, are two of multiple contaminants of concern that may be negatively impacting delta smelt habitats. One of the largest known sources of ammonia/um is the Sacramento Regional Wastewater

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Treatment Plant (SRWTP) which discharges into the lower Sacramento River, ultimately leading to delta smelt spawning and larval nursery areas. Ammonium in the Sacramento River, downstream of the SRWTP point of discharge has been recorded at concentrations up to 1.0 mg/L and 0.6 mg/L in the years 2007 and 2008 respectively and at concentrations of 0.28 mg/L directly upstream from the aforementioned delta smelt spawning and nursery areas (Werner et al., 2010).

The term ammonia/um refers to two chemical species which are in equilibrium in water, according to $NH_3 + H_2O \rightleftarrows NH_4^+ – OH^-$ (NH_3, un-ionized and NH_4^+, ionized or nitrogenous ammonia). Toxicity to aquatic organisms is primarily attributable to the un-ionized form (Vines and Wedding, 1960). Previous work in fish species has shown that ammonia/um affects cell membrane permeability leading to membrane transport deficiencies and increases in energy consumption (Martinelle and Haggstrom, 1993). Membrane integrity is the first line of defense against toxicants entering a cell, thus sublethal ammonia/um may lead to increased susceptibility to other contaminants, or contaminant mixtures, increasing the threat to aquatic organisms. Sublethal concentrations of ammonia/um have also been shown to cause histological alterations in fish, leading to immune system impairments and can result in increased susceptibility to bacteria, fungi and parasites (Evans et al., 2006). Reported histological effects include gill lamellae fusions and deformities, along with liver hydropic degenerations and glomerular nephritis (Benli et al., 2008). Ammonia is also known to affect the nervous and muscular systems in fish impacting behavioral performance eventually leading to mortality (McKenzie et al., 2009).

Given the impacts of ammonia/um on fish health, behavior and survival it is important to understand the potential sublethal impacts on the endangered delta smelt and to develop interrogative field-applicable tools to successfully monitor contaminant induced population decline in this species. Genomic tools can be cost effective and sensitive methods to assess sublethal contaminant impacts, and can aid regulatory toxicology by providing insights on the biological consequence of contaminant exposures through the identification of toxicity pathways and chemical modes of action (Ankley et al., 2006). Microarray gene profiling is a powerful tool for defining genome-wide effects and mechanistic changes caused by contaminant exposure. We have developed a cDNA microarray for delta smelt (Connon et al., 2009) and present here the application of this tool to investigate genome-wide effects of ammonium chloride on the delta smelt. We further investigate specific genomic responses utilizing quantitative PCR (q-PCR), and discuss their use as biomarkers and in terms of assessing the potential effects of ammonia exposure on juvenile delta smelt health and survival.

2. Materials and methods

2.1. Test organisms

Delta smelt were obtained from the UC Davis Fish Conservation and Culture Laboratory (FCCL) and maintained for 48 h in experimental conditions prior to test initiation. 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. The Assurance Number is A3433-01.

2.2. Exposures

Juvenile delta smelt (57-day old) were exposed for 4 days to 2.5, 5, 10, 20, 40 and 80 mg/L total ammonium by adding sodium chloride (Sigma-Aldrich, ACS reagent grade >99%) to culture water obtained from the FCCL. Controls were maintained in culture facility water with electric conductivity (EC) of 733 μS/cm and pH of 7.6. Replicate experimental treatments ($n = 4$) were initiated with 10 juveniles in 7 L of water at 15.5–16.5 °C. Fish were fed twice daily with <48 h old, *Artemia franciscana* (Argent Chemical Laboratories, Richmond, WA, USA) and held at a 16:8 h light:dark photoperiod. Approximately 80% of the water in each replicate was renewed at test initiation and on day 2. At test end, surviving fish were euthanized with MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO, USA), rinsed in de-ionized water, snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analyses. Comprehensive Environmental Toxicity Information System (CETIS) by Tidepool Scientific Software, (CA, USA) was used to calculate nominal lethal concentrations. Surviving juveniles from 10 mg/L NH_4^+–N were assessed against controls, utilizing the developed microarrays (see below). Due to high mortality at 80 mg/L NH_4^+–N, gene transcription assessments (described below) were not conducted on surviving fish from this concentration.

2.3. Experimental physicochemistry

Water temperature, pH, and dissolved oxygen (DO) were measured daily, and conductivity (EC) was measured at test initiation. Ammonia nitrogen (NH_4^+–N) concentrations were measured prior to each water renewal and at test termination. Ammonia nitrogen was measured using a Hach (Loveland, CO) AmVer Ammonia Test'N Tube Reagent Set, "low range" test kit (0–2.5 mg/L N) and "high range" test kit (0–50 mg/L N) for respective concentrations (estimated detection limit 0.7 mg/L). Un-ionized ammonia concentrations were calculated based on the measured total ammonia–N, temperature, EC and pH. pH was measured with a Beckman 240 pH meter, and DO and EC were measured using Yellow Springs Instruments (YSI) 85 meters.

2.4. Microarray assessments

We utilized a cDNA microarray with 8448 non-sequenced expressed sequence tags (ESTs) which were pin-printed in duplicate onto epoxysilane coated glass slides. Development of the delta smelt microarray is described in Connon et al. (2009). Genomic assessments were carried out between juveniles exposed to 10 mg/L; the estimated lowest observed effect concentration (LOEC), and controls. RNA was extracted from frozen whole, individual organisms, using Trizol Reagent (Invitrogen, Carlsbad, CA) as per manufacturer's guidelines. RNA from 5 fish, per replicate, per treatment was pooled and cDNA was synthesized from a total of 500 ng total RNA, amplified using a SuperScript™ Indirect RNA Amplification System (Invitrogen, Carlsbad, CA). Resulting cDNA was labeled with Alexa fluor dyes (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. Two color microarray assessments were carried out on quadruplicate treatments, using 1 μg of amplified cDNA for each control and exposed sample, including dye swaps for each (total 8 slides). Microarray hybridizations were performed using an automated Tecan HS4800 hybridization station. Slides were scanned using a GenePix 4000B scanner (Axon Instruments, Foster City, CA). Microarray data are available for download through the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov) accession number GSE29089.

Data were analyzed using LIMMA GUI (Linear model for microarray analysis graphical user interface; (Smyth, 2005)), written in the R-programming language available through Bioconductor http://www.Bioconductor.org. Data was normalized within using print-tip Lowess and between arrays applying average intensity quantile normalization methods, with background correction (Smyth, 2005). A linear model fit was computed using the

Table 1
Quantitative polymerase chain reaction primer/probe systems designed for genomic assessments on delta smelt (*Hypomesus transpacificus*).

Gene name	Gene code	Primer left	Primer right	Roche probe No.
Adenylate kinase	Adk	ctgtcttctggggacctgttg	ctcctttctgcataattgcctgt	36
Calmodulin	Calm	ttccttattcgacatggatggc	gcagacccagtgactgcatg	17
Claudin-10	Cldn10	ctgcctcggattctttggtg	cctccaattttggtgcacttc	140
Keratin-15	Krt15	ccagcaaaaccagttactcctcc	cctgatgagcctccatacctca	38
Myosin regulatory light chain 2	Mlc2	catgggagaccgcttcacc	tgtcgatgggagcttcacg	10
Septin-3	Sept3	ggctttgacctcaacattatggt	cttgagcagagtgttgaccagagt	60
Sirtuin-6	Sirt6	gaagccgacaggacgctact	ttccctctgcaggctctgag	1
Transmembrane4-l6-family-member-4	Tm4sf4	ccctggctctcatctccatc	ccatctttggcatacttcacc	64
Tropomyosin	Tpm	tcccttaacagacgcatccag	cagtagccagacgctcctgtg	101
Tubulin folding cofactor beta	Tbcb	gactcctgcagctggtatgga	ccagcttctgcaggaacttgtc	78
Beta Actin	β-Actin	tgccacaggactccatacc	catcggcaacgagaggtt	12
Ammonium transporter, Rhesus-associated type C glycoprotein 1	Amt-RhCG1	caggctgtcttatcgcttacgg	cagcgtcatgactaacagctgaa	61

duplicates on the arrays and least-squares method, with Benjamini and Hochberg (1995) false discovery rate adjustment.

Sequencing of differentially expressed features was conducted post microarray analysis, and carried out at the Genomic Facility, College of Agriculture and Environmental Sciences, UC Davis, thus only genes that were differentially expressed following exposure were sequenced. Basic Local Alignment Search Tool; translated nucleotide (BLASTx) searches were performed on specific fragments that responded significantly to the exposure treatments. 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 they were found to have a BLASTx match with the expect value smaller than 1×10^{-5} and a score above 50.

Differentially expressed genes were classified according to the Kyoto Encyclopedia of Genes and genomes (KEGG – http://www.genome.jp/kegg/kegg2.html), Gene Ontology (GO – http://www.uniprot.org/uniprot), and information gathered from literature, into functional groups. Classification was carried out based on gene expression changes in respect of control subjects. Specific genes of interest were selected for further investigation using quantitative PCR (see below).

2.5. Quantitative polymerase chain reaction (PCR) assessments

Genes for q-PCR assessments 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 (https://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). Respective primers and probe systems for investigated genes are detailed in Table 1, including a gene encoding for an ammonium transporter; *Rhesus-associated type C glycoprotein 1* (Amt-RhCG1), which was selected from related studies (unpublished data). Control fish and those exposed to concentrations between 2.5 and 40 mg/L were assessed using q-PCR. Complementary cDNA was synthesized using 1.0 μg total RNA, with random primers and SuperScript® III reverse transcriptase (Invitrogen, Carlsbad, CA), 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, Foster City, CA) was used in q-PCR amplifications. SDS 2.2.1 software (Applied Biosystems, Foster City, CA) was used to quantify transcription. We used the geNorm algorithm (Vandesompele et al., 2002) to estimate the variability of the reference genes, and to determine an optimal normalization gene. This approach failed to identify any suitable genes for normalization purposes, therefore quantitative PCR data was analyzed using the log 2(−Delta C_t) method (Livak and Schmittgen, 2001). The use of internal reference genes is not always appropriate (Tricarico et al., 2002), therefore expression differences were calculated relative to test controls, based on total RNA concentration normalization conducted prior to cDNA synthesis. Differences in gene expression, relative to the unexposed controls, were assessed

Table 2
Mean ammonia/um concentrations and physicochemical parameters from 96-h exposure of 57-day old delta smelt (*Hypomesus transpacificus*) to ammonium chloride.

Treatment	Temperature (°C)			EC (μS/cm)			DO (mg/L)		
	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
0.0 mg/L	16.5	0.5	4	733	–	1	8.9	0.4	4
2.5 mg/L	16.5	0.6	4	748	–	1	9.2	0.7	4
5.0 mg/L	16.5	0.8	4	769	–	1	9.2	0.6	4
10.0 mg/L	16.4	0.7	4	789	–	1	9.1	0.7	4
20.0 mg/L	16.5	0.7	4	847	–	1	9.3	0.3	4
40.0 mg/L	16.5	0.6	4	961	–	1	9.4	0.3	4
80.0 mg/L	15.5	0.3	2	1216	–	1	9.5	0.4	2

Treatment	Ammonia nitrogen (mg/L)			Un-ionized ammonia (mg/L)			pH		
	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
0.0 mg/L	0.11	0.05	4	0.001	0.001	4	7.58	0.13	4
2.5 mg/L	1.90	0.03	3	0.023	0.009	3	7.64	0.13	4
5.0 mg/L	5.00	0.00	2	0.066	0.034	2	7.62	0.13	4
10.0 mg/L	9.00	0.00	3	0.105	0.033	3	7.62	0.10	4
20.0 mg/L	17.67	0.58	3	0.228	0.120	3	7.63	0.16	4
40.0 mg/L	36.33	2.08	3	0.439	0.247	3	7.59	0.18	4
80.0 mg/L	72.00	5.66	2	0.526	0.133	2	7.47	0.16	2

EC: electric conductivity; DO: dissolved oxygen; SD: standard deviation; *N*: number of replicates.

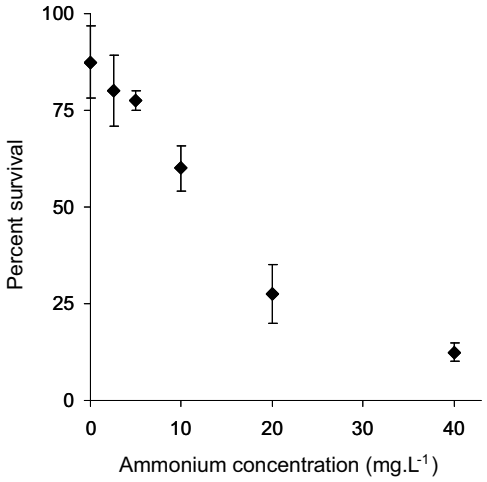

Fig. 1. Mean survival (±standard errors) of 57-day old juvenile delta smelt (*Hypomesus transpacificus*) after 96-h exposure to ammonium chloride.

using Kruskal–Wallis statistical tests on log 2(−Delta C_t) data, followed by Dunn's multiple comparison to controls. Quantitative PCR expression profiles were clustered with Genesis software (Sturn et al., 2002), using Pearsons' correlation *K*-means analysis, having previously determined 4 principal components by correspondence analysis.

3. Results and discussion

3.1. Experimental physicochemistry

Temperature, DO and pH remained stable throughout the test duration (Table 2). An increase in electric conductivity (733–1216 μS/cm) was measured with increasing total ammonium concentrations ($R^2 = 0.998$). This is resultant from the increase in chlorides originating from the ammonium chloride spike solution and likely reducing the overall ammonia toxicity in this euryhaline fish, as an increase in electric conductivity would lead to a decrease in ammonium uptake (Lin and Chen, 2001).

3.2. Acute toxicity

Four-day exposure of 57-day old juveniles to ammonium chloride (Fig. 1) resulted in a nominal median lethal concentration (LC50) of 13 mg/L, a no observable effect concentration (NOEC) of 5 mg/L and LOEC of 10 mg/L NH_4^+–N, corresponding to a calculated un-ionized ammonia (NH_3) LC50 of 147 μg/L, a NOEC of 66 μg/L and LOEC 105 μg/L. Comparatively, the 96-h LC50 for rainbow trout is reported to vary between 32 and 207 μg/L NH_3 for actively swimming and resting fish respectively (Wicks et al., 2002), significantly more toxic for active fish. Ammonium chloride is thus almost three-fold more toxic to delta smelt which were maintained in an environment, free from stressors that would enhance swimming, thus the determined LC50 is considered to be a conservative estimate for this species. Exposure duration is an important factor influencing ammonia toxicity. Four-day toxicity tests, as performed in this study, are unable to detect the potential chronic effects of ammonia/um exposure on delta smelt, such as upon behavior, reproduction and long-term survival, that may occur at far lower concentrations than those tested herein.

3.3. Microarray assessments

A wide variety of genes from a number of functional pathways were affected by exposure to ammonium chloride (Table 3, and Supplementary Table 3). However, the majority of responding genes were predominantly associated with membrane integrity and function (56%); such as membrane bound proteins responsible for ion transport and ionic exchange. Membrane disruption from ammonia exposure has previously been reported and attributed to changes in cellular pH resulting from ammonium gradients (Randall and Tsui, 2002; Wicks et al., 2002). Ammonium ions compete with membrane associated potassium ions and are transported into the cell, affecting mitochondrial ATP production, that is dependent on the ionic gradient across their membrane (Martinelle and Haggstrom, 1993). Other disrupted pathways affected by ammonium exposure, which are directly associated with cellular membranes, are energy metabolism through oxidative phosphorylation, cellular responses to environmental stimuli, highlighted through signal transduction and molecular interactions, cellular processes encompassing transport and catabolism, along with cell motility, development, communication and cell death. Inhibition of catabolic transport endocytosis and exocytosis, has been previously reported as resulting from proton gradient changes associated with ammonia/um exposure (Docherty and Snider, 1991).

Another common pathway disrupted by ammonia/um was highlighted by genes involved in calcium signaling. Decreased transcription of FK506, 1a (also known as FKBP1a or FKBP12) a gene involved in the regulation of the ryanodine receptor, and one of the key players of the calcium release unit responsible for excitation–contraction coupling in skeletal muscle (Zalk et al., 2007) strongly implies negative effects of ammonia exposure on muscle contraction. Effects upon other genes involved in calcium signaling, including tropomyosin and calmodulin, were also identified and further indicate probable neurological and muscular activity impairments. The microarray findings support work by Randall and Tsui (2002) who showed that displacement of potassium ions by elevated ammonium can depolarize neurons and lead to an excessive calcium influx, resulting in nerve cell death. In addition, calcium signaling is implicated in numerous organismal systems such as endocrine pathways, development, digestive and sensory systems and cell apoptosis and necrosis (Berridge et al., 2000). These neuromuscular impacts along with ammonia/um differential regulation of genes associated with neuroactive ligand-receptor interaction, long-term potentiation, and axon guidance pathways, indicate effects that could result in behavioral performance impairments. In fact effects of ammonia on swimming performance have been reported in past studies (Wicks et al., 2002; McKenzie et al., 2009). Although swimming performance was not assessed in the present study, hyperactive behavior and unsynchronized swimming performance in exposed individuals was observed at test termination, in this and other exposure tests (unpublished data) at similar concentrations.

3.4. Quantitative polymerase chain reaction (PCR) assessments

To elucidate the genomic dose response to varying ammonia/um concentrations and to aid in biomarker development, several genes that showed significant changes on the microarray, along with an ammonium transporter identified in a prior study (unpublished), were further investigated through q-PCR (Table 1). Dose dependent transcriptional responses to ammonium chloride exposure are presented in Fig. 2, clustered based on Pearson's correlations of profile similarity. Corresponding means, standard errors, Kruskal–Wallis statistical significance and multiple comparison of transcription data are presented in Table 4. The levels of gene transcription, as assessed by q-PCR, at 10 mg/L NH_4^+–N (LOEC), were non-significant to controls. The q-PCR assessments confirmed differential transcription of genes identified through

Table 3
Microarray assessment and functional classification of delta smelt (*Hypomesus transpacificus*) genes responding to 10 mg/L total ammonia (nominal concentration), as determined by Kyoto Encyclopedia for Genes and Genomes (KEGG) pathway analysis.

Functional category	Upregulated transcripts	Downregulated transcripts
1. Metabolism		
1.1 Carbohydrate metabolism		
Arginine and proline metabolism [PATH:00330]		Gamt*
Glycolysis/Gluconeogenesis [PATH:00010]		GAPDH*
Pyruvate metabolism [PATH:00620]	Glo1	
1.2 Energy metabolism		
Oxidative phosphorylation [PATH:00190]	ATPF2†, ATP6v0d*,†	ATP5c1†, ND1†, ND2†, ATP6†, Ndufa3†
Methane metabolism [PATH:00680]	ATP6v0d*,†	
1.3 Lipid metabolism		
Sphingolipid metabolism [PATH:00600]	Galac†	
Glycerophospholipid metabolism [PATH:00564]		ApoD
1.4 Nucleotide metabolism		
Purine metabolism [PATH:00230]		Adk*
1.5 Amino acid metabolism		
Glycine, serine and threonine metabolism [PATH:00260]		Gamt*
1.6 Metabolism of other amino acids	Rnpep#,†	
1.7 Glycan biosynthesis and metabolism	ST8Sia4#,†, Spock2#,†	
Glycosaminoglycan degradation [PATH:00531]	Zpa†, Zpc†	
1.8 Metabolism of terpenoids and polyketides		
Terpenoid backbone biosynthesis [PATH:00900]	Fdps*	
1.9 Biosynthesis of other secondary metabolites	Fdps*	GAPDH*, Adk*
2. Genetic information		
2.1 Transcription	Zfp572*,#, Ef1g#	Tpt1#
Spliceosome [PATH:03040]	Strap*, Ccdc827, Ccdc107	
Ribosome [PATH:03010]		Rpsa, Rps26, Rps7, Rppoc, Rpl10, Rpl3l
2.2 Folding, sorting and degradation	Pwap#,†	
Protein processing in endoplasmic reticulum [PATH:04141]		Rbp1†
Ubiquitin mediated proteolysis [PATH:04120]		Ubc
SNARE interactions in vesicular transport [PATH:04130]	Scoc†	
Chaperones and folding catalysts		Fkbp1a
2.3 Replication and repair	Bora#, Sirt6#, Tpase#,†	
DNA replication [PATH:03030]	Rnaset2	
3. Environmental processing information		
3.1 Membrane transport	Mfsd4#,†	
3.2 Signal transduction		
Calcium signaling pathway [PATH:04020]	Efha2†	ANTs250†, Calm*
ErbB signaling pathway [PATH:04012]		Eif4ebp1*
TGF-beta signaling pathway [PATH:04350]		Pp2Ab
Phosphatidylinositol signaling system [PATH:04070]		Calm*
mTOR signaling pathway [PATH:04150]		Eif4ebp1*
3.3 Signaling molecules and interaction	Gtpbp1#	Tspan8†
Cytokine–cytokine receptor interaction [PATH:04060]	Tpte†	CxC*,†
Cell adhesion molecules [PATH:04514]	Ctnt1a†, Tax1bp3†, Cldn10*,†, Cd99*,†	Tm4sf4†
Neuroactive ligand-receptor interaction [PATH:04080]	Plg*, Vps4b*,†	
4. Cellular processes		
4.1 Transport and catabolism		
Endocytosis [PATH:04144]	Vps4b*,†, Ap2s1†	
Lysosome [PATH:04142]	CtsD†	
Peroxisome [PATH:04146]	Pex11a†	
Phagosome [PATH:04145]	Tbcb*,†	
4.2 Cell motility	CSTB#,†	
Regulation of actin cytoskeleton [PATH:04810]		Krt15†, Krt5†, upk3l†, Mlc3, Mlc2*, Cfl2*, Tb4
4.3 Cell growth and death		
Cell cycle [PATH:04110]	Chk1*, sept3*,†	Ywhab, Ppdpfb
p53 signaling pathway [PATH:04115]	Chk1*, sept3*,†	
Apoptosis [PATH:04210]	Strap*	
4.4 Cell communication		
Gap junction [PATH:04540]	Tbcb*,†, Vps4b*,†	
Focal adhesion [PATH:04510]		Mlc2*
Tight junction [PATH:04530]	Cldn10*,†	Mlc2*
5. Organismal systems		
5.1 Immune system		
Antigen processing and presentation [PATH:04612]		HLA†
Chemokine signaling pathway [PATH:04062]		CxC*,†, Gnb2†
Leukocyte transendothelial migration [PATH:04670]	Cldn10*,†, Cd99*,†	Mlc2*
Fc gamma R-mediated phagocytosis [PATH:04666]		Cfl2*
Complement and coagulation cascades [PATH:04610]	Plg*	
Intestinal immune network for IgA production [PATH:04672]		CxC*,†
Toll-like receptor signaling pathway [PATH:04620]	Strap*	
RIG-I-like receptor signaling pathway [PATH:04622]	Strap*	
Cytosolic DNA-sensing pathway [PATH:04623]	Strap*	

Table 3 (*Continued*)

Functional category	Upregulated transcripts	Downregulated transcripts
5.2 Endocrine system		
Melanogenesis [PATH:04916]		Calm*
GnRH signaling pathway [PATH:04912]		Calm*
Insulin signaling pathway [PATH:04910]		Calm*, Eif4ebp1*
5.3 Circulatory system		
Cardiac muscle contraction [PATH:04260]		Tpm
Vascular smooth muscle contraction [PATH:04270]		Calm*
5.4 Digestive system		
Gastric acid secretion [PATH:04971]		Calm*
5.5 Nervous system		
Neurotrophin signaling pathway [PATH:04722]	Zfp572*	Ywhab, Calm*
Long-term potentiation [PATH:04720]		Calm*
5.6 Sensory system		
Olfactory transduction [PATH:04740]		Calm*
Phototransduction [PATH:04745]		Calm*
5.7 Development		
Axon guidance [PATH:04360]	Gpsm1†	Cfl2*

Abbreviations: SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptors; ErbB: Erythroblastic leukemia viral oncogene homolog; TGF-beta: Transforming growth factor beta; mTOR: Mammalian target of rapamycin; p53: Protein 53 kDa; Fc gamma R: Fragment crystalizable gamma receptor; IgA: Immunoglobulin A; RIG-I: Retinoic-acid-inducible gene I; GnRh: Gonadotropin-releasing hormone.

Gene names: Adk: Adenylate kinase; ANTs250: adenine nucleotide translocator s254; Ap2s1: AP-2 complex subunit sigma-1; ApoD: Apolipoprotein D precursor; ATP5c1: ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1; ATP6: ATP synthase F0 subunit 6; ATP6v0d1: ATPase, H+ transporting, V0 subunit D isoform 1; ATPF2: ATP synthase mitochondrial F1 complex assembly factor 2, mitochondrial precursor; Bora: protein aurora borealis; Calm: Calmodulin; Ccdc107: Coiled-coil domain-containing protein 107; Ccdc827: Coiled-coil domain-containing protein 827; Cd99: CD99 antigen precursor; Cfl2: Cofilin-2; Chk1: CHK1 checkpoint homolog; Cldn10: Claudin-10; CSTB: Cystatin-B; Ctnt1a: contactin 1a precursor; CtsD: Cathepsin D precursor; CxC: C–C motif chemokine 28 precursor; Ef1g: Elongation factor 1-gamma; Efha2: E F-hand domain family, member A2; Eif4ebp1: Eukaryotic translation initiation factor 4E-1A-binding protein; Fdps: Farnesyl pyrophosphate synthetase; Fkbp1a: FK506-binding protein 1A; Galac: Galactocerebrosidase precursor; Gamt: guanidinoacetate N-methyltransferase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Glo1: Lactoylglutathione lyase; Gnb2: guanine nucleotide binding protein beta 2; Gpsm1: G-protein-signaling modulator 1; Gtpbp1: Nucleolar GTP-binding protein 1; HLA: HLA class II histocompatibility antigen gamma chain; Krt15: keratin-15; Krt5: type II keratin E3; Mfsd4: Major facilitator superfamily domain-containing protein 4; Mlc2: Myosin regulatory light chain 2, smooth muscle isoform; Mlc3: Myosin light chain 3; ND1: NADH dehydrogenase subunit 1; ND2: NADH dehydrogenase subunit 2; Ndufa3: NADH dehydrogenase 1 alpha subcomplex subunit 3; Pex11a: Peroxisomal membrane protein 11A; Plg: plasminogen; Pp2Ab: serine/threonine-protein phosphatase 2A catalytic subunit beta isoform; Ppdpfb: Pancreatic progenitor cell differentiation and proliferation factor b; Pwap: Perlwapin; Rbp1: Plasma retinol-binding protein I; Rnaset2: Ribonuclease T2; Rnpep: Aminopeptidase B; Rpl10: 60S ribosomal protein L10; Rpl3l: Ribosomal protein L3-like; Rppoc: 60S acidic ribosomal protein P0; Rps26: Ribosomal protein S26; Rps7: Ribosomal protein S7; Rpsa: 40S ribosomal protein SA; Scoc: Short coiled-coil protein; sept3: Septin-3; Sirt6: Sirtuin (silent mating type information regulation 2 homolog) 6; Spock2: Novel protein similar to *H. sapiens* SPOCK2, sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2 (SPOCK2); ST8Sia4: Alpha-2,8-polysialyltransferase IV; Strap: Serine/threonine kinase receptor associated protein; Tax1bp3: Tax1 binding protein 3; Tb4: Thymosin beta-4; Tbcb: Tubulin folding cofactor B; Tm4sf4: Transmembrane 4 L6 family member 4; Tpase: Transposase; Tpm: Tropomyosin; Tpt1: Translationally-controlled tumor protein; Tpte: Transmembrane phosphatase with tensin homology; Tspan8: Tetraspanin-8; Ubc: Ubiquitin C; upk3l: Uroplakin 3l; Vps4b: Vacuolar protein sorting-associated protein 4B; Ywhab: Tyrosine 3-onooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide 2; Zfp572: Zinc finger protein 572; Zpa: ZPA domain containing protein; Zpc: ZPC domain containing protein 1.

* Indicates genes present in more than one functional category.

# Denotes genes for which no KEGG pathways were identified, and where functional categories were attributed from KEGG Brite functional hierarchies, gene ontology and related literature.

† Denotes genes associated with cell membrane integrity and function.

microarray assessments, though strangely not at the evaluated 10 mg/L NH_4^+–N, however the difference in normalization steps used on microarray and q-PCR data may account for discrepancies between the two approaches.

Quantitative PCR results indicate biphasic responses pivoting around the estimated NOEC (5.0 mg/L NH_4^+–N) and/or 2.5 mg/L NH_4^+–N, suggesting thresholds that correspond with sublethal and acute toxicity. Biphasic genomic responses measured following exposure to contaminants have been described in detail in a number of studies (Korsloot et al., 2004; Heckmann et al., 2008) and have been postulated to be indicative of compensatory response thresholds or tolerance to exposure. The biphasic responses could thus be indicative of detrimental limits of exposure to ammonium chloride.

Genes with similar functions were seemingly grouped within respective clusters however these clusters do not represent distinct functional classifications:

Cluster 1, is comprised of *Tropomyosin* (Tpm) which acts in association with the troponin complex in striated muscle contraction which is dependent on calcium release from the sarco endoplasmic reticulum (Lehman et al., 2009), and *tubulin cofactor beta* (Tbcb), a cytoskeleton-associated protein which has been reported to control directional growth and development of nerve axons (Grynberg et al., 2003; Lopez-Fanarraga et al., 2007). All genes in this cluster were significantly upregulated following exposure to ammonium chloride ($p < 0.01$).

Cluster 2, consists of *Sirtuin-6* (Sirt6) a gene involved in DNA replication and repair (Schwer et al., 2010), it responded significantly to ammonium chloride exposure ($p < 0.01$), and is likely involved in cell differentiation and development (Mahlknecht et al., 2006). A further three genes involved in cell membrane structure and integrity were clustered in this group: *Claudin-10* (Cldn10), a component of tight junction strands that serve as a cell membrane barrier, maintaining cell polarity and signal transduction (Kiuchi-Saishin et al., 2002); *Keratin-15* (Krt15), a gene encoding for an intermediate filament protein in epithelial cells (Badock et al., 2001); and *Septin*-3 (Sept3), a neuronal specific septin required for cytokinesis and has a suggested role in nerve development (Xue et al., 2008). All three genes were significantly upregulated at sublethal concentrations ($p < 0.05$, $p < 0.001$ and $p < 0.01$, respectively).

Cluster 3, includes *Adenylate kinase* (Adk), an ATP–AMP transphosphorylase involved in energy metabolism and nucleotide synthesis (Ravera et al., 2007) and an ammonium transporter, *Rhesus-associated type C glycoprotein 1* (Amt-RhCG1). ADK did not respond significantly to ammonia/um however, it followed the biphasic dose response seen in other responding genes. Interestingly, ammonium is produced in the contractile muscle through the breakdown of adenosine monophosphate (AMP), which affects

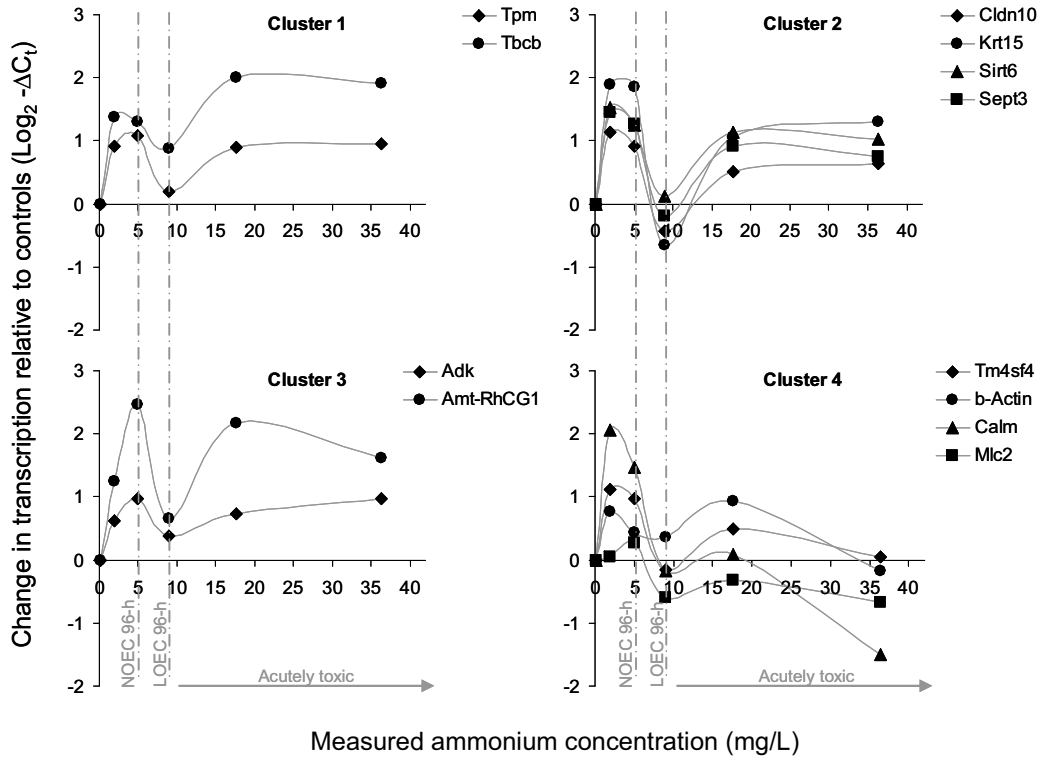

Fig. 2. Pearson's *K*-means cluster analysis of quantitative PCR assessed genes responding in 57-day old delta smelt (*Hypomesus transpacificus*) exposed for 96-h to ammonium chloride. No observable effect concentrations (NOEC) and the lowest observable effect concentration (LOEC) for 96-h mortality are noted, differentiating between assessed sublethal and acute toxicity ranges. (Data points have been connected to aid visualization).
Tpm: *Tropomyosin*; Tbcb: *Tubulin folding cofactor beta*; Cldn10: *Claudin-10*; Krt15: *Keratin-15*; Sirt6: *Sirtuin-6*; Sept3: *Septin-3*; Adk: *Adenylate kinase*; Amt-RhCG1: *Ammonium transporter, Rhesus-associated type C glycoprotein 1*; Tm4sf4: *Transmembrane 4-l6-family-member-4*; β-Actin: *Beta Actin*; Calm: *Calmodulin*; Mlc2: *Myosin regulatory light chain 2*.

the equilibrium of adenylate kinase (Adk) creating additional ATP and ADP to maintain contraction under stress (Wilkinson et al., 2010). ADK is likely to be affected by increased ammonium in muscle at sublethal concentrations, which is supported by the observable biphasic response. Amt-RhCG1, responsible for excess ammonia/um elimination across the gills and to a lesser extent the skin (Hung et al., 2007), was significantly upregulated ($p < 0.05$) upregulated, suggesting that the fish maybe compensating for the increased toxicant load. Amt-RhCG1 correlated significantly ($R^2 = 1$) with ammonium chloride dose at the tested sublethal concentrations, further supporting the overall compensatory threshold hypothesis of transcription response profiles observed. This dose-dependent increase in Amt-RhCG1 has been reported on studies on killifish (Hung et al., 2007) and an $R^2 = 1$ correlation was also determined in prior studies conducted in our laboratory (as yet unpublished).

Cluster 4, incorporates four genes; a *Transmembrane protein 4*, *superfamily 4* (*Tm4sf4*), a cell-surface glycoprotein, which was significantly upregulated by ammonium chloride ($p < 0.01$). The protein encoded by this gene mediates signal transduction events regulating cell proliferation (Diosdado et al., 2004), playing a role in cell development, activation, growth and motility. Beta actin (*β-Actin*) is a structural component of the cytoskeleton, and was included in this study as an analytical reference gene for expression normalization, however, it responded significantly to ammonium chloride exposure ($p < 0.01$), as did numerous genes associated with cell structure and integrity, and was therefore not applicable as a reference gene. *Calmodulin* (*Calm*) mediates the control of proteins that are regulated by calcium (Klee et al., 1980), and *Myosin light chain-2* (*Mlc2*) which is a *Calmodulin*-activated cardiac myosin regulatory light chain that triggers muscle contraction through phosphorylation by calcium (Ding et al., 2010). Interestingly, both of these genes were upregulated at sublethal ammonium chloride concentrations, but significantly downregulated by acutely toxic concentrations ($p < 0.01$ and $p < 0.05$, respectively).

The present study was primarily aimed at identifying suitable molecular biomarkers in the delta smelt, to assess the effects of exposure to ammonia/um, and it is important to point out that concentrations of ammonia present at delta smelt spawning and nursery sites are lower than those assessed in this study. Nevertheless, our findings suggest that short-term effects of ammonia exposure may impact growth and development, and neuromuscular activity, as well as affect membrane permeability, directly impinging on the protective barriers against pollutant routes of uptake and subsequent mechanisms of action. Ammonia/um is a pseudo-persistent pollutant, as it is continuously introduced into the aquatic environment. Fate and transport of the wastewater effluent, as well as seasonal variations of environmental conditions likely affect concentrations and potential toxicity of ammonia/um discharged into the Sacramento River. The toxicity of other contaminants, such as urban pesticides, pharmaceuticals and personal care products, not fully removed during the wastewater treatment process, are likely to be synergized by the presence of ammonia. Additionally, other environmental factors can alter the toxicity of ammonia/um including pH and temperature leading to increased threats in certain environments (Randall and Tsui, 2002). Pre-exposure or simultaneous exposure to multiple contaminants, state of disease or other stressful environmental conditions may considerably alter the physiological condition and therefore susceptibility of species of concern, such as the delta smelt.

Interestingly a prevalent upregulation in the selected genes was observed at sublethal exposure concentrations; below the

Table 4
Differences in gene expression, relative to the unexposed controls, of 57-day old delta smelt (*Hypomesus transpacificus*). Differences between treatments were determined using a Kruskal–Wallis statistical tests on quantitative polymerase chain reaction log 2(−Delta C_t) data, followed by Dunn's multiple comparison to controls ($n = 4$).

Gene	*p*-value summary	Kruskal–Wallis statistic: H	Dunnett's test (relative to control)						
			(mg/L)	0.11	1.90	5.00	9.00	17.67	36.33
Adk	0.124	8.6	Mean	0.000	0.613	0.976	0.380	0.733	0.965
			SE	0.250	0.166	0.258	0.146	0.255	0.255
			p-value		>0.05	>0.05	>0.05	>0.05	>0.05
Calm	0.003	18.3	Mean	0.000	2.055	1.469	−0.171	0.094	−1.506
			SE	0.339	0.182	0.065	0.119	0.126	0.739
			p-value		>0.05	>0.05	>0.05	>0.05	>0.05
Cldn10	0.018	13.6	Mean	0.000	1.136	0.912	−0.436	0.510	0.644
			SE	0.242	0.247	0.106	0.157	0.372	0.318
			p-value		>0.05	>0.05	>0.05	>0.05	>0.05
Krt15	0.001	21	Mean	0.000	1.897	1.863	−0.653	1.058	1.302
			SE	0.342	0.082	0.116	0.266	0.052	0.109
			p-value		>0.05	>0.05	>0.05	>0.05	>0.05
Mlc2	0.013	14.5	Mean	0.000	0.057	0.277	−0.593	−0.326	−0.676
			SE	0.390	0.066	0.075	0.271	0.058	0.184
			p-value		>0.05	>0.05	>0.05	>0.05	>0.05
Sept-3	0.008	15.6	Mean	0.000	1.444	1.263	−0.193	0.909	0.751
			SE	0.325	0.126	0.102	0.110	0.194	0.284
			p-value		>0.05	>0.05	>0.05	>0.05	>0.05
Sirt6	0.005	16.7	Mean	0.000	1.530	1.247	0.127	1.136	1.033
			SE	0.500	0.155	0.112	0.211	0.088	0.096
			p-value		>0.05	>0.05	>0.05	>0.05	>0.05
Tms4sf4	0.009	15.4	Mean	0.000	1.120	0.966	−0.161	0.485	0.045
			SE	0.301	0.101	0.078	0.190	0.277	0.298
			p-value		<0.05	>0.05	>0.05	>0.05	>0.05
Tpm	0.008	15.6	Mean	0.000	0.910	1.084	0.196	0.892	0.959
			SE	0.223	0.139	0.100	0.181	0.072	0.204
			p-value		>0.05	>0.05	>0.05	>0.05	>0.05
Tbcb	0.004	17.4	Mean	0.000	1.379	1.308	0.878	2.002	1.904
			SE	0.219	0.080	0.231	0.185	0.196	0.283
			p-value		>0.05	<0.05	>0.05	>0.05	<0.05
β-Actin	0.002	19.2	Mean	0.000	0.766	0.431	0.362	0.933	−0.178
			SE	0.144	0.150	0.065	0.028	0.080	0.215
			p-value		>0.05	>0.05	>0.05	<0.01	>0.05
Amt-RhCG1	0.016	13.89	Mean	0.000	1.242	2.472	0.650	2.168	1.624
			SE	0.258	0.377	0.517	0.184	0.557	0.204
			p-value		>0.05	<0.05	>0.05	>0.05	>0.05

Adk: *Adenylate kinase*; Calm: *Calmodulin*; Cldn10: *Claudin-10*; Krt15: *Keratin-15*; Mlc2: *Myosin regulatory light chain 2*; Sept3: *Septin-3*; Sirt6: *Sirtuin-6*; Tm4sf4: *Transmembrane4-l6-family-member-4*; Tpm: *Tropomyosin*; Tbcb: *Tubulin folding cofactor beta*; β-Actin: *Beta Actin*; Amt-RhCG1: *Ammonium transporter, Rhesus-associated type C glycoprotein 1*.

determined NOEC. The measured up-regulation may be a resulting compensation to acute ammonia/um exposure, but could also be indicative of detrimental effects that may result following chronic sublethal exposure. This hypothesis is further supported by the inflexion in genomic responses at sublethal and lethal concentrations, representing the concentration at which organisms can no longer contend with exposure. Although a full range of exposure concentrations assessed by q-PCR are presented, the sublethal responses convey some environmental relevance for the application of molecular biomarkers as a monitoring tool for the assessment of ammonia/um toxicity. Chronic exposure effects at concentrations of 1.0 mg/L that have been measured in the Sacramento River (Werner et al., 2010), could be mechanistically comparable to those determined in the 96 h exposure assessment conducted in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2011.07.002.

References

Ankley, G.T., Daston, G.P., Degitz, S.J., Denslow, N.D., Hoke, R.A., Kennedy, S.W., Miracle, A.L., Perkins, E.J., Snape, J., Tillitt, D.E., Tyler, C.R., Versteeg, D., 2006. Toxicogenomics in regulatory ecotoxicology. Environmental Science and Technology 40, 4055–4065.

Badock, V., Steinhusen, U., Bommert, K., Wittmann-Liebold, B., Otto, A., 2001. Apoptosis-induced cleavage of keratin 15 and keratin 17 in a human breast epithelial cell line. Cell Death and Differentiation 8, 308–315.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289–300.
Benli, A.C., Koksal, G., Ozkul, A., 2008. Sublethal ammonia exposure of Nile tilapia (*Oreochromis niloticus* L.): effects on gill, liver and kidney histology. Chemosphere 72, 1355–1358.
Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. Nature Reviews. Molecular Cell Biology 1, 11–21.
Brown, L.R., Kimmerer, W., Brown, R., 2009. Managing water to protect fish: a review of California's Environmental Water Account, 2001–2005. Environmental Management 43, 357–368.
Bryant, M., Souza, K., 2004. Summer townet and fall midwater trawl survey status and trends. Interagency Ecological Program Newsletter, 14–17.
Connon, R.E., Geist, J., Pfeiff, J., Loguinov, A.V., D'Abronzo, L., Wintz, H., Vulpe, C.D., Werner, I., 2009. Linking mechanistic and behavioral responses to sublethal esfenvalerate exposure in the endangered delta smelt; *Hypomesus transpacificus* (Fam. Osmeridae). BMC Genomics, 608.
Connon, R.E., Beggel, S., D'Abronzo, L.S., Geist, J.P., Pfeiff, J., Loguinov, A.V., Vulpe, C.D., Werner, I., 2011. Linking molecular biomarkers with higher level condition indicators to identify effects of copper exposures on the endangered delta smelt (*Hypomesus transpacificus*). Environmental Toxicology and Chemistry 30, 290–300.
Ding, P., Huang, J., Battiprolu, P.K., Hill, J.A., Kamm, K.E., Stull, J.T., 2010. Cardiac myosin light chain kinase is necessary for myosin regulatory light chain phosphorylation and cardiac performance in vivo. Journal of Biological Chemistry, 40819–40829.
Diosdado, B., Wapenaar, M.C., Franke, L., Duran, K.J., Goerres, M.J., Hadithi, M., Crusius, J.B.A., Meijer, J.W.R., Duggan, D.J., Mulder, C.J.J., Holstege, F.C.P., Wijmenga, C., 2004. A microarray screen for novel candidate genes in coeliac disease pathogenesis. Gut, 944–951.
Docherty, P.A., Snider, M.D., 1991. Effects of hypertonic and sodium-free medium on transport of a membrane glycoprotein along the secretory pathway in cultured mammalian cells. Journal of Cellular Physiology 146, 34–42.
Evans, J.J., Pasnik, D.J., Brill, G.C., Klesius, P.H., 2006. Un-ionized ammonia exposure in Nile Tilapia: toxicity, stress response, and susceptibility to Streptococcus agalactiae. North American Journal of Aquaculture, 23–33.
Feyrer, F., Nobriga, M.L., Sommer, T.R., 2007. Multidecal trends for three declining fish species: habitat patterns and mechanisms in the San Francisco Estuary, California, USA. Journal of Fisheries and Aquatic Sciences 64, 723–734.
Grynberg, M., Jaroszewski, L., Godzik, A., 2003. Domain analysis of the tubulin cofactor system: a model for tubulin folding and dimerization. BMC Bioinformatics 4, 46.
Heckmann, L.H., Sibly, R.M., Connon, R., Hooper, H.L., Hutchinson, T.H., Maund, S.J., Hill, C.J., Bouetard, A., Callaghan, A., 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. Genome Biology 9, R40.
Hieb, K., Bryant, M., Souza, K., Greiner, T., Slater, S., 2005. Place holder for bay and estuary species 2004 status and trends report. Interagency Ecological Program Newsletter, 6–10.
Hung, C.Y., Tsui, K.N., Wilson, J.M., Nawata, C.M., Wood, C.M., Wright, P.A., 2007. Rhesus glycoprotein gene expression in the mangrove killifish Kryptolebias marmoratus exposed to elevated environmental ammonia levels and air. The Journal of Experimental Biology 210, 2419–2429.
Kiuchi-Saishin, Y., Gotoh, S., Furuse, M., Takasuga, A., Tano, Y., Tsukita, S., 2002. Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. Journal of the American Society of Nephrology, 875–886.
Klee, C.B., Crouch, T.H., Richman, P.G., 1980. Calmodulin. Annual Review of Biochemistry 49, 489–515.
Korsloot, A., van Gestel, C.A.M., van Straalen, N.M., 2004. Environmental Stress and Cellular Response in Arthropods. CRC Press, Boca Raton, FL.
Lehman, W., Galinska-Rakoczy, A., Hatch, V., Tobacman, L.S., Craig, R., 2009. Structural basis for the activation of muscle contraction by troponin and tropomyosin. Journal of Molecular Biology 388, 673–681.
Lin, Y.-C., Chen, J.-C., 2001. Acute toxicity of ammonia on Litopenaeus vannamei Boone juveniles at different salinity levels. Journal of Experimental Marine Biology and Ecology 259, 109–119.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta]CT Method. Methods 25, 402–408.
Lopez-Fanarraga, M., Carranza, G., Bellido, J., Kortazar, D., Villegas, J.C., Zabala, J.C., 2007. Tubulin cofactor B plays a role in the neuronal growth cone. Journal of Neurochemistry 100, 1680–1687.
Mahlknecht, U., Ho, A.D., Voelter-Mahlknecht, S., 2006. Chromosomal organization and fluorescence in situ hybridization of the human Sirtuin 6 gene. International Journal of Oncology 28, 447–456.
Martinelle, K., Haggstrom, L., 1993. Mechanisms of ammonia and ammonium ion toxicity in animal cells: transport across cell membranes. Journal of Biotechnology 30, 339–350.
McKenzie, D.J., Shingles, A., Claireaux, G., Domenici, P., 2009. Sublethal concentrations of ammonia impair performance of the teleost fast-start escape response. Physiological and Biochemical Zoology 82, 353–362.
Randall, D.J., Tsui, T.K., 2002. Ammonia toxicity in fish. Marine Pollution Bulletin 45, 17–23.
Ravera, S., Calzia, D., Panfoli, I., Pepe, I.M., Morelli, A., 2007. Simultaneous detection of molecular weight and activity of adenylate kinases after electrophoretic separation. Electrophoresis 28, 291–300.
Schwer, B., Schumacher, B., Lombard, D.B., Xiao, C., Kurtev, M.V., Gao, J., Schneider, J.I., Chai, H., Bronson, R.T., Tsai, L.-H., Deng, C.-X., Alt, F.W., 2010. Neural sirtuin 6 (Sirt6) ablation attenuates somatic growth and causes obesity. Proceedings of the National Academy of Sciences of the United States of America.
Smyth, G.K., 2005. Limma: linear models for microarray data. In: Gentleman, R., Carry, V., Dudoit, S., Irizarry, R., Huber, W. (Eds.), Bioinformatics and Computational Biology Solutions Using R and Bioconductor. Springer, New York, NY, pp. 397–420.
Sommer, T., Armor, C., Baxter, R., Breuer, R., Brown, L., Chotkowski, M., Culberson, S., Feyerer, F., Gingras, M., Herbold, B., Kimmerer, W., Mueller-Solger, A., Nobriga, M., Souza, K., 2007. The collapse of pelagic fishes in the upper San Francisco estuary. Fisheries 32, 270–277.
Sturn, A., Quackenbush, J., Trajanoski, Z., 2002. Genesis: cluster analysis of microarray data. Bioinformatics (Oxford, England) 18, 207–208.
Tricarico, C., Pinzani, P., Bianchi, S., Paglierani, M., Distante, V., Pazzagli, M., Bustin, S.A., Orlando, C., 2002. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Analytical Biochemistry 309, 293–300.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, R34.
Vines, H.M., Wedding, R.T., 1960. Some effects of ammonia on plant metabolism and a possible mechanism for ammonia toxicity. Plant Physiology 35, 820–825.
Werner, I., Deanovic, L.A., Markiewicz, D., Khamphanh, M., Reece, C.K., Stillway, M., Reece, C., 2010. Monitoring acute and chronic water column toxicity in the Northern Sacramento-San Joaquin Estuary, California, USA, using the euryhaline amphipod, Hyalella azteca: 2006–2007. Environmental Toxicology and Chemistry/SETAC 29, 2190–2199.
Wicks, B.J., Joensen, R., Tang, Q., Randall, D.J., 2002. Swimming and ammonia toxicity in salmonids: the effect of sub lethal ammonia exposure on the swimming performance of coho salmon and the acute toxicity of ammonia in swimming and resting rainbow trout. Aquatic Toxicology (Amsterdam, Netherlands) 59, 55–69.
Wilkinson, D.J., Smeeton, N.J., Watt, P.W., 2010. Ammonia metabolism, the brain and fatigue; revisiting the link. Progress in Neurobiology 91, 200–219.
Xue, J., Anggono, V., Robinson, P.J., 2008. Septins and the Synapse. John Wiley & Sons, Ltd.
Zalk, R., Lehnart, S.E., Marks, A.R., 2007. Modulation of the ryanodine receptor and intracellular calcium. Annual Review of Biochemistry 76, 367–385.