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

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Abstract:

The delta smelt (*Hypomesus transpacificus*) is a pelagic fish species endemic to the Sacramento-San Joaquin Estuary in Northern California, listed as endangered under both the USA Federal and Californian State Endangered Species Acts and 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 larvae resulted in a measured un-ionized ammonia (NH_3) LC_{50} of 147$\mu g.L^{-1}$, a NOEC of 66 $\mu g.L^{-1}$ and LOEC 105 $\mu g.L^{-1}$. We assessed genome-wide expression at 105 $\mu g.L^{-1}$ and selected genes were further investigated as molecular biomarkers using quantitative PCR analyses on exposures to 23, 66, 105, 228 and 439 $\mu g.NH_3.L^{-1}$. Genes predominantly encoding for membrane bound proteins responded significantly to ammonia exposure, however, neurological and muscular activity were also impaired. We present here our functional gene classification and further investigations into neurological, muscular, immune, growth and development responses significantly affected by exposure to this contaminant.

Keywords: 'Hypomesus transpacificus', 'delta smelt', microarray, biomarker, ammonia

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 a possible step decline in numbers of several pelagic fish species in the years 2000-2001 (Bryant and Souza, 2004; Feyrer et al., 2007; Hieb et al., 2005; 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 several years (Brown et al., 2009; Connon et al., 2009; Sommer et al., 2007). Delta smelt (*Hypomesus transpacificus*) is one of the species of concern. It is endemic to the Delta and has been listed as endangered under both the USA Federal and Californian State Endangered Species Acts.

Ammonia (NH_3) originating from municipal wastewater treatment plants, agricultural activity and numerous other sources, is one of multiple contaminants of concern in delta smelt habitat.
The term ammonia/um refers to two chemical species which are in equilibrium in water (NH_3, un-ionized and NH^{4+}, ionized or nitrogenous ammonia) according to $NH_3 + H^+ \leftrightarrows NH^{4+}$. Tests for ammonia/um usually measure total ammonia plus ammonium, while the toxicity is primarily attributable to the un-ionized form. In general, more un-ionized ammonia and greater toxicity exist at higher pH, because its relative proportion increases with increasing pH according to the following equations (USEPA, 1985):

$$1 / (1 + 10^{pKa-pH}) = \% NH_3$$
$$\text{where: } pKa = 0.0902 + [2729.9/(°C+273.2)]$$

Temperature will affect this equilibrium, but to a far lesser extent than pH. Acute fish toxicity of ammonia decreases with increasing temperature, but toxicity of total ammonia/um shows no correlation with temperature (USEPA, 1999). This is probably due to an increase in the permeability of biological membranes such as gills by a factor of 2-3 for each 10°C increase in water temperature (Eddy et al., 1995).

The Sacramento River drains into delta smelt spawning and larval nursery areas, thus toxicants present in river water could potentially affect early life stages of delta smelt found downstream. Werner et al. (2010), found that ambient ammonia concentrations were greatest in Cache Slough (≤0.025 mg/L nitrogenous ammonia), and near the Sacramento River confluence with the Deep Water Shipping Channel (≤0.021 mg/L nitrogenous ammonia). Ammonia concentrations in the Sacramento River, downstream from the regional wastewater treatment plant were generally lower (≤0.019 mg/L nitrogenous ammonia), likely due to the lower pH of the river water at this location.

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. Microarray gene profiling is a powerful tool for defining genome-wide effects of environmental change on biological function. We have developed a microarray for delta smelt (Connon et al., 2009) and present here the application of this tool to investigate genome-wide effects in delta smelt exposed to ammonia/um. We further assess specific genomic responses utilizing quantitative PCR, within functional gene pathways, and assess the validity of using molecular biomarkers as monitoring tools of individual and population damage.

Materials and Methods.

Test organisms: Delta smelt were obtained from the Fish Conservation and Culture Laboratory (FCCL), UC Davis and maintained for a minimum of 24 hours 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 (AAALAC) and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance Number is A3433-01.

Exposures: Larval delta smelt (57-d old) were exposed for 4 days to 2.5, 5. 10. 20, 40 and 80 $mg.L^{-1}$ ammonium chloride prepared in culture water obtained from the FCCL, concentrations that correspond to 23, 66, 105, 228 and 439 µg NH_3 L^{-1} (un-ionized ammonia). Controls were maintained in culture facility water with specific conductance (SC) of 930 $\mu S.cm^{-1}$ and pH of 7.9. Larvae were acclimated to control water for 24 h prior to test initiation. Replicate experimental treatments (n=4) were initiated with 10 larvae in 7L of water at 20°C. Fish were fed twice daily with artemia (<48 h old). The light:dark cycle was 16h:8h. Approximately 80% of the water in each replicate container 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 and snap-frozen in liquid nitrogen and stored at –80°C for subsequent analyses.

Experimental physicochemistry: Water temperature, pH, and DO were measured daily. Conductivity was measured at test initiation. Ammonia nitrogen (NH_4^+-N) concentrations were measured prior to each water renewal and at test termination.

Genomic assessments - microarrays: Development of the delta smelt microarray was described in Connon et al. (2009) (Connon et al., 2009), briefly we utilized a cDNA microarray with 8,448 expressed sequence tags (ESTs) which were pin-printed in duplicate onto epoxysilane coated glass slides. RNA was extracted from frozen whole, individual organisms, using Trizol Reagent (Invitrogen) as per manufacturer's guidelines. cDNA was synthesized from a total of 1ug total RNA, and amplified using a SuperScript^{tm} Indirect RNA Amplification System (Invitrogen) and labeled with and labeled with Alexa fluor dyes (Invitrogen) as per manufacturer's instructions. Microarray assessments were carried out using quadruplicate treatments. Microarray hybridizations were performed using an automated Tecan HS4800 hybridization station. Slides were scanned using a GenePix 4000B scanner (Axon Instruments). Microarray images and data from exposed delta smelt can be accessed at under the pelagic organism decline (POD) section at: http://www.vetmed.ucdavis.edu/apc/WernerLab/subpage/pelagic_organism_decline.html.

Data was 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 arrays using print-tip Lowess and between arrays applying aquantile normalization methods (Livak and Schmittgen, 2001). A linear model fit was computed using the duplicates on the arrays and the least-squares method, no multiple assessment methods were applied to eliminate false positives as our aim was to increase the number of genes available for biomarker assessment, and qualify these through quantitative PCR.

Sequencing of differentially expressed features was carried out at the CA&ES Genomic Facility, UC Davis. 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 they were found to have a BLASTx match with the expect value smaller than $1x10^{-5}$ 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) and 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, regardless of whether these were up- or downregulated. Specific genes of interest were selected for further investigation using quantitative PCR (see below).

Genomic assessments – qPCR: Genes for quantitative 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 biomarkers are detailed in Table 1. 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. SDS 2.2.1 software (Applied Biosystems) 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. Quantitative PCR data was analyzed using the relative quantification 2(-Delta Delta CT) method (Livak and Schmittgen, 2001). 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.

Table 1. Primer probe systems designed from microarray assessments on larval delta smelt.

Gene Name	Gene Code	Primer Left	Primer Right	Probe No.
adenylate_kinase	ADK	ctgtcttctggggacctgttg	ctcctttctgcataattgcctgt	36
calmodulin	CaM	ttccttattcgacatggatggc	gcagacccagtgactgcatg	17
claudin-10	CLDN10	ctgcctcggattctttggtg	cctccaattttggtgcacttc	140
epimorphin	EpiM	ctttcgggaaaggaccaaaac	tgcttgtcacttttcccagttatc	94
hla	HLA	atcgtgtctgtggagaaacaggt	ggaagctctggttgaactcgg	25
keratin-15	Ker15	ccagcaaaaccagttactcctcc	cctgatgagcctccatacctca	38
myosin-regulatory-light-chain-2	MYL2	catgggagaccgcttcacc	tgtcgatgggagcttcacg	10
septin-3	SEPT3	ggctttgacctcaacattatggt	cttgagcagagtgttgaccagagt	60
sirtuin-6	SIRT6	gaagccgacaggacgctact	ttccctctgcaggctctgag	1
transmembrane-4-l6-family-member-4	Tm4sf4	ccctggctctcatctccatc	ccatctttggcatacttcacc	64
tropomyosin	TPM	tcccttaacagacgcatccag	cagtagccagacgctcctgtg	101
tubulin-folding-cofactor-b	TBCB	gactcctgcagctggtatgga	ccagcttctgcaggaacttgtc	78
Alpha-Actin	A-Actin	cctgcctcgtcgtactcctg	catcctggcttccctgtcc	11
Amylase	Amy	gatcaccatgttcttgatctgacg	ccatcaatcctgaccaaacctg	99
Beta-Actin	B-Actin	tgccacaggactccatacc	catcggcaacgagaggtt	12
Creatine Kinase	CK	cgatcggcgttggagatg	gccaagttcaacgagattctgg	163
Myozenin	MyoZ	ccaatgtcgtgctggtacacc	ctgccagacattgatgtagcca	106
SER-Ca	SER CA	catgatcattgggggagca	tgctgtgatgacaacgaggac	148
TGF-B	TGF-b	caacggcatagtgcatgtgg	gaatgtgtgcacgttgttggt	76
Tumor Necrosis Factor	TNF	ctttttccgctgttccatgttc	gttaccagcatacgcagtgtcc	2
Aspartoacylase	ASPA	ggaggcacacatgggaatg	cttcctctgaatctctgttccattatc	109
Hemopexin	HPEX	catgcactacgaggacgacaag	tggtagtagctgaacaccttgctg	143
Titin-a	Titin	tgatcactggcgtgaaagagg	caagctcattggacagtttgagg	159
Zona Pellucida	ZPA	catgcggctgagtttggataa	tgccattgatagcatcaacttca	106

Results and Discussion.

Acute toxicity: 4-day exposure of 57-day old larvae to ammonium chloride resulted in a nominal LC_{50} of 13 $mg.L^{-1}$, a NOEC of 5 $mg.L^{-1}$ and LOEC 10$mg.L^{-1}$, corresponding to measured un-ionized ammonia (NH_3) LC_{50} of 147$\mu g.L^{-1}$, a NOEC of 66 $\mu g.L^{-1}$ and LOEC 105 $\mu g.L^{-1}$ (Table 2 and Fig 1).

Table 2. Ammonium chloride toxicity data on 57-d old larval delta smelt (96-h exposure). Calculated and measured ammonia/um concentrations.

	Survival			
	LC50 (mg/L)		NOEC (mg/L)	LOEC (mg/L)
	Estimate	95% C.I.		
Ammonia Nitrogen (Nominal)	13.0	9.3 - 16.5	5	10
Mean Ammonia Nitrogen (measured)	12.0	8.8 - 15.0	5	9
Mean Unionized Ammonia (measured)	0.147	0.109 - 0.182	0.066	0.105

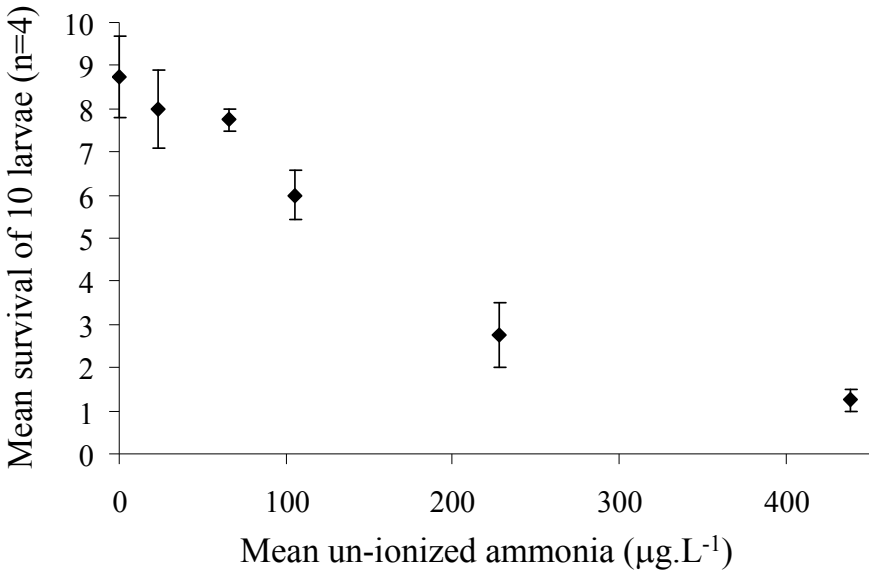

Figure 1. Mean survival (± standard errors) of larval delta smelt exposed to ammonium chloride (96-h exposure). Data expressed as un-ionized ammonia.

Experimental physicochemistry: Temperature, DO and pH remained stable throughout the test duration. Mean data for water temperature, conductivity, DO, pH, ionized and un-ionized ammonia data is shown in table 3.

Table 3. Mean ammonia/um concentrations and physicochemical parameters from 96-h exposure of larval delta smelt to ammonium chloride.

Treatment	Temp (°C)			EC (uS/cm)			DO (mg/L)		
	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
Hatchery Water	16.5	0.5	8	733	-	1	8.9	0.4	8
2.5 mg/L Ammonia	16.5	0.6	4	748	-	1	9.2	0.7	4
5 mg/L Ammonia	16.5	0.8	4	769	-	1	9.2	0.6	4
10 mg/L Ammonia	16.4	0.7	4	789	-	1	9.1	0.7	4
20 mg/L Ammonia	16.5	0.7	4	847	-	1	9.3	0.3	4
40 mg/L Ammonia	16.5	0.6	4	961	-	1	9.4	0.3	4
80 mg/L Ammonia	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*
Hatchery Water	0.11	0.05	5	0.001	0.001	5	7.58	0.13	8
2.5 mg/L Ammonia	1.90	0.03	3	0.023	0.009	3	7.64	0.13	4
5 mg/L Ammonia	5.00	0.00	2	0.066	0.034	2	7.62	0.13	4
10 mg/L Ammonia	9.00	0.00	3	0.105	0.033	3	7.62	0.10	4
20 mg/L Ammonia	17.67	0.58	3	0.228	0.120	3	7.63	0.16	4
40 mg/L Ammonia	36.33	2.08	3	0.439	0.247	3	7.59	0.18	4
80 mg/L Ammonia	72.00	5.66	2	0.526	0.133	2	7.47	0.16	2

Genomic assessments: A wide variety of genes from a number of functional pathways were affected by exposure to ammonium chloride (Fig. 2a), of which genes encoding for membrane bound proteins were prominent (56%) (Fig 2b).

Microarray assessment of identified a number of genes that were predominantly related to membrane integrity, membrane bound proteins responsible for ion transport and ionic exchange. This has previously been reported and is attributed to changes in cellular pH resulting from ammonium gradients (Martinelle and Haggstrom, 1993; Randall and Tsui, 2002; Wicks et al., 2002). Neurological and muscular activity was also affected by exposure to ammonium chloride, suggesting possible effects on swimming performance however this was not assessed in this study. Effects of ammonia on swimming performance has been reported in past studies (McKenzie et al., 2009; Wicks et al., 2002).

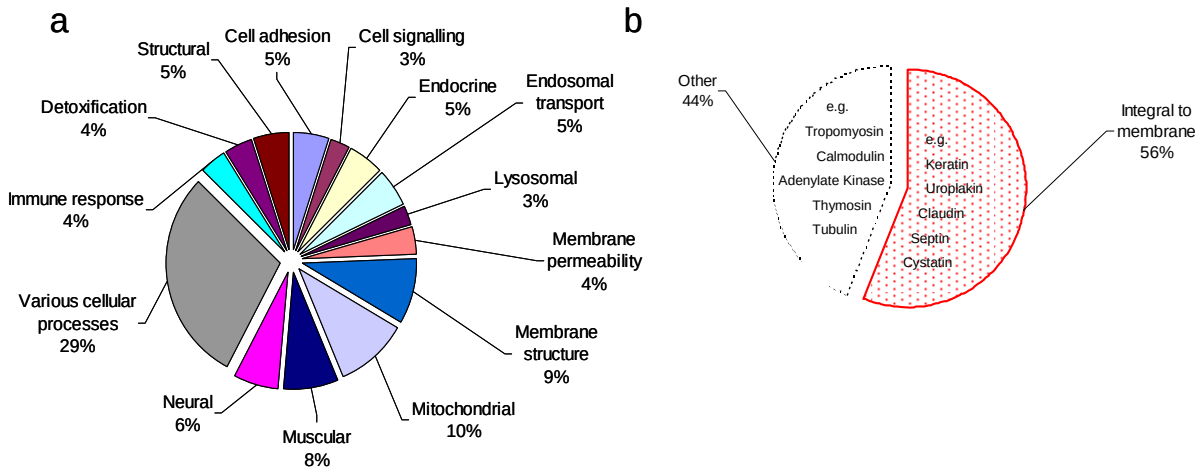

Figure 2. Functional classification of microarray assessed genes responding to 105 µg.L^{-1} ammonium chloride (a) and percentage of genes encoding for membrane proteins (b).

Interestingly, the number, and significance level of genes responding at the assessed concentration (105 µg NH_3 L^{-1}) was low, however the levels of gene expression at this concentration were supported by quantitative PCR assessments. Dose responses of gene expression assessed using qPCR and clustered based on Pearson's correlations of profile similarity, are shown in Figure 3. Responses were predominantly biphasic, 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 (Heckmann et al., 2008; Korsloot et al., 2004) and have been postulated to be indicative thresholds of compensatory responses, or tolerance to exposure. The biphasic responses, measured by quantitative PCR, correspond with NOEC and LOEC determined in this study. From a sublethal perspective, that is concentrations at and below NOEC, there is a predominant upregulation of genes concerning membrane proteins (cluster 1), neuromuscular activity (cluster 1 and 2), immune response and digestion (cluster 2), calcium regulation (cluster 4) and of particular interest is Tubulin Cofactor Beta (cluster 5), which has been reported to control directional growth and development of nerve axons (Grynberg et al., 2003; Lopez-Fanarraga et al., 2007). This gene was significantly upregulated in a dose response manner, beyond the biphasic response observed in other gene candidates. Neuromuscular related genes in cluster 3 are highly variable in response, but display an overall downregulation trend.

Effects upon neurological and muscular activity were supported by quantitative PCR assessments. Other studies on larval delta smelt exposed to ammonium chloride, as yet unpublished, have resulted in a decrease in swimming activity. Though not conclusive, the differential responses in creatine kinase, SERCa ATPase and Aspartoacylase could be indicative of swimming performance thresholds. To corroborate this, studies combining genomic assessments and swimming performance would need to be conducted on the same set of organisms. Titin and Tropomyosin were also affected by ammonium chloride exposure, indicating likely effects on muscle structure and development.

Genomic responses at sublethal concentrations of ammonium chloride indicate that membrane systems are being affected by exposure, affecting overall osmoregulation capacity. The biphasic response, observed primarily at 105 μg NH_3 L^{-1} could indicate a threshold beyond which organisms can no longer compensate for exposure.

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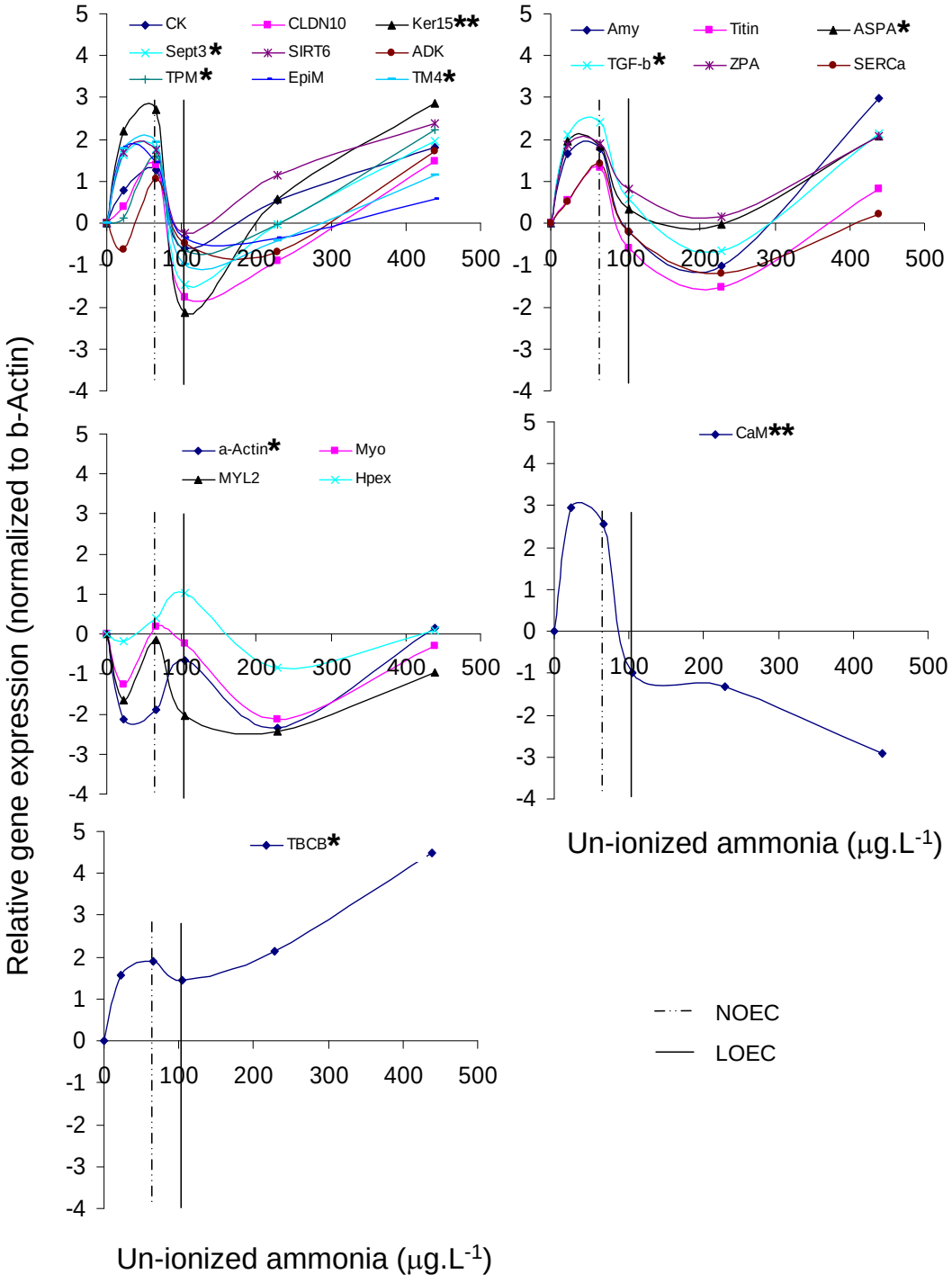
Figure 3. Pearson Correlation cluster analysis of quantitative PCR assessed genes responding in larval delta smelt to 96-h ammonium chloride exposure. Significance levels are displayed for NOEC at 66 µg NH_3 L^{-1} (* = p<0.05 and ** = p<0.01).



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