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MOLECULAR AND CELLULAR EFFECTS OF CONTAMINATION IN AQUATIC ECOSYSTEMS

Chronic exposures to low and high concentrations of ibuprofen elicit different gene response patterns in a euryhaline fish

Ken M. Jeffries · Susanne M. Brander · Monica T. Britton · Nann A. Fangue · Richard E. Connon

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Abstract Ibuprofen is one of the most commonly detected pharmaceuticals in wastewater effluent; however, the effects of ibuprofen on aquatic organisms are poorly understood. This study presents the transcriptome-wide response of the inland silverside, *Menidia beryllina*, to chronic exposure to ibuprofen. At the lowest exposure concentration (0.0115 mg/L), we detected a downregulation of many genes involved in skeletal development, aerobic respiration, and immune function. At the highest exposure concentration (1.15 mg/L), we detected increased expression of regulatory genes in the arachidonic acid metabolism pathway and several immune genes involved in an inflammatory response. Additionally, there was differential expression of genes involved in oxidative stress responses and a downregulation of genes involved in osmoregulation. This study provides useful information for monitoring the effects of this common wastewater effluent contaminant in the environment and for the generation of biomarkers of exposure to ibuprofen that may be transferable to other fish species.

Keywords Inland silverside · NSAIDs · Transcriptomics · Microarray · Gene expression · qPCR · Ecotoxicology

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K. M. Jeffries (✉) · R. E. Connon
Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
e-mail: kenmjeffries@gmail.com

K. M. Jeffries · N. A. Fangue
Wildlife, Fish and Conservation Biology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

S. M. Brander
Biology and Marine Biology, University of North Carolina, Wilmington, 601 South College Road, Wilmington, NC 28403, USA

M. T. Britton
Bioinformatics Core Facility, Genome Center, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

Introduction

Municipal wastewater effluents are major point sources of numerous industrial contaminants, personal care products, hormones, and pharmaceuticals to aquatic systems (Kolpin et al. 2002; Metcalfe et al. 2003; Fernandez et al. 2007; Jeffries et al. 2010; reviewed in Brander 2013; Yu et al. 2013). Many of the compounds introduced to the environment via wastewater effluent have unknown or poorly understood consequences on aquatic organisms. Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of widely used pharmaceuticals that are routinely detected in wastewater effluent (ng/L to μg/L range; Metcalfe et al. 2003; reviewed in Fent et al. 2006; Yu et al. 2013) and receiving waters (pg/L to ng/L range; Kolpin et al. 2002; Brozinski et al. 2013), with detections reaching up to 5 μg/L at sites downstream from wastewater effluent discharge points (Ashton et al. 2004). Ibuprofen is one of the most commonly detected NSAIDs in the environment due to its high consumption (reviewed in Fent et al. 2006) and because much of the dose is unmetabolized (Pounds et al. 2008). Ibuprofen is not very persistent in the environment (Buser et al. 1999); however, its continual reintroduction into aquatic systems allows it to exist in a "pseudo-persistent" state. The concentrations of ibuprofen in water samples may be misleading as unmetabolized ibuprofen levels in wild fish plasma and bile samples can be 100 to 1000 times higher, respectively, than those found in surrounding water samples (Brozinski et al. 2013). Therefore, understanding how aquatic organisms respond to chronic exposure to low

concentrations of ibuprofen in systems that receive wastewater effluent is critical for determining the consequences of ibuprofen in the environment.

Ibuprofen is a nonselective cyclooxygenase (COX) inhibitor that causes decreased production of prostaglandins, a group of compounds derived from arachidonic acid metabolism that contribute to inflammation, to produce the desired anti-inflammatory effect. The COX enzymes involved in prostaglandin synthesis, PTGS1 and PTGS2 (prostaglandin G H synthase 1 and 2, respectively; PTGS2 is in vertebrates only), also known as COX1 and COX2, are relatively evolutionarily conserved, and therefore, the effects of ibuprofen exposure have been detected in many diverse aquatic organisms, such as cladocerans (Heckmann et al. 2008; Han et al. 2010), molluscs (Pounds et al. 2008; Milan et al. 2013), and several fish species (David and Pancharatna 2009; Gravel et al. 2009; Bhandari and Venables 2011; Nallani et al. 2011; Morthorst et al. 2013; Ji et al. 2013; Islas-Flores et al. 2014). Consequently, understanding the effects of ibuprofen on aquatic life has gained significant research attention in recent years. Ibuprofen has been shown to reduce prostaglandin levels in fishes [e.g., bluntnose minnow (*Pimephales notatus*; Bhandari and Venables 2011) and zebrafish (*Danio rerio*; Morthorst et al. 2013)]. Prostaglandins also influence steroid synthesis in fishes and act as pheromones (reviewed in Munakata and Kobayashi 2010). Therefore, a reduction in prostaglandin production as a result of exposure to ibuprofen can affect the synthesis of hormones involved in reproduction (Ji et al. 2013); however, this may be dose- and species-dependent (Morthorst et al. 2013). An ibuprofen-induced reduction in reproductive hormones has been linked with embryonic developmental issues and delayed hatching, as well as reduced egg production in spawning adult zebrafish (David and Pancharatna 2009; Ji et al. 2013) and decreased numbers of spawning events in medaka (*Oryzias latipes*; Flippin et al. 2007). Ibuprofen exposure may also influence the synthesis of cortisol, a multipurpose hormone involved in many physiological processes that include stress responses and salinity tolerance. An ibuprofen-induced alteration in cortisol production was hypothesized to have contributed to an inhibition of N^+/K^+-ATPase activity in rainbow trout (*Oncorhynchus mykiss*; Gravel et al. 2009), an effect that could lead to reduced salinity tolerance in fishes. Although studies exist that demonstrate the effects of ibuprofen exposure on reproduction and osmoregulation in fishes, to our knowledge, a transcriptome-wide assessment on the effects of ibuprofen on fishes is lacking.

The goal of the present study was to examine the transcriptome-wide effects of exposure to ibuprofen on an environmentally relevant nonmodel fish species, the inland silverside (*Menidia beryllina*). Silverside species (*Menidia* spp.) are highly abundant in estuaries throughout North America (Moyle 2002) and have been suggested to be useful bioindicator species in estuaries and coastal systems (Doyle et al. 2011; Brander et al. 2013). The inland silverside is also a commercially available *Menidia* species used in whole effluent toxicity testing by the United States Environmental Protection Agency (http://water.epa.gov/). Although an invasive species in California, USA, the inland silverside habitat overlaps with many native pelagic fishes that have been listed as threatened or endangered (Moyle 2002), such as the critically endangered delta smelt (*Hypomesus transpacificus*). Therefore, the inland silverside may be a particularly useful, environmentally relevant bioindicator species of exposure to contaminants in California surface waters.

Genomics tools are valuable for identifying cellular responses in toxicological studies (Ankley et al. 2006; Villeneuve et al. 2011; Connon et al. 2012) and are increasingly being used in the study of nonmodel fishes (e.g., Connon et al. 2009, 2011; Jeffries et al. 2012, 2014). We developed a *M. beryllina* 4x44K probe oligonucleotide microarray and used it to examine the chronic effects of ibuprofen in an environmentally relevant fish species in North America. Ibuprofen is regularly detected in aquatic systems and has recently been identified as a “contaminant of emerging concern” in California (Maruya et al. 2014). We also examined the expression of specific genes involved in osmoregulatory function and prostaglandin synthesis, known to be affected by exposure to ibuprofen, to further identify some of the cellular responses in inland silversides. To the best of our knowledge, this is the first transcriptome-wide assessment of the effects of ibuprofen on a nonmodel fish.

Materials and methods

Ibuprofen exposures

Inland silversides were ordered from Aquatic Biosystems (Ft. Collins, CO, USA) and were allowed 4 days to acclimatize to holding conditions at the Aquatic Toxicology Laboratory at the University of California Davis. Fish were fed ad libitum with *Artemia franciscana* throughout the acclimatization and exposure period. Acute toxicity tests were conducted over 96-h exposures with nine nominal exposure concentrations ranging from 1.2 to 300 mg/L ibuprofen, along with a control. The actual exposure concentration was measured for two of the exposures, and the effective concentration was then estimated for the other treatment groups and used to determine the 10, 50, and 90 % lethal concentrations (LCs) for *M. beryllina*. Analytical chemistry resulted in a recovery of 56 %; thus, concentrations are presented as measured values. Reduced ibuprofen recovery, due to adsorption to glass, has previously been reported (Nallani et al. 2011). A nonlinear regression curve was fitted to the cumulative mortality data using a Weibull distribution, and the fit was confirmed by a goodness

of fit test. A three-parameter regression model was fitted assuming a lower limit of 0.

Transcriptome profiling was used to assess the chronic effects of ibuprofen exposure. For this, another subset of fish was exposed for 14 days to nominal concentrations of 0.025, 0.25, and 2.5 mg/L (which will subsequently be referred to as the low, medium, and high concentrations, respectively), along with a control group handled identically to the other treatments. The highest concentration was analyzed for the actual ibuprofen level, and recovery was 46 % of the 2.5-mg/L nominal concentration. Therefore, the measured concentration was 1.15 mg/L for the high treatment, followed by effective concentrations for the low and medium treatments estimated to be 0.0115 and 0.115 mg/L, respectively. At the termination of the chronic 14-day exposure period, fish were sacrificed by a lethal dose of tricaine methanesulfonate (MS-222), and whole fish were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

For both the acute (n=10 fish/container) and chronic exposures (n=8 fish/container), there were four replicate 2-L containers for each treatment. All exposures were conducted in 1.5 L of freshwater on fish that were 66 days posthatch (dph) at the initiation of the experiment. Water was changed (75 % renewal), and the nominal exposure concentrations were renewed daily. Tanks were monitored for temperature (19.6 °C±SD 0.9), electrical conductivity (729.4 μS/cm±SD 21.5), dissolved oxygen (8.84 mg/L±SD 0.4), total ammonia as nitrogen (0.23 mg/L±SD 0.07), and pH (7.58±SD 0.3). There were no differences in these parameters between the treatment tanks. This research was approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC #16845).

Microarray development

We generated the data to construct an oligonucleotide microarray (Agilent technologies) using RNA from tissues and whole body homogenates from laboratory-reared *M. beryllina* (Aquatic Biosystems) that were exposed to a number of physiological (temperature, salinity, handling) and chemical stressors (ibuprofen, bifenthrin), as well as wild-caught individuals, in order to capture a diverse suite of mRNA transcripts. Total RNA was extracted using an RNeasy kit (Qiagen) with on-column DNAse digestion following manufacturer's guidelines. Total RNA integrity was verified using an Agilent Technologies 2100 Bioanalyzer that determined that the samples were high quality [RNA integrity numbers (RIN) values >9]. Samples were pooled to represent an equal proportion of RNA per tissue and whole organisms (larvae/juveniles) to normalize and maximize the identification of rare transcripts. Transcriptome sequencing was conducted at the University of California, Davis Genome Center DNA Technologies Core Facility. A library was constructed using a TruSeq RNA Sample Prep kit (Illumina) with poly-A selection. The library was sequenced on an Illumina HiSeq2000 sequencer, using a 100-bp paired-end read protocol, to generate 183 million pairs of reads.

Sequencing data were processed in collaboration with the University of California, Davis Genome Center Bioinformatics Core Facility. Raw reads were trimmed to remove adapter contamination and low-quality sequences using Scythe and Sickle (https://github.com/ucdavis-bioinformatics). The trimmed reads were used for a de novo transcriptome assembly with Trinity version r2013-02-16 (Grabherr et al. 2011; Haas et al. 2013), which included read normalization, open reading frame (ORF) predition, and isoform abundance filtering steps. Annotation was conducted by Basic Local Alignment Search Tool (BLAST) comparisons of all contigs against a custom subset of the nr database representing all teleost species using ncbi-blast-2.2.28+ (Altschul et al. 1997). The BLAST results were consolidated, and Gene Ontology (GO) terms were assigned using Blast2GO (Conesa et al. 2005). Additional BLASTs were made between the *M. beryllina* transcriptome assembly and the Ensembl protein sets for medaka and zebrafish. Custom microarrays GE 4x44K, designed utilizing Agilent specific software (https://earray.chem.agilent.com/earray/), consisted of 14,393 unique *M. beryllina* genes and nonspecific Agilent control probes in triplicate. Each gene was printed with three different overlapping probes.

RNA extraction and microarray analysis

Whole fish from the chronic 14-day exposures were homogenized using a pestle in 1 mL of Trizol reagent following manufacturer's protocols. Concentration and purity (260/280, 260/230) were assessed using a Nanodrop spectrophotometer (Thermo Scientific), and RNA integrity was determined through electrophoresis. Two hundred nanograms of total RNA was amplified and labeled with Cy3 using the Low Input Quick Amp Labeling kit (Agilent Technologies). A total of 24 single color microarray hybridizations (6 fish per treatment) were performed with the custom-designed 4x44K oligonucleotide microarrays using Agilent Gene Expression Hybridization Kits (Agilent Technologies). To minimize technical artefacts, labeling reactions were performed simultaneously, and individuals from each treatment were randomized between slides. Prior to hybridization, 1.65 μg of dye-labeled complementary RNA for each sample was combined with 2.2 μL of 25× fragmentation buffer, 11 μL of 10× Gene Expression Blocking Agent, and nuclease-free water to bring the final volume to 55 μL. The fragmentation mix was incubated at 60 °C for 30 min, cooled on ice for 1 min, and the fragmentation was stopped by adding 55 μL of 2× Hi-RPM hybridization buffer. Samples were centrifuged for 1 min, placed on ice, and 100 μL of the mix was loaded onto arrays,

and each microarray slide was placed in a single hybridization chamber. Samples were hybridized to the arrays for 17 h at 65 °C, followed by a wash with Gene Expression Wash Buffer 1 at room temperature and a wash with Gene Expression Wash Buffer 2 heated to 37 °C. Fluorescent images were scanned using an Axon GenePix 4000B Scanner and the analysis software GenePix Pro (Molecular Devices, LLC, Sunnyvale, CA). The images were quantified using Feature Extraction 11.5.1.1 (Agilent Technologies).

Normalization of the microarray data and statistical analysis was performed in Genespring v12.6 (Agilent Technologies). Data was quantile-normalized and $\log_2$-transformed prior to statistical analysis. Probes that had a raw signal less than 80 (~2 times the intensity of the background) were filtered out of the statistical analysis to eliminate sources of background noise, but still retain probes with low intensity (Sifakis et al. 2012). After the filtering step, there were 8789 probes remaining to be used for the statistical comparisons. Differences in the expression of probes between treatment groups were assessed by ANOVA followed by Student-Newman-Keuls (SNK) post hoc tests at α=0.01. It is important to note that there are three different overlapping probes for the same gene that were treated as separate genes in the statistical analyses. Therefore, multiple copies of each gene that were significant in our analyses were used to validate the results and identify true patterns from background noise in the data. The microarray data have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE64967.

Functional analysis was performed using Blast2GO. Due to a relatively low number of significant genes detected in the ANOVA, functional enrichment analysis was performed on significant gene lists generated by separate *t* tests between each exposure treatment and the control group (α=0.05). Redundancies in the GO term lists were reduced using ReVigo (Supek et al. 2011). Functional groups were considered significantly enriched in gene lists using Fisher exact tests at a FDR corrected α=0.001.

cDNA synthesis and qPCR

We selected 28 genes to examine responses in greater scrutiny by qPCR (Table 1; *n*=20 per treatment that included the same individuals from the microarray analysis), for microarray validation, and to examine the changes in osmoregulatory function and arachidonic acid metabolism expected to be affected by exposure to ibuprofen. Primers were designed using the Universal Probe Library Assay Design Center (Roche). Primer sequences and Roche probe numbers are available in Table 1.

One microgram of total RNA was used for cDNA synthesis. For each sample, nuclease-free water was added to bring the total volume up to 12 μL followed by the addition of 2 μL of gDNA wipeout buffer (Qiagen) and an incubation at 42 °C for 2 min. Each cDNA synthesis reaction consisted of 4-μL 5× Quantiscript RT Buffer, 1-μL RT Primer Mix, 1-μL Quantiscript RT (Qiagen), and 14 μL of diluted sample for a total reaction volume of 20 μL. Reactions were carried out at 42 °C for 30 min, followed by 95 °C for 3 min, and an end hold at 4 °C. The cDNA was stored at −20 °C until qPCR analysis.

A 1:6 dilution of cDNA was used as a template in the qPCR reactions. Assays were performed on an automated fluorometer (ABI HT 7900 A FAST Sequence Detection System, Applied Biosystems) in 384-well plates using 12-μL reaction volumes composed of 6 μL of PCR Master Mix (Qiagen), 0.6-μL primer probe mix (forward primer, reverse primer, the appropriate fluorescent probe, and nuclease-free water), 2.4 μL of nuclease-free water, and 3 μL of diluted cDNA. Cycling 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, and amplifications were analyzed using *SDS 2.4* software (Applied Biosystems). Relative expression of target genes was determined using the $2^{-\Delta\Delta Ct}$ method and are presented as relative fold change (Livak and Schmittgen 2001). Target gene expression was normalized to four reference genes: beta-actin (actin, cytoplasmic; ACTB), elongation factor 1-alpha-like (EEF1A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 60s ribosomal protein l7 (RPL7). Stability of the reference genes across experimental treatments was assessed using the program geNorm (Vandesompele et al. 2002). Statistical differences were determined via two different approaches. First, individual treatments were compared using one-factor ANOVAs on $\log_2$-scaled ΔΔCt's, followed by SNK post hoc tests at α=0.05. We then fit dose-response curves using maximum likelihood estimates, similar to previous toxicological studies (Brander et al. 2009; Brander et al. 2012) that fit two different curves to each gene's dataset: a sigmoidal curve (four parameter logistic model) and a nonmonotonic curve (a modified four parameter logistic model; Brain and Cousens 1989). We used a likelihood ratio test (LRT) with a significance level of α=0.05 (Bolker 2008) to test whether each model fit significantly better than a flat line (i.e., no dose-response) and whether the nonmonotonic curve afforded a significantly better fit than the sigmoidal curve. Curves were fit by maximum likelihood using the mle2 algorithm (Bolker 2008) in R 3.1.1 (R Core Development Team 2010). All curves were fit to $\log_{10}(x+0.025)$ transformed concentration data to avoid taking the log of concentration 0 (control).

Results

The LC10, LC50, and LC90 of ibuprofen over 96-h exposures were estimated to be 17.0 (SE±0.59), 34.35 (SE±0.40), and 53.77 mg/L (SE±1.06), respectively, for *M. beryllina*. There was no mortality observed at any of the ibuprofen concentrations in the 14-day chronic exposures used in the transcriptomic assessments.

Table 1 Primer sequences and efficiencies for the genes selected for quantitative real-time polymerase chain reaction (qPCR)

Function	Symbol	Name	Amplicon length (nt)	Roche probe #	Efficiency (%)	Primer sequence
Apoptosis	BMF	bcl-2-modifying factor-like	60	78	102.1	F: tggaggcttgcattggaca R: gaaggtgttcccggtggaat
Apoptosis	CASP3	caspase-3 precursor	68	34	109.1	F: gggcaagtttcggagaggat R: agtagtagcctggagtggtggagt
Apoptosis	PSMA7	proteasome subunit alpha type-7	72	21	103.2	F: agcagatgcccgtattgtgat R: actgggtcctcaactgttagcc
Immune Function	CCL28	c-c motif chemokine 28 precursor	65	151	101.4	F: gctctggccatcaccttcac R: aaattaacgggccttatgctga
Immune Function	MMP9	matrix metalloproteinase 9	65	123	98.6	F: caaccagcagctttgacagtg R: tccgattacagcagtgtcacg
Immune function	STAT1	signal transducer and activator of transcription 1	60	7	107.1	F: cggctccatcatgggct R: gggcacttatcgctcagcag
Metabolism	ACAD11	acyl- dehydrogenase family member 11	65	24	98.9	F: gaggcacgtgtgatagcagtgt R: ggccaggtctatgaatgggtt
Metabolism	ARG	arginase- mitochondrial-like	63	80	107	F: actttggcgacctgagcttc R: ggggaaacttcacgtccatgt
Metabolism	COI	cytochrome c oxidase subunit mitochondrial-like	84	94	104.8	F: actggcctccagatgttactcc R: tcgggcttgctgaagtaagtg
Metabolism	UQCRB	cytochrome b-c1 complex subunit 7	72	81	102	F: gagggcacttacaacgacagg R: gggaggacctgctgcttca
Oxidative stress	CP	ceruloplasmin precursor	61	71	99.9	F: actgctgaaatggtggctgac R: agatggtcattgacggtgcat
Oxidative stress	CYBA	cytochrome b-245 light chain-like	65	85	105.7	F: cacgacctcctgcggattat R: catagggttgtcgtaggctgct
Oxidative stress	GR	glutathione reductase	72	4	99.9	F: ctccaatgtatcacgccatcac R: ttctcctctttgccaacacaca
Structural	C1QTNF5	complement c1q tumor necrosis factor-related protein 5-like	80	52	100.1	F: agtactgaagtttcgcaatgtgct R: gccatccaagggacaagtga
Structural	KRT13	type i cytoskeletal 13-like	63	19	96.7	F: agatgctgctcgacatcaagac R: catccagcagcctcctgtattc
Prostaglandin synthesis	CBR1	carbonyl reductase	63	120	105.1	F: tggccaaagagggtctgaag R: ggtgatgctgctcaggtcgt
Prostaglandin synthesis	HPGD	15-hydroxyprostaglan-din dehydrogenase	68	52	100.2	F: gcatatgaacaggctgactggag R: gggccgatacctgccatag
Prostaglandin synthesis	PTGES3	prostaglandin e synthase 3	61	86	103.7	F: aacagacgacatcaaacaccaga R: ttagagtctttggggtcaatctcc
Prostaglandin synthesis	PTGR1	prostaglandin reductase 1-like	62	39	101.4	F: tttgggagaatagctgtttgtgg R: ggccctgtttggggtgtagtat
Prostaglandin synthesis	PTGS1	prostaglandin g h synthase 1-like	60	10	100.7	F: aaatatcagatgataaacggcgaga R: caccatgtggacaggaacctc
Prostaglandin synthesis	PTGS2	prostaglandin g h synthase 2-like	60	22	94.5	F: ggtgactgagcacggcataag R: cccgtcctgcaatctgctt
Leukotriene synthesis	ALOX5AP	arachidonate 5-lipoxygenase-activating protein	61	62	103.3	F: gatgtcctctgtgggcatcttt R: gtactctttgtagtcgctgccgta
Leukotriene synthesis	LTA4H	leukotriene a-4 hydrolase	65	65	98.6	F: cgtgatcagcgggaaggt R: caaagtcagggcggagagg
Osmoregulation	ATP1A1		60	33	100.4	F: tgatctggtggaggtgaaagg

Table 1 (continued)

Function	Symbol	Name	Amplicon length (nt)	Roche probe #	Efficiency (%)	Primer sequence
		sodium potassium-transporting atpase alpha-1 subunit				R: tgggcggagatgattctca
Osmoregulation	ATP1A1b	sodium potassium-transporting ATPase alpha 1b subunit	65	76	99.9	F: ggttatcatggtgacgggtga R: agatgatacccacgcctttagc
Osmoregulation	ATP1A2	sodium potassium-transporting atpase subunit alpha-2-like	64	71	105.5	F: gccaacgacaacctgtacctg R: ggagaaacagccggtgatgat
Osmoregulation	ATP1A3	sodium potassium-transporting atpase subunit alpha-3-like	61	49	101	F: aagtgcagatcaacgctgaaga R: ccggtctcctcccttcactt
Osmoregulation	ATNB233	sodium potassium-transporting atpase subunit beta-233	64	162	96.8	F: ggccctacagttcgtcaacct R: ccgccgtaaactttgcactc
Reference gene	ACTB	beta-actin (actin, cytoplasmic)	64	11	95.4	F: gcaatgagaggttccgttgc R: cgcaggactccataccaagg
Reference gene	EEF1A1	elongation factor 1-alpha-like	88	65	95.7	F: catcgcctgcaagttcagc R: cccagacttcagcgcctt
Reference gene	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	67	38	98.8	F: cagtggtggacctgacatgc R: ttcttgacggcgtccttgat
Reference gene	RPL7	60s ribosomal protein l7	76	31	95.1	F: aacttcttgtggccgttcaag R: tcgcctccctccacaaagt

Microarray and functional analysis results

At the highest exposure concentration, genes associated with functional categories involved in proteolysis (e.g., catalytic activity), metabolism (e.g., metabolic process), and cell structure (e.g., intracellular part) were differentially expressed in the 387 significant gene list (665 microarray probes; *t* test, $P<0.05$; Table 2). Many genes associated with aerobic respiration and protein synthesis [e.g., oxidative phosphorylation, respiratory electron transport chain, and NADH dehydrogenase (ubiquinone) activity; ribonucleoprotein complex biogenesis, ribosome, and translation] were differentially expressed in the 481 and 525 (828 and 913 microarray probes) significant gene lists (*t* test, $P<0.05$) for the medium and low concentration treatments, respectively. Similar to the high treatment, genes associated with cell structure (e.g., structural molecule activity) were differentially expressed at the lowest exposure concentration.

Overall responses to the ibuprofen exposures were relatively subtle, as only 57 different genes (96 microarray probes; see Table 3) were statistically significant across all treatments (ANOVA, significant at $P<0.01$; Fig. 1). The relative fold change differences between each treatment group and the control for the 96 probes are presented in Table S1. There were two distinct responses occurring as 59.4 % of the genes significantly differing between the high exposure concentration, and control groups were upregulated, compared with only 6.7 and 8.0 % significantly upregulated in the medium and low exposure concentration groups, respectively.

There were ten genes significantly downregulated compared with the control group that were common between all three treatment groups (Fig. 2). These included genes involved in apoptosis [caspase-3 precursor (CASP3)], an immune response [c-c motif chemokine 28 precursor (CCL28)], and cell proliferation [proline-rich protein 15-like protein a-like (PRR15LA). There was a downregulation of transcriptional enhancer factor tef-5-like (TEAD3), also involved in cell proliferation; however, this was not significant in the medium exposure group. There was also a downregulation of genes associated with cellular structural components [complement c1q tumor necrosis factor-related protein 5-like (C1QTNF5), type i enveloping like (CYT1L), type i cytoskeletal 13-like (KRT13), and muscle m-line assembly protein unc-89-like (UNC-89)], lipid metabolism [sphingomyelin phosphodiesterase 3-like (SMPD3)] and cell adhesion [fermitin family homolog 1-like (FERMT1)] at all exposure concentrations. Last, there was a downregulation of a gene involved in ion transport [zinc transporter zip14-like (SLC39A14)].

Differentially expressed genes at the high exposure concentration

There were several genes that were upregulated at the highest exposure concentration (i.e., 1.15 mg/L) involved in

Table 2 Gene ontology (GO) terms that were enriched at FDR corrected $P<0.001$ in the significant gene lists generated from t tests ($P<0.05$) between each ibuprofen concentration treatment and the control group

Treatment	GO category	GO ID	Go term	Corrected *P* value
High	Biological Process	GO:0008152	metabolic process	9.73E-04
High	Cellular Component	GO:0044424	intracellular part	9.73E-04
High	Molecular Function	GO:0003824	catalytic activity	9.73E-04
Medium	Biological Process	GO:0022904	respiratory electron transport chain	4.09E-07
Medium	Biological Process	GO:0022613	ribonucleoprotein complex biogenesis	1.94E-06
Medium	Biological Process	GO:0042254	ribosome biogenesis	3.40E-06
Medium	Biological Process	GO:0006818	hydrogen transport	1.76E-04
Medium	Biological Process	GO:0015992	proton transport	1.76E-04
Medium	Biological Process	GO:0006412	translation	3.47E-04
Medium	Cellular Component	GO:0005840	ribosome	3.40E-06
Medium	Cellular Component	GO:0030529	ribonucleoprotein complex	1.11E-05
Medium	Molecular Function	GO:0003735	structural constituent of ribosome	1.94E-06
Medium	Molecular Function	GO:0005198	structural molecule activity	9.99E-06
Medium	Molecular Function	GO:0008137	NADH dehydrogenase (ubiquinone) activity	5.89E-04
Low	Biological Process	GO:0006119	oxidative phosphorylation	3.57E-04
Low	Biological Process	GO:0022613	ribonucleoprotein complex biogenesis	4.53E-04
Low	Cellular Component	GO:0030529	ribonucleoprotein complex	5.63E-04
Low	Cellular Component	GO:0005840	ribosome	6.10E-04
Low	Molecular Function	GO:0005198	structural molecule activity	5.39E-04

High, medium, low treatments refer to the 1.15, 0.115, and 0.0115 mg/L ibuprofen exposure concentrations, respectively

extracellular matrix and protein breakdown [matrix metalloproteinase 9 (MMP9; also involved in an inflammatory response), proteasome subunit alpha type-7 (PSMA7; also associated with an apoptotic response), and 26S protease regulatory subunit 6B (PSMC4)]. There was also an upregulation of several genes involved in cell structure [pdz and lim domain protein 1 (PDLIM1), pleckstrin homology-like domain family a member 2-like (PHLDA2), and von willebrand factor type egf and pentraxin domain-containing protein 1-like (SVEP1)]. This was in contrast to the downregulated genes associated with the cytoskeleton and cell structure (e.g., KRT13, CYT1L, FERMT1, and UNC-89) that were common at all treatment concentrations.

There was a significant upregulation of genes involved in an inflammatory response [signal transducer and activator of transcription 1 (STAT1; however, STAT1 has also been identified as potentially important in bone development in fish; Vieira et al. 2013) and signal transducer and activator of transcription 2 (STAT2)], apoptosis [dna damage-inducible transcript 3 (DDIT3)], RNA processing [thioredoxin-like protein 4a (TXNL4A)], and DNA repair [protein bccip homolog (BCCIP)]. There was also an upregulation of genes involved in fatty acid beta-oxidation [acyl-dehydrogenase family member 11 (ACAD11)], regulating cell signaling pathways [14-3-3 protein beta alpha (YWHAB)], the secretory pathway [transmembrane emp24 domain-containing protein 10 precursor (TMED10)], protein folding [calreticulin precursor (CALR)], and two genes with unknown function [plac8-like protein 1 (PLAC8L1) and SI:DKEY-6 N6.2]. Interestingly, tributyltin binding protein type 1 (TBT-BP1), discovered because of its ability to bind to the marine pollutant tributyltin, may also play a role in osteoblast activity and development in fishes in addition to aiding in detoxification (Satone et al. 2011) and was upregulated at the highest exposure concentration. There was variable expression of genes involved in an oxidative stress response as cytochrome b-245 light chain-like (CYBA), and glutathione reductase (GR) were upregulated while manganese superoxide dismutase (SOD2) was downregulated. There was also decreased expression of thyroid hormone receptor beta (THRb; ANOVA $P<0.05$]; however, this was not significant at the $P<0.01$ threshold.

Differentially expressed genes at the two lower exposure concentrations

There was a downregulation of genes involved in lipid metabolism [fatty acid-binding protein 6 (FABP6; that binds to bile salts and bilirubins) and 5-aminolevulinate mitochondrial-like (ALAS1)], proteolysis [serine protease htra3-like (HTRA3)], translation [40s ribosomal protein s13 (RPS13)], cell proliferation [beta-galactoside-binding lectin (GALECTIN) and non-specific cytotoxic cell receptor protein 1 homolog (NCCRP1)], and with an unknown function [upf0139 membrane protein c19 or f56 homolog (CS056)], which was common at the two lowest exposure concentrations. There was

Table 3 Genes grouped by biological function that were differentially regulated in *Menidia beryllina* following 14-day exposures to ibuprofen as determined by the microarray analysis (57 significant at $P<0.01$; ANOVA)

Function	Symbol	Gene	Specific function	Regulation (relative to control)	Ibuprofen treatment		
					High	Medium	Low
Apoptosis	CASP3	caspase-3 precursor	Activates apoptosis cascade	Down	X	X	X
Apoptosis	DDIT3	dna damage-inducible transcript 3	Apoptosis; Response to endoplasmic reticulum stress	Up	X		
Cell Proliferation	GALECTIN-1	beta-galactoside-binding lectin	May regulate apoptosis, cell proliferation and cell differentiation	Down		X	X
Cell Proliferation	NCCRP1	non-specific cytotoxic cell receptor protein 1 homolog	Promotes cell proliferation	Down		X	X
Cell Proliferation	PPDPF-B	pancreatic progenitor cell differentiation and proliferation factor b	Involved in cell differentiation	Down		X	
Cell Proliferation	PRR15LA	proline-rich protein 15-like protein a-like	May have a role in cell proliferation or differentiation	Down	X	X	X
Cell Proliferation	TEAD3	transcriptional enhancer factor tef-5-like	Hippo signaling pathway	Down	X		X
Cell Proliferation	TXNL4A	thioredoxin-like protein 4a	RNA processing and cell cycle	Up	X		
Cell Signaling	YWHAB	14-3-3 protein beta alpha	Regulates many signaling pathways	Up	X		
DNA Repair	BCCIP	protein bccip homolog	May promote cell cycle arrest and DNA repair	Up	X		
Immune Response	AIMP1	aminoacyl trna synthase complex-interacting multifunctional protein 1-like	Inflammation	Down		X	X
Immune Response	C4BPA	c4b-binding protein alpha chain precursor	Controls complement activation	Up			X
Immune Response	CCL28	c-c motif chemokine 28 precursor	Cellular calcium regulation	Down	X	X	X
Immune Response	GIMAP4	gtpaseimap family member 4-like	GTPase activity; Involved in immunity	Down		X	
Immune Response	STAT1	signal transducer and activator of transcription 1	Inflammation	Up	X		
Immune Response	STAT2	signal transducer and activator of transcription 2	Inflammation	Up	X		
Ion Transport	SLC39A14	zinc transporter zip14-like	Preferentially binds to zinc	Down	X	X	X
Metabolism	ACAD11	acyl- dehydrogenase family member 11	Fatty acid beta-oxidation	Up	X		
Metabolism	ALAS1	5-aminolevulinate mitochondrial-like	Lipid metabolism	Down		X	X
Metabolism	ARG	arginase- mitochondrial-like	Arginine metabolism associated with the urea cycle and nitric oxide biosynthetic process	Down		X	
Metabolism	FABP6	fatty acid-binding protein 6	Lipid metabolism	Down		X	X
Metabolism	NAG1	glucosamine-6-phosphate isomerase	Carbohydrate metabolism	Down		X	
Metabolism	SMPD3	sphingomyelin phosphodiesterase 3-like	Lipid metabolism; mediates many cellular processes	Down	X	X	X
Oxidative Stress	CYBA	cytochrome b-245 light chain-like	Component of membrane-bound oxidase of phagocytes that generates superoxide	Up	X		
Oxidative Stress	GR	glutathione reductase	Cell redox homeostasis	Up	X		
Oxidative Stress	SOD2	manganese superoxide dismutase	Mitochondrial superoxide response	Down	X		
Protein Binding and Transport	CALR	calreticulin precursor	Protein folding; Calcium binding	Up	X		
Protein Binding and Transport	RAB21	ras-related protein rab-21-like	Protein transport; May regulate cell adhesion	Up		X	X
	TBT-BP1	tributyltin binding protein type 1		Up	X		

Table 3 (continued)

Function	Symbol	Gene	Specific function	Regulation (relative to control)	Ibuprofen treatment		
					High	Medium	Low
Protein Binding and Transport			Protein binding; Binds endogenous and exogenous compounds				
Protein Binding and Transport	TMED10	transmembrane emp24 domain-containing protein 10 precursor	Protein trafficking; Secretory pathway	Up	X		
Protein Metabolic Process	AAD-A	alpha-aspartyl dipeptidase	Proteolysis	Down			X
Protein Metabolic Process	COPS6	cop9 signalosome complex subunit 6	Proteosomal degradation; Regulator of the ubiquitin conjugation pathway	Down		X	
Protein Metabolic Process	FAU	ubiquitin-like protein fubi	Proteosomal degradation; Belongs to the ubiquitin family	Down		X	
Protein Metabolic Process	FBXL18	f-box lrr-repeat protein 18-like	Proteosomal degradation; Component of ubiquitin ligase complex	Up		X	
Protein Metabolic Process	HTRA3	serine protease htra3-like	Proteolysis; Cleaves several extracellular matrix proteins	Down		X	X
Protein Metabolic Process	PSMA7	proteasome subunit alpha type-7	Proteosomal degradation; Protein polyubiquination	Up	X		
Protein Metabolic Process	PSMC4	26S protease regulatory subunit 6B	Proteosomal degradation	Up	X		
Protein Metabolic Process	USP42	ubiquitin carboxyl-terminal hydrolase 42-like	Proteosomal degradation; Deubiquitinating enzyme	Down		X	
Protein Synthesis	CNOT6	ccr4-not transcription complex subunit 6-like	Transcription	Down		X	
Protein Synthesis	EEF1D	elongation factor 1-delta	Translation	Down		X	
Protein Synthesis	NOA1	nitric oxide-associated protein 1-like	Regulates mitochondrial protein translation	Down			X
Protein Synthesis	NR0B2	nuclear receptor subfamily 0 group b member 2	Transcription	Down			X
Protein Synthesis	RPS13	40s ribosomal protein s13	Translation	Down		X	X
Protein Synthesis	RPS16	40s ribosomal protein s16	Translation	Down		X	
Structural	APP1	protein app1-like	Cytoskeleton	Down			X
Structural	C1QTNF5	complement c1q tumor necrosis factor-related protein 5-like	Collagen	Down	X	X	X
Structural	CYT1L	type i enveloping like	Cytoskeleton	Down	X	X	X
Structural	FERMT1	fermitin family homolog 1-like	Cell adhesion	Down	X	X	X
Structural	KRT13	type i cytoskeletal 13-like	Cytoskeleton	Down	X	X	X
Structural	MMP9	matrix metalloproteinase 9	Gelatinase	Up	X		
Structural	PDLIM1	pdz and lim domain protein 1	Cytoskeleton	Up	X		
Structural	PHLDA2	pleckstrin homology-like domain family a member 2-like	Binds phosphoinositides; Associated with cell membranes	Up	X		
Structural	SVEP1	von willebrand factor type egf and pentraxin domain-containing protein 1-like	Cell adhesion	Up	X		
Structural	UNC-89	muscle m-line assembly protein unc-89-like	Structural component of muscle	Down	X	X	X

Table 3 (continued)

Function	Symbol	Gene	Specific function	Regulation (relative to control)	Ibuprofen treatment		
					High	Medium	Low
Uknown	CS056	upf0139 membrane protein c19orf56 homolog		Down		X	X
Uknown	PLAC8L1	plac8-like protein 1		Up	X		
Uknown	SI:DKEY-6 N6.2	Unknown		Down	X		

The exposure concentration where the gene was differentially expressed from the control is indicated with an x. High, medium, low treatments refer to the 1.15, 0.115, and 0.0115 mg/L ibuprofen exposure concentrations, respectively

also a downregulation of a nonenzymatic cofactor of the multisynthase complex, aminoacyl trna synthase complex-interacting multifunctional protein 1-like (AIMP1), which can act as a cytokine in an immune response, however can also interact with components of the cytoskeleton (Jackson et al. 2011). The only gene that was upregulated in both the medium and low concentration exposures was ras-related protein rab-21-like (RAB21).

Differentially expressed genes at the medium exposure concentration

Unique to the medium exposure treatment was the upregulation of f-box lrr-repeat protein 18-like (FBXL18). However, there was a downregulation of several other genes associated with ubiquitin [cop9 signalosome complex subunit 6 (COPS6), ubiquitin-like protein fubi (FAU), and ubiquitin carboxyl-terminal hydrolase 42-like (USP42)]. Collectively, these results suggest that proteasomal breakdown was affected by exposure to ibuprofen. There was also a downregulation of genes involved in transcription and translation [ccr4-not transcription complex subunit 6-like (CNOT6), elongation factor 1-delta (EEF1D), 40s ribosomal protein s16 (RPS16)], metabolism [arginase- mitochondrial-like (ARG) and glucosamine-6-phosphate isomerase (NAG1)], immune function [gtpaseimap family member 4-like (GIMAP4)], and cell proliferation [pancreatic progenitor cell differentiation and proliferation factor b (PPDPF-B)].

Differentially expressed genes at the low exposure concentration

Unique to the low exposure concentration was the upregulation of the immune response gene c4b-binding protein alpha chain precursor (C4BPA). As in the responses detected at the other concentrations, there was a downregulation of genes involved in proteolysis [alpha-aspartyl dipeptidase (AAD-A)], transcription and translation [nitric oxide-associated protein 1-like (NOA1, which is also associated with mitochondria-mediated cell death) and nuclear receptor subfamily 0 group b member 2 (NR0B2) that inhibits receptor-dependent signaling pathways], and the cytoskeleton [protein app1-like (APP1)].

Quantitative PCR results

Several qPCR assays were designed to examine responses to ibuprofen exposure of genes specifically associated with general stress and metabolism, arachidonic acid metabolism (prostaglandin and leukotriene synthesis), and osmoregulatory function. Among the genes assayed, most were significant only at the highest exposure concentration in the ANOVA tests; however, several additional genes had a

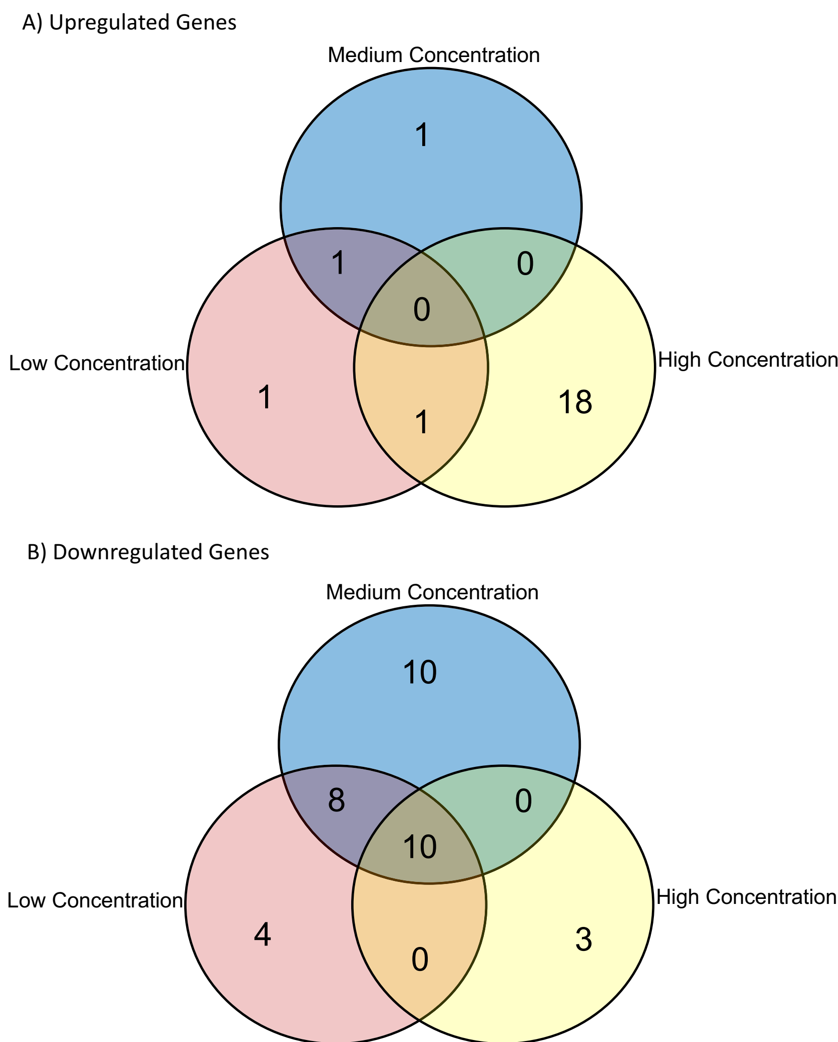

Fig. 1 Venn diagram of **a** upregulated and **b** downregulated genes that differed significantly from the control group in *M. beryllina* exposed to different concentrations of ibuprofen as determined by a one-factor ANOVA (P<0.01). High, medium, low treatments refer to the 1.15, 0.115, and 0.0115 mg/L ibuprofen exposure concentrations, respectively

significant sigmoidal dose-response when curves were fit using an LRT indicating that these genes respond in a dose-dependent manner. Although both sigmoidal and nonmonotonic dose-response curves were fit for each gene, the sigmoidal curve was the best fit for all (Supplemental Table 2). We report P values from the ANOVA and also from the LRTs used to fit dose-response curves.

There was a significant upregulation of MMP9 (ANOVA, P<0.00001; LRT, P<0.0001), a pattern detected in both the qPCR and microarray results (Fig. 3). Also associated with an immune response was a downregulation of CCL28 at the highest exposure concentration (ANOVA, P<0.01; LRT, P<0.01), similar to the pattern detected in the microarray. We also detected an upregulation of STAT1 in the microarray results; however, this pattern was not evident in the qPCR assay. We confirmed a significant downregulation of C1QTNF5 (detected in both the qPCR and microarray analysis) at the highest exposure concentration and a nearly significant negative sigmoidal dose-response in the qPCR results (ANOVA, P<0.05; LRT, P=0.056). Similar to the microarray analysis, decreased expression of KRT13 was present in the qPCR results; however, it was not statistically significant.

The upregulation of GR and CYBA detected in the microarray results was confirmed in the qPCR assessment (ANOVA, P<0.05; LRT, P<0.05, 0.0001, respectively). Additionally, the iron and copper transport protein ceruloplasmin precursor (CP), which may be associated with an antioxidant and inflammatory response, was significant in the microarray (at P<0.05) and was also significantly upregulated in the qPCR assay (ANOVA, P<0.01; LRT, P<0.01). There was a decrease in the expression of genes involved in aerobic metabolism as cytochrome b-c1 complex subunit 7 (UQCRB), and cytochrome c oxidase subunit mitochondrial-like (COI) were downregulated (P<0.05) in the microarray, and this response pattern was confirmed by the qPCR results as we detected a significant decrease in UQCRB levels (ANOVA, P<0.05; LRT, P<0.05). The decrease in COI was not statistically significant (ANOVA, P>0.05). The expression of ARG was also significantly downregulated in the qPCR (ANOVA, P<0.05; LRT, P<0.01), similar to the patterns detected in the

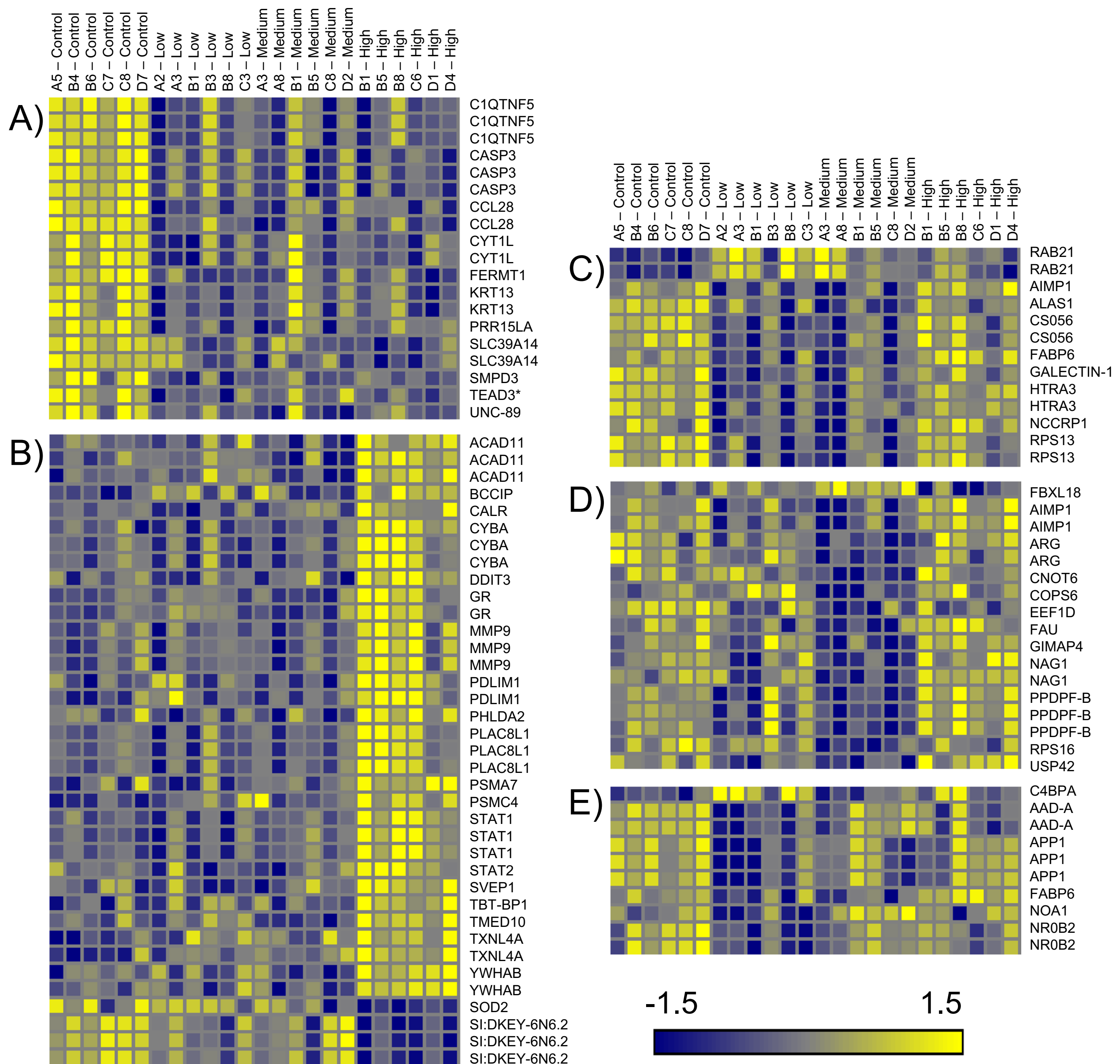

Fig. 2 Heat map showing the 96 microarray probes that were statistically different from the control group (57 different genes; ANOVA, $P<0.01$) in *M. beryllina* following 14-day exposures to ibuprofen. Microarray probes are organized by differences in expression from the control group as determined by post hoc analysis of the ANOVA: **a** common at all exposure concentrations (note that TEAD3 was only significant in the low and high concentration treatments); **b** differentially expressed in the high concentration treatment; **c** differentially expressed in the two lower exposure concentration treatments; **d** differentially expressed in the medium concentration treatment; **e** differentially expressed in the low concentration treatment. High, medium, and low treatments refer to the 1.15, 0.115, and 0.0115 mg/L ibuprofen exposure concentrations, respectively. Expression levels are presented as normalized $\log_2$ values with *yellow* indicating upregulation and *blue* indicating downregulation. Gene symbols are presented along the right side of the heat map

microarray. Additionally, ACAD11 was upregulated at the lower exposure concentrations in the microarray, a trend that was present in the qPCR assessment; however, this was not statistically significant ($P>0.05$). The response for PSMA7, which was significantly upregulated in the microarray, was not confirmed in the qPCR assessment. A downregulation trend was observed in the qPCR assays for genes associated with apoptosis [bcl-2-modifying factor-like (BMF) and CASP3]; however, these were not statistically significant.

There was a significant upregulation of PTGS2 and arachidonate 5-lipoxygenase-activating protein (ALOX5AP) assays detected in the qPCR (ANOVA, $P<0.001$; LRT, $P<1.0\text{E}^{-08}$, 0.0001, respectively; Fig. 4). Involved in ion regulation, sodium potassium-transporting atpase subunit alpha-2-

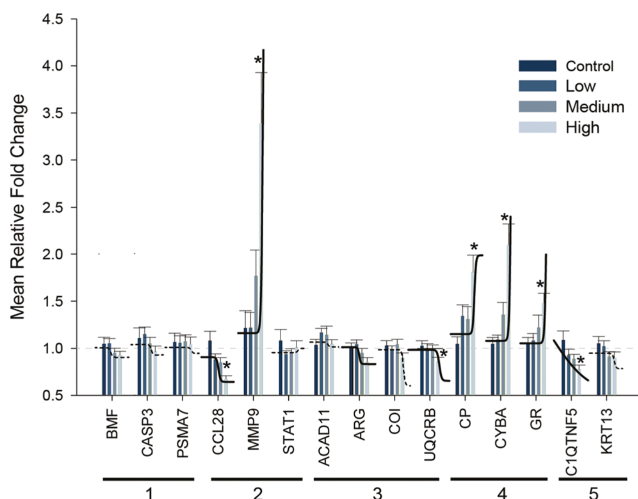

Fig. 3 Changes in the expression of *M. beryllina* genes involved in *1* apoptosis, *2* immune function, *3* metabolism, *4* oxidative stress, and *5* structural components due to ibuprofen exposure as determined by quantitative PCR. These genes were selected to verify expression patterns observed in the microarray analysis. High, medium, and low treatments refer to the 1.15, 0.115, and 0.0115 mg/L ibuprofen exposure concentrations, respectively. There is a reference line at one because a negative mean relative fold change is impossible using the $2^{-\Delta\Delta Ct}$ method for qPCR analysis; therefore, values greater than one indicate an upregulation and values below one indicate a downregulation of a gene. For each gene, differences in expression from the control, as determined by ANOVA ($P<0.05$), are indicated by an *asterisk* (*). The sigmoidal dose-response curves for each gene fit using a likelihood ratio test ($P<0.05$) are indicated using a *solid line*. Nonstatistically significant dose-response trends are indicated with a *dashed line*

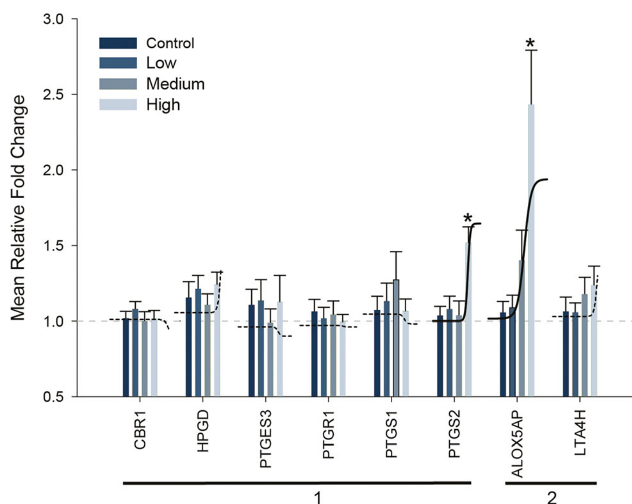

Fig. 4 Changes in the expression of *M. beryllina* genes involved in arachidonic acid metabolism, specifically *1* prostaglandin and *2* leukotriene synthesis, due to ibuprofen exposure as determined by quantitative PCR. High, medium, and low treatments refer to the 1.15, 0.115, and 0.0115 mg/L ibuprofen exposure concentrations, respectively. There is a reference line at one because a negative mean relative fold change is impossible using the $2^{-\Delta\Delta Ct}$ method for qPCR analysis; therefore, values greater than one indicate an upregulation and values below one indicate a downregulation of a gene. For each gene, differences in expression from the control, as determined by ANOVA ($P<0.05$), are indicated by an *asterisk* (*). The sigmoidal dose-response curves for each gene fit using a likelihood ratio test ($P<0.05$) are indicated using a *solid line*. Nonstatistically significant dose-response trends are indicated with a *dashed line*

like (ATP1A2) was significantly downregulated (ANOVA, $P<0.05$; LRT, $P<0.05$), and sodium potassium-transporting atpase subunit alpha-3-like (ATP1A3) was near significantly downregulated in the ANOVA, but had a highly significant negative sigmoidal dose response (ANOVA, $P=0.076$; LRT, $P<1.0E^{-11}$; Fig. 5) in the qPCR analyses. Similarly, sodium potassium-transporting atpase alpha-1 subunit (ATP1A1) had a significant negative sigmoidal dose response (LRT, $P<1.0E^{-07}$). There appeared to be slight downregulation of sodium potassium-transporting atpase subunit beta-233 (ATNB233); however, this pattern was not statistically significant.

Discussion

The goal of the present study was to determine the transcriptome-wide effects of ibuprofen exposure on the inland silverside, a nonmodel fish species with specific ecological relevance to the assessment of contaminants in California surface waters (e.g., Brander et al. 2013). The 96 h LC50 of ibuprofen was 34.35 mg/L for the inland silverside. While the acutely toxic concentration is quite high relative to environmental concentrations, inland silversides are more sensitive to ibuprofen than the Indian major carp (*Cirrhinus mrigala*) with a 24 h LC50 of 142 mg/L (Saravanan et al. 2012) or the common carp (*Cyprinus carpio*) with a 96 h LC50 of 175.6 mg/L (Islas-Flores et al. 2014). We also chronically exposed inland silversides to ibuprofen and detected different transcriptomic responses based on exposure concentration. At the lowest exposure concentration (0.0115 mg/L), consistent with those detected in undiluted wastewater effluent (e.g., Metcalfe et al. 2003; reviewed in Fent et al. 2006; Yu et al. 2013) and ocassionally downstream of effluent discharge points (Ashton et al. 2004), we detected a downregulation of many processes including those involved in skeletal development. At the highest exposure concentration (1.15 mg/L), we detected an inflammatory response that included increased expression of regulatory genes in the arachidonic acid metabolism pathway. There are numerous examples in the literature that address nonlinear relationships between exposure concentration and measured endpoint (reviewed in Vandenberg et al. 2012), where functional responses to exposure at higher concentrations, upon which modes of action are generally determined, differ to responses observed at lower, more environmentally realistic concentrations. The microarray analysis in the present study showed that at the low concentration, there was a downregulation of several biological processes, whereas at the higher exposure concentration, there was an upregulation of several biological processes, patterns that were not

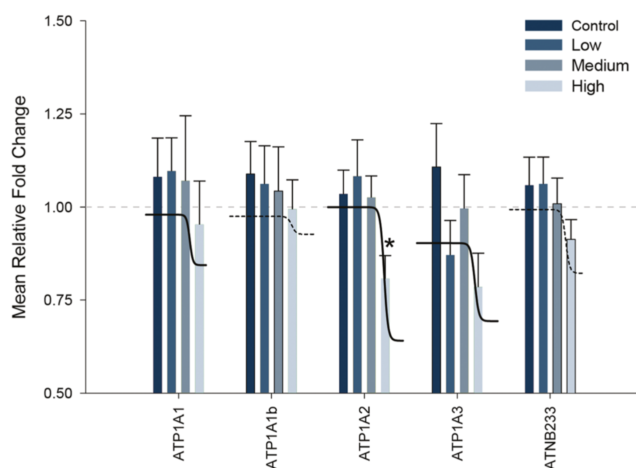

Fig. 5 Changes in the expression of *M. beryllina* genes involved in osmoregulation due to ibuprofen exposure as determined by quantitative PCR. High, medium, and low treatments refer to the 1.15, 0.115, and 0.0115 mg/L ibuprofen exposure concentrations, respectively. There is a reference line at one because a negative mean relative fold change is impossible using the $2^{-\Delta\Delta Ct}$ method for qPCR analysis; therefore, values greater than one indicate an upregulation and values below one indicate a downregulation of a gene. For each gene, differences in expression from the control, as determined by ANOVA ($P<0.05$), are indicated by an *asterisk* (*). The sigmoidal dose-response curves for each gene fit using a likelihood ratio test ($P<0.05$) are indicated using a *solid line*. Nonstatistically significant dose-response trends are indicated with a *dashed line*

detected at the lower exposure concentrations. This difference in responses, and the subtle response patterns described herein, highlight the importance of measuring transcriptome-wide cellular responses to a range of concentrations to identify the diversity of cellular mechanisms that underlie responses to this common wastewater contaminant.

Arachidonic acid metabolism is vital for many physiological processes in fishes. There are two main branches of arachidonic acid metabolism: one branch involves the production of prostaglandins and thromboxanes that are used in many physiological functions including inflammation and bone formation; the second branch involves the production of leukotrienes that are used in inflammatory responses and paradoxically, regulating prostaglandin and thromboxane activity (Lutz and Cornett 2013). Both PTGS2 and ALOX5AP are highly inducible and are key enzymes in regulating inflammatory responses in vertebrates. Consequently, they are highly regulated through a variety of cellular mechanisms to maintain tight control of arachidonic acid metabolism (Lutz and Cornett 2013). We observed an upregulation of PTGS2 and ALOX5AP after 14-day exposure to the highest concentration of ibuprofen. This pattern was not observed at the lower exposure concentrations, although both genes followed a sigmoidal dose-response pattern. The activation of the leukotriene branch of arachidonic acid metabolism is consistent with responses observed in *Daphnia magna* exposed to ≥20 mg/L of ibuprofen (Heckmann et al. 2008). Because the mode of action of ibuprofen is to inhibit PTGS1 and PTGS2 activity, the increased abundance of transcripts for the inducible PTGS2 and ALOX5AP may indicate a compensatory mechanism to counteract PTGS1 and PTGS2 enzyme inhibition at the protein level to maintain arachidonic acid metabolism-dependent processes. Therefore, future research should examine PTGS2 activity along with mRNA patterns to determine whether increased PTGS2 transcript abundance maintains sufficient PTGS2 enzyme activity and successfully counteracts the effects of exposure to ibuprofen.

Exposure to ibuprofen led to the differential expression of several genes associated with an immune response, mostly involved in an inflammatory response. Differential regulation of genes involved in inflammation could be expected based on ibuprofen's intended anti-inflammatory mode of action in humans. The inducible gelatinase MMP9 can be regulated by PTGS2 and was significantly upregulated at the highest exposure concentration of ibuprofen, directly linking exposure to an inflammatory response. Interpretation of the MMP9 induction, however, can be complicated because of its crucial role in bone matrix degradation and skeletal development (Vieira et al. 2013), in addition to its association with a response to, and recovery from, inflammation (Chadzinska et al. 2008). Similarly, CP is generally associated with an inflammation response (Gitlin 1988); however, it has also been linked to antioxidant responses in fishes (Liu et al. 2011) and was significantly upregulated due to exposure to ibuprofen. Because these and many other genes involved in an inflammatory response (e.g., STAT1 and STAT2), along with PTGS2 and ALOX5AP, were still upregulated after 14 days of exposure to the highest concentration of ibuprofen, it suggests maintenance of an inflammatory response due to chronic exposure to high concentrations of ibuprofen.

There was differential regulation of several genes involved in oxidative stress, which include the downregulation of SOD2 and the upregulation of GR, CYBA, and CP. The downregulation of SOD2 is consistent with a decrease in SOD activity detected in clams (Milan et al. 2013), however is in contrast to an increase in SOD activity in common carp acutely exposed to ibuprofen (Islas-Flores et al. 2014). Additionally, the upregulation of GR and downregulation of SOD2 observed in the present study is contradictory to the patterns observed in Mediterranean mussel (*Mytilus galloprovincialis*) that were chronically exposed to 250 ng/L of ibuprofen for 14 days (Gonzalez-Rey and Bebianno 2011). Because SOD2 is located in the mitochondrion and responds to superoxides produced through aerobic respiration (Robb et al. 2014), the decrease in SOD2 transcript abundance detected in the present study may be associated with the overall decrease in the expression of genes involved in aerobic metabolism. However, the upregulation of other genes involved in an oxidative stress response suggests that the fish in the present study were responding to other oxidative stress conditions related to ibuprofen exposure.

Collectively, these studies indicate that exposure to ibuprofen affects the oxidative stress response of aquatic organisms; however, the patterns of the response appear to be dependent on species, exposure duration, and concentration.

There was evidence of differential expression of genes involved in bone development and structure due to exposure to ibuprofen. Previous work has confirmed a downregulation of numerous hormone receptors in response to ibuprofen exposure in zebrafish, which was linked to reduced hormone levels due to an inhibition of prostaglandin production (Ji et al. 2013). There was a decrease in THRb in the present study (although not significant at the $P<0.01$ threshold), which is critical to growth and development, and may be associated with an ibuprofen-induced decrease in hormone levels. Decreases in sex steroids can lead to bone loss in humans (reviewed in Nelson et al. 2013), and a potential endocrine regulation of bone has been identified in gilthead seabream (*Sparus auratus*; Vieira et al. 2013). In the present study, at all exposure concentrations, there was an upregulation of genes involved in extracellular matrix degradation and calcium binding, variable regulation of genes associated with cell adhesion, and a downregulation of genes involved in collagen and cytoskeleton structure, along with calcium metabolism, which collectively, may be associated with ibuprofen effects on skeletal development. The fish used in the present study were ~66 dph and consequently were in a critical stage of skeletal growth and development. Disruption in bone developmental processes may have implications on overall fish growth throughout the life cycle. Due to the importance of fish size in predator avoidance, a decrease in skeletal growth may result in reduced survival in fish chronically exposed to ibuprofen in aquatic systems. Future research should examine the effects of exposure to low concentrations of ibuprofen across the entire life cycle of fishes (e.g., Han et al. 2010) to examine potential long-term decreases in growth and development.

Various genes involved in aerobic metabolism were downregulated in the inland silverside in the present study. This is in contrast to previous studies that detected increased expression of genes involved in glycolysis, the TCA cycle and oxidative phosphorylation in clams (Milan et al. 2013), and increased gill glycolytic capacity and reduced glycogen content in the livers of rainbow trout, which was suggested to be related to maintaining energetically costly ion pumps (Gravel et al. 2009). Both studies reported these responses to ibuprofen following exposures conducted in salt water. Interestingly, there was a trend of higher glycogen content in the livers of rainbow trout exposed to ibuprofen in freshwater; however, this was not statistically significant (Gravel et al. 2009). There was a downregulation of CYBA in inland silverside exposed to ibuprofen in freshwater, and functional categories associated with metabolism were significantly enriched in the functional analysis. Additionally, there was a downregulation of genes involved in proteolysis and protein synthesis, and several functional categories associated with protein synthesis were enriched in the significant gene lists that collectively suggest a reduction in protein turnover.

A downregulation of genes involved in osmoregulation at the highest exposure concentration was suggestive of an ibuprofen-dependent decrease in osmoregulatory ability. This is consistent with previous work on rainbow trout that showed an inhibition of the required elevated Na^+/K^+-ATPase activity in salt water (Gravel et al. 2009). We found decreased expression of Na^+/K^+-ATPase genes (ATP1A1, ATP1A2, ATP1A3) at the highest exposure concentration, genes required for ion regulation in fishes (Liao et al. 2009). Downregulation of Na^+/K^+-ATPases may be associated with the decreased expression of genes involved in aerobic metabolism observed in the present study, as this physiological process can use a significant portion of the cellular energy budget (Staples and Buck 2009). These results suggest an overall disturbance in osmoregulatory mechanisms and reduced metabolism in inland silversides. An ibuprofen-dependent decrease in these genes may affect the osmoregulatory capability of fishes in salt water, which could have significant consequences in fishes that are chronically exposed to ibuprofen in an estuary or marine environment, such as amphidromous fishes like the inland silverside.

Many of the cellular responses in the present study occurred at ibuprofen exposure concentrations that exceed those detected in the aquatic environment. While the responses detected at 1.15 mg/L provide valuable insight into the mode of action of ibuprofen in fish, it may be difficult to directly extrapolate these results to the environment. At the most environmentally relevant concentration (0.0115 mg/L) used in this study, the fish were still responding to the ibuprofen exposure at the level of the transcriptome. There was evidence of differential expression of genes involved in immune function and a downregulation of genes associated with cell structure, and many functional categories associated with aerobic respiration were significantly enriched. Disturbances in these physiological processes over exposures longer than 14 days may have significant consequences on the health of wild fishes, especially those with limited home ranges (e.g., inland silverside; Brander et al. 2013) at locations where they may be continually exposed to wastewater effluents. Additionally, the exposure concentration in the environment likely fluctuates seasonally and may spike randomly due to changes in water usage or ibuprofen consumption that lead to variable ibuprofen concentrations over time; therefore, it is difficult to predict true environmental exposure concentrations. Given that ibuprofen levels can often be orders of magnitude higher in bile and plasma samples (Brozinski et al. 2013), dissolved ibuprofen concentrations measured in individual water samples may not accurately reflect the concentrations relevant to fishes in a system. Last, it is critical to note that ibuprofen is often detected along with many other NSAIDs and pharmaceuticals in systems that receive wastewater effluent (e.g., Ashton et al.

2004), and therefore, it is the response to the entire suite of compounds that is ecologically relevant and not necessarily the response to a single compound.

This study presents the transcriptome-wide response of an environmentally relevant fish species to chronic exposure to ibuprofen. Interestingly, some of the response patterns are similar to those of previous transcriptome-wide assessments of the effects of ibuprofen exposure on evolutionarily distant invertebrate species (Heckmann et al. 2008; Milan et al. 2013). The similarity of the responses across taxa suggests that these results collectively may be useful for generating biomarkers of exposure to ibuprofen for various aquatic species for monitoring the effects of this common wastewater effluent contaminant. Additionally, this study significantly expands on the current suite of molecular tools available for studying inland silversides, a highly abundant and widely distributed fish species that can potentially be used as a bioindicator of contaminant exposure in coastal ecosystems in North America.

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