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Identification of biomarkers indicative of barotrauma and recovery in black rockfish *Sebastes melanops*

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A *Sebastes*-specific complementary DNA (cDNA) microarray was developed to identify potential biomarkers involved in the capture stress and recovery of *Sebastes* species if they are assisted in returning to their original depth of capture following barotrauma. Black rockfish *Sebastes melanops* were exposed to simulated decompression from 450 kPa (*c*. 35 m depth) (which resulted in barotrauma) and subsequent recompression. *Sebastes melanops* were sampled for liver tissue at days 3, 15 and 31 post-barotrauma. Potential candidate genes were identified from the microarray and then quantitative real-time PCR (qrt-PCR) was used to validate expression levels in biological replicates. Six potential biomarkers associated with the innate immune system were identified that were up-regulated in liver tissue at 3 days post-barotrauma: *complement C1q-like protein 2, complement component C3*, *complement regulatory plasma protein*, *serum amyloid A-5*, *c-type lysozyme* and *hepcidin precursor type I*. In addition, complement *c1q* was correlated to the presence of a ruptured swimbladder, providing further support that this gene may be a good biomarker of injury and recovery. Immune genes were no longer up-regulated at day 31 post-barotrauma, a good indication of recovery in *S. melanops*.

Key words: fish; gene expression; microarray; non-model organism.

INTRODUCTION

Recent advances in gene sequencing and the development of tools to analyse gene expression on a large scale have allowed studies of gene expression to expand to non-model organisms, including non-model fish species. In the past decade, custom microarrays have been developed for a variety of non-model fish species such as the African cichlid *Astatotilapia burtoni* (Gunther 1894), (Renn *et al*., 2004), European flounder *Platichthys flesus* (L. 1758), (Williams *et al*., 2003; Diab *et al*., 2008), annual killifish *Austrofundulus limnaeus* Schultz 1949, (Podrabsky & Somero, 2004), rainbow trout *Oncorhynchus mykiss* (Walbaum 1792), (Krasnov *et al*., 2005),

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Atlantic salmon *Salmo salar* L. 1758, (Rise *et al*., 2004), carp *Cyprinus carpio* L. 1758, (Reynders *et al*., 2006) and largemouth bass *Micropterus salmoides* (Lacépède 1802), (Garcia-Reyero *et al*., 2008). The custom microarrays in fishes have provided valuable insight into the molecular mechanisms that result from exposure to different environmental toxicants (Williams *et al*., 2003; Reynders *et al*., 2006; Garcia-Reyero *et al*., 2008), diseases (Kurobe *et al*., 2005; Diab *et al*., 2008), changing temperatures (Podrabsky & Somero, 2004; Kassahn *et al*., 2007) and physical stressors (Krasnov *et al*., 2005). In this study, a custom complementary DNA (cDNA) microarray for black rockfish *Sebastes melanops* Girard 1856 was designed to identify biomarkers and gain insight into the molecular mechanisms governing pressure-related injuries incurred during capture in *Sebastes* species in the Pacific Ocean.

Genes involved in the response to capture in fishes can be an important tool for the management of overfished fisheries where discard is required. By identifying genes that are affected by capture stress and following the expression of these genes over time, scientists and fishery managers can better assess the potential for a fish species to recover from a capture event. For example, if a gene is up-regulated following a capture and expression of that gene returns to control levels within a certain amount of time, it could be an indicator of recovery. The *Sebastes* genus forms an important commercial and recreational fishery off the west coast of North America. Over 65 species occur between Baja California to Alaska but since the 1980s there has been a widespread decline in many populations (Love *et al*., 2002). Currently seven species have been declared depleted by the Pacific Fishery Management Council (PFMC, 2008). Because of long generation times, late maturation and sporadic recruitment, recovery of these populations is expected to take a long time. Bycatch of depleted *Sebastes* species is a common problem because different species often have similar feeding habits and tend to aggregate in mixed-species assemblages. Current management tools for *Sebastes* species are limited to restricting fishing effort, location and requiring discard of depleted species. Discarding these species is required so they will not be targeted. Unfortunately, most *Sebastes* species have high mortality rates when discarded because of pressure-related injuries incurred during capture, referred to as barotrauma.

Barotrauma is a condition that commonly arises in physoclist fishes (*i.e.* fishes that have a closed swimbladder). When such fishes are captured and brought to the surface, gas in the closed swimbladder expands as pressure is decreased (Boyle's Law), resulting in excess gas inside the fishes. This excess gas causes a variety of conditions collectively called barotrauma. Depending on the degree of pressure change, barotrauma in physoclist fishes can involve bloating, ruptured swimbladder, crushed organs, eversion of the oesophagus or stomach and exopthalmia (Gotshall, 1964; Rummer & Bennett, 2005; Parker *et al*., 2006; Hannah *et al*., 2008; Jarvis & Lowe, 2008; Pribyl *et al*., 2009). Increasing the length of time it takes to reel a fish to the surface with a closed swimbladder will not reduce barotrauma injuries because excess gas must be resorbed by the swimbladder; it would take days to reel up species of *Sebastes* at a rate where the excess gas could be eliminated, avoiding barotrauma injury (Parker *et al*., 2006). *Sebastes* species that are discarded with barotrauma often have high mortality rates because they are too buoyant from barotrauma to submerge on their own (Hannah *et al*., 2008); if not assisted in re-submerging, these fishes often suffocate or succumb to thermal shock and predation at the surface. Research has demonstrated that if excessively buoyant *Sebastes* species can be assisted in

submerging as soon as possible after capture, excess gases recompress and external barotrauma indicators disappear allowing survival in the short-term (Hannah & Matteson, 2007; Jarvis & Lowe, 2008).

More recently, research using *S. melanops* as a model for other species of *Sebastes* has demonstrated survival, for at least 1 month post-barotrauma in the laboratory, if individuals are recompressed shortly after being decompressed from 35 m (Pribyl *et al*., 2012). During this month-long recovery period, *S. melanops* resumed feeding, showed no changes in blood chemistry due to barotrauma (there were changes due to handling stress) and had no cellular injury in the heart, liver, head kidney, gonad or eye that could be detected at the histological level. Approximately 35% of *S. melanops*, however, sustained a ruptured swimbladder after the 31 day recovery period and 7% of *S. melanops* had a severely injured rete mirabile (gas-concentrating organ in the swimbladder). Although these injuries are not fatal, it is unknown to what degree these injuries will affect *S. melanops* survival in the wild. Recent research has identified nociceptors (pain receptors) in fishes that are similar to those found in mammals (Sneddon *et al*., 2003) and an increase in brain activity in response to painful stimuli (Dunlop & Lamin, 2005; Nordgreen *et al*., 2007). Studies in *O. mykiss* found that individuals injected with dilute acid in their lips exhibited reduced activity and a reduced anti-predator response (Ashley *et al*., 2009). Thus, there may be additional behavioural responses in *Sebastes* species if pain receptors are activated in response to a ruptured swimbladder. The life history traits of different *Sebastes* species will probably play a role in determining to what extent a ruptured swimbladder will affect survival. *Sebastes* species that are active in the water column are more likely to be affected by this injury compared to *Sebastes* species that are benthic and not very active. Many *Sebastes* species, including the species listed as depleted, exhibit benthic and sedentary life-history characteristics (Love *et al*., 2002), thus it is unclear to what degree a ruptured swimbladder will affect survival.

Research has been conducted on how barotrauma affects physoclist fishes at the macroscopic and cellular level (Pribyl *et al*., 2011), but no work has yet been conducted at the genomic level. In order to gain a better understanding of the potential for *S. melanops* to recover from forced decompression, the objective of this study was to analyse patterns of gene expression in liver tissue from *S. melanops* at three time points during a 31 day recovery period post-barotrauma. Only a limited number of genes have been sequenced for *S. melanops* and, as it is unknown which genes may be activated as a result of barotrauma, an anonymous custom cDNA microarray specific for *S. melanops* was developed to identify candidate genes. Once candidate genes were identified, expression levels of candidate genes were determined using quantitative real-time PCR (qrt-PCR). By observing recovery from barotrauma at the genomic level, a better understanding of the recovery process in *S. melanops* and their potential for long-term survival if they undergo an assisted release following capture can be gained.

METHODS

FISH COLLECTION AND RECOMPRESSION EXPERIMENTS

Collection and maintenance of *S. melanops* prior to the recompression experiments and the recompression experiments themselves are described in detail in Pribyl *et al*. (2012).

Briefly, for each recompression experiment, six *S. melanops* were placed in each of two large flow-through hyperbaric aquaria (1302 l) and adjusted to 450 kPa (*c*. 35 m depth). The 35 m simulated depth is within the depth range that *S. melanops* are commonly captured in the recreational fishery. One chamber served as a treatment chamber and the other chamber served as a control chamber. Once neutrally buoyant, *S. melanops* in the treatment chamber were exposed to a simulated capture event by decreasing pressure to 100 kPa over a 90 s period to induce decompression and barotrauma. *Sebastes melanops* were then held at surface pressure for 3 min to simulate the time it would take to unhook an individual and place it on a descending device and then were immediately recompressed to 450 kPa. Shortly after being recompressed, treatment *S. melanops* showed no outward signs of stress. Control *S. melanops* remained at 450 kPa during this time. After a minimum recovery time of 6 h post-barotrauma for treatment *S. melanops*, both treatment and control *S. melanops* were slowly brought to surface pressure with a 10% pressure reduction every 2–3 h over a period of 3 days. Once at surface pressure, two *S. melanops* from each chamber were immediately removed from the pressure chambers, examined for external barotrauma indicators, sampled for blood, euthanized and dissected. Portions of liver tissue were immediately removed and flash frozen in liquid nitrogen. Tissue samples were later stored at -80° C until they could be processed. The remaining four *S. melanops* from each chamber were transported to recovery tanks. Two treatment and two *S. melanops* were subsequently sampled as described above at 15 days post-barotrauma and 31 days post-barotrauma. This experiment was replicated five times, for a total of 30 treatment and 30 control *S. melanops*, *i.e.* a total of 10 *S. melanops* sampled per treatment and sample day.

All *S. melanops* were assessed in a previous study for plasma cortisol, plasma insulin-like growth factor 1 (IGF-1), feeding behaviour and internal injuries (Pribyl *et al*., 2012). Internal injuries assessed for included ruptured swimbladder and injury to the liver, heart, head kidney and rete mirabile at the histological level. The only internal injuries observed from the previous study included ruptured swimbladders and rete mirabile injury (Pribyl *et al*., 2012). For this study, parasite load was assessed in the heart, liver and head kidney using the same histology slides from the previous study to determine if parasite load affected gene expression. All three tissues showed evidence of parasite infection. Parasite infections were categorized as no infection, a light infection (one to three parasites in a section) or a heavy infection (more than three parasites in a section).

This protocol was reviewed and approved by Oregon State University's Institutional Animal Care and Use Committee (ACUP #: 3238).

CONSTRUCTION OF cDNA MICROARRAY

Total RNA was extracted from liver tissue using Trizol according to the manufacturer's instructions (Invitrogen; www.invitrogen.com). All total RNA samples from the liver ($n = 60$) were pooled and messenger (m)RNA was isolated from total RNA using Ambion's MicroPoly(A) kit (Applied Biosystems; www.appliedbiosystems.com). The pooled liver mRNA from both treatment and control *S. melanops* was used to create a liver cDNA library using Stratagene's Lambda ZAP-CMV XR library construction kit (Agilent Technologies; www.agilent.com). The pCMV-Script EX phagemid vector was mass excised from the Lambda Zap-CMV XR vector and the plasmid library was plated onto LB-kanamycin agar plates. A total of 5000 colonies were picked and grown in LB-kanamycin overnight in 384 well plates. These plates were replicated and replicate plates were supplemented with 10% glycerol and stored at -80° C. The cDNA inserts were amplified by PCR using T3 forward and T7 reverse primers. Approximately 5% of the amplified cDNA inserts were randomly checked with gel electrophoresis to ensure proper amplification and to determine the approximate size of cDNA inserts. The size of cDNA inserts varied between 0·4 and 2·0 kb. Fifty-four of the inserts (11%) were randomly selected and sequenced to determine redundancy. Sequences were identified using BLASTx (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi/). Of the 54 sequences, 13 represented vector (24%), six represented unknown genes (11%) and 35 (65%) represented known genes. Of the known genes, 16 genes (46%) were represented once and five genes (54%) were represented multiple times. The most abundant genes were for ribosomal proteins (19%) and apolipoproteins (19%).

The PCR-amplified cDNA was buffered in 3× saline-sodium citrate (SSC) and 1·5 M betaine and spotted on Corning UltraGAPS coated slides (Corning Inc.; www.corning.com) in two replicate blocks (each block contained 16, 18 × 18 spot minor blocks). Slides were printed at Oregon State University's Center for Genome Research and Biocomputing (CGRB) Core laboratory using a MicroGrid II (Digilab, Inc; www.digilabglobal.com). Once printed, the array slides were dried for 48 h in a vacuum desiccator and UV cross linked at 300 mJ. Arrays were then stored in a vacuum desiccator until hybridization.

HYBRIDIZATION OF cDNA MICROARRAY

Probes for hybridization were constructed by pooling total RNA according to treatment group and sample day for a total of six experimental pools of mRNA. A reference pool of mRNA was created by combining total RNA from all 60 *S. melanops*.

For probe synthesis and hybridization, Genisphere's Array 900 microarray kit was used according to the manufacturer's protocol (Genisphere LLC.; www.genisphere. com). Complementary DNA was synthesized for each probe from 0·7 μg of total RNA. All array slides were prewashed and prehybridized prior to hybridization with cDNA. Once array slides were ready, LifterSlips (Thermo Scientific; www.thermoscientific.com) were applied to each array and experimental and reference cDNA in enhanced cDNA hybridization buffer was applied to each slide and incubated overnight at 55° C in sealed hybridization chambers. The following day, after post-hybridization washes, LifterSlips were again applied to each array and the arrays were hybridized with Cy3 and Cy5 3DNA Capture Reagents in an sodium dodecyl sulphate (SDS)-based hybridization buffer at 55° C in sealed hybridization chambers. After hybridization, slides were washed and dried according to the manufacturer's instructions, and immediately scanned on an Axon GenePix 4200A scanner (Molecular Devices, LLC.; www.moleculardevices.com). The software GenePix Pro 5 was used to align slides, apply quality control to individual spots and extract data from all slides.

MICROARRAY EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

For this experiment a reference design with a dye swap was utilized to determine changes in gene expression between treatment and control pools within days 3, 15 and 31 post-barotrauma. Each experimental pool was compared to the reference pool for a total of 12 arrays hybridized in total, including the dye swap. Fluorescence intensity values were normalized based on a ratio of medians relative to 32 control spots placed in each 18 spot × 18 spot minor block. Control spots consisted of a pool of all 60 samples, thus it was assumed experimental and reference samples would bind to these spots equally; the ratio of medians for these features was set to 1·0. To determine changes in gene expression between treatment and control pools within each sample day, normalized intensity values from the two blocks on each slide were averaged, and the Cy3:Cy5 ratio representing the treatment pool relative to the reference pool (Cy5:Cy3 for the dye swap) was determined. The treatment ratio was then normalized relative to the control ratio to determine the fold change in gene expression between treatment and control pools. To determine genes for sequencing, ratios $>2{\cdot}0$ in both dye swaps from day 3 hybridizations were used. Biological replicates were pooled for the microarray because the main purpose of the microarray was to identify candidate genes and not to quantify their expression. This meant, however, that statistical analyses could not be run on the microarray data because there were no d.f. All analyses were conducted on Acuity 4.0 (Molecular Devices; www.moleculardevices.com).

SEQUENCING

Differentially expressed cDNA inserts were sequenced using the T3 forward primer. Sequencing was conducted at Oregon State University's CGRB Core laboratory using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems). Vector fragments were manually removed from the sequence and, if necessary, low quality sections of the sequence as well.

Sequences were then identified by homology to known sequences using a BLASTx search of the GenBank database. All identified sequences had an expected value of $<1 \times 10^{-6}$ and thus were considered homologs to this sequence.

QUANTITATIVE REAL TIME PCR

Several genes of interest shown to be differentially regulated by the microarray data were chosen and primers that amplified 150–200 bp fragments were designed for them using Primer3 (http://frodo.wi.mit.edu/primer3/input.htm/). All primers were checked for specific amplification of appropriately sized bands using PCR and gel electrophoresis prior to use in rt-PCR reactions.

Total RNA was pooled from the two pseudoreplicates within each experiment for a total of 30 pools (2 treatments $\times$ 3 sample days $\times$ 5 replicates $=$ 30). This resulted in five biological replicates per sample day and treatment group. Complementary DNA was synthesized from pseudoreplicate pools of total RNA (5·0 μg) using Invitrogen's SuperScript III First Strand Synthesis System for rt-PCR (Invitrogen) according to the manufacturer's instructions. All cDNA samples were then diluted 1:100. Quantitative rt-PCR reactions were carried out using 4 μl of cDNA in a 20 μl reaction with 5 μM primers and SYBR Premix Ex Taq (Takara Bio Inc; www.takara-bio.com) according to the manufacturer's instructions. All samples were measured in triplicate with no-template controls using an ABI PRISM 7400 Fast Real-Time PCR System (Applied Biosystems) for a total of 40 cycles. A melting disassociation curve was run after each reaction to confirm the amplification of specific product only.

The comparative cycle-threshold (CT) method corrected for actual PCR efficiency was used to determine relative quantities of mRNA transcripts in each cDNA sample. Actual PCR efficiencies were determined using LinRegPCR (Ramakers *et al*., 2003; Ruijter *et al*., 2009). Gene expression data were normalized with two housekeeping genes; *60S ribosomal protein L13* and *ubiquitin*. The first housekeeping gene, *60S ribosomal protein L13*, was identified using the microarray data, and the second housekeeping gene, *ubiquitin*, was found by attempting to generate degenerate primers for several potential housekeeping genes for fishes from a literature search. Potential housekeeping genes tested included *acidic ribosomal protein*, *ribosomal protein L37* (Olsvik *et al*., 2008) and *ubiquitin* (Geist *et al*., 2007; Olsvik *et al*., 2008). *Ubiquitin* was the only gene with enough conserved sequence where a primer could be developed that worked in *S. melanops*. Expression stability of housekeeping genes was analysed using geNorm (Vandesompele *et al*., 2002) and was 0·116 for both genes. The normalization factor calculated by geNorm was used to normalize each cDNA sample.

QUANTITATIVE REAL-TIME PCR STATISTICAL ANALYSES

The non-parametric Mann–Whitney *U*-test was used to identify expression differences between treatment and control *S. melanops* within each sample day because of small sample sizes and non-normal distributions. Because multiple tests were conducted on data from the same experimental unit a false discovery rate correction as described in Waite & Campbell (2006) was applied at a level of 0·05.

Mann–Whitney *U*-tests were also used to determine if there was a difference in gene expression levels between the presence and absence of internal injury or feeding in *S. melanops*. Presence or absence injuries included a ruptured swimbladder and rete mirabile inflammation (Pribyl *et al*., 2012). A false discovery rate correction was applied to these *P*-values. Differences in gene expression values as a result of parasite load in the heart, liver and head kidney was also tested. Pseudoreplicate values for parasite loads were averaged and a Kruskal–Wallace test was used to determine if there were differences in gene expression between each parasite load category. Finally, a general linear model (GLM) was used to determine if there was a relationship between plasma cortisol or plasma IGF-1 values and gene expression values. All statistical analyses were conducted with SPSS v.17.0 (IBM; www-01.ibm.com/software/analytics/spss).

TABLE I. Differentially regulated genes that were sequenced and identified from the *Sebastes melanops*-specific cDNA microarray

Gene	Genbank Accession ID	dbEST ID	Reps	Direction
Hepcidin precursor type I	GT617666	67785081	4	Up
Serum amyloid A-5	GT617654	67785069	2	Up
c-type lysozyme	GT617660	67785075	4	Up
Complement regulatory plasma protein	GT617661	67785076	3	Up
MID1 interacting-like protein	GT617659	67785074	3	Down
Glyceraldehyde-3-phosphate dehydrogenase	GT617674	67785089	2	Down
Fructose-bisphosphate aldolase B	GT617672	67785087	2	Down
Complement C1q-like protein 2	GT617675	67785090	1	Up
Complement component C3	GT617665	67785080	1	Up
Warm temp acclimation related-like 65kDa protein	GT617671	67785086	1	Up
NADH dehydrogenase subunit 5	GW603858	68981602	1	Up
Haptoglobin	GW603857	68981601	1	Up
Inter-alpha (globulin) inhibitor H4 isoform 1	GT617669	67785084	1	Up

Reps, the number of times each gene was represented; Direction, the direction of differential regulation.

RESULTS

MICROARRAY

A total of 44 genes were up-regulated >two-fold and 23 genes down-regulated >1·6 fold relative to the control samples for day 3. These genes were no longer differentially regulated at day 31. Approximately half of the differentially expressed genes from day 3 were randomly chosen and sequenced (due to financial constraints, it was not possible to sequence all genes); this included 19 of the up-regulated and 10 of the down-regulated genes. All microarray files are available in the MIAME database (ArrayExpress accession #: E-MEXP-3087).

Of the 29 genes sequenced (Table I), two contained no inserts, seven were represented multiple times and seven were represented once. Nine genes of interest were chosen for validation with qrt-PCR (Table II).

QUANTITATIVE REAL-TIME PCR

The qrt-PCR results generally supported the trends observed from the microarray analyses for up-regulated genes in the liver (Table III). Genes identified from the microarray that were down-regulated in the liver (Table III), however, did not support the trends observed from the microarray analyses. Genes from the microarray that were down-regulated in the liver all showed a high level of variation between biological replicates when checked with qrt-PCR. Some replicates were up-regulated while other replicates were down-regulated within the same gene. All genes from the microarray that showed up-regulation in the liver at day 3, however, were consistently up-regulated at the biological replicate level and showed significant differences between treatment and control groups (Table III). By days 15 and 31, these same genes showed a trend towards returning to control levels (Fig. 1). The up-regulated

TABLE II. Forward and reverse primers for *Sebastes melanops* genes of interest used in quantitative real-time PCR

Gene	Primer sequence
Complement Clq-like protein 2	Fwd: TCTGCTGACCCTAAGCCTGT Rev: ACAGAGAAGGCCACTTTGGA
Complement component c3	Fwd: CGGAGGCTATGGATCAACTC Rev: GATGTTCTGGTGGCGTAGTG
Complement regulatory plasma protein	Fwd: ATGGTGAATGGGTTGGAGAG Rev: ATCGTGTCTTCGTCCACCTT
c-type lysozyme	Fwd: TGGAACCTCCTTATCGAACG Rev: ATCCTGCCACATAGGAGCTG
Serum amyloid A-5	Fwd: ATGATATGAGGGACGCCAAC Rev: ATGCTCATTTGCCCTCTGAT
Hepcidin precursor type I	Fwd: CCGTCCGAAGATGAAGACAT Rev: TCAGCAGCAACTGGATTGTC
MID-1 interacting-like protein	Fwd: ACAGCCAACCACATCCAACT Rev: TCACAGTCCCACATCTCATCA
Fructose bisphosphate aldolase	Fwd: CGTGACCTCCTCTTCTCCAC Rev: GTGCCTTTGTCCACCTTGAT
Gylceraldeyhde-3-phosphate dehydrogenase	Fwd: CCAGGTCGTCTCCACAGACT Rev: GCGGGTCAGTTTACTCCTTG
*60S ribosomal protein L13**	Fwd: TGGAACCTCCTTATCGAACG Rev: ATCCTGCCACATAGGAGCTG
*Ubiquitin**	Fwd: TGAGCCCAGTGACACTATCG Rev: GAGAGAAGGCTCGATGATGC

*Housekeeping genes.

genes included *complement C1q-like protein 2*, *complement component C3*, *complement regulatory plasma protein*, *serum amyloid A-5*, *c-type lysozyme* and *hepcidin precursor type I*. None of these genes were significantly different from controls by day 31.

MODELLING EXPRESSION VALUES

Complement component C1q-like protein 2 had higher expression levels in *S. melanops* that had a ruptured swimbladder compared to *S. melanops* that did not (Mann–Whitney U-test, d.f. $= 1$, $P < 0{\cdot}01$). No other statistical differences in gene expression values and presence or absence of a ruptured swimbladder, rete mirabile inflammation or feeding after correcting for false discovery rates were found. Additionally, no statistical relationship was found between gene expression values and plasma cortisol or plasma IGF-1 values after correcting for false discovery rates.

Several parasites were identified in the heart, liver and head kidney of *S. melanops*. In the heart atrium and ventricle, several *S. melanops* had light to heavy infections of blood flukes (both adults and eggs). In the head kidney, several *S. melanops* had light to heavy infections of *Ichthyophonous* sp. and granulomas possibly indicative of a bacterial infection. In the liver, a large proportion of *S. melanops* had light to heavy infections by a myxozoan parasite and two *S. melanops* had encysted tapeworms and

TABLE III. Genes of interest from *Sebastes melanops* that were quantified using quantitative real-time polymerase chain reaction qrt-PCR. Ratios for qrt-PCR are mean ± S.E. of the five biological replicates

Gene (function)	Genbank accession ID	dbEST ID	Sample day	Ratio of treatment:control	
				Microarray†	qrt-PCR
Complement c1q-like protein 2 (innate immune system)	GT617675	67785090	3	4·94	20·27 ± 3·30*
			15	1·47	6·51 ± 2·11
			31	1·07	2·37 ± 0·34
Complement component c3 (innate immune system)	GT617665	67785080	3	2·27	3·81 ± 0·77*
			15	1·06	1·61 ± 0·42
			31	1·25	1·05 ± 0·44
Complement regulatory plasma protein (innate immune system)	GT617661	67785076	3	2·36	2·90 ± 0·28*
			15	1·29	1·37 ± 0·34
			31	1·11	1·32 ± 0·10
c-type lysozyme (innate immune system)	GT617660	67785075	3	2·48	5·49 ± 1·93*
			15	1·31	5·73 ± 4·32
			31	1·29	1·19 ± 0·74
Serum amyloid A-5 protein (innate immune system)	GT617654	67785069	3	3·12	6·94 ± 2·23*
			15	4·93	26·64 ± 17·20
			31	1·66	1·56 ± 0·66
Hepcidin precursor type I (iron regulation/innate immune system)	GT617666	67785081	3	3·76	20·42 ± 9·25*
			15	1·25	2·50 ± 1·64
			31	1·32	1·46 ± 0·68
MID-1 interacting-like protein (microtubule stabilization; cell division)	GT617659	67785074	3	0·45	0·48 ± 0·32
			15	1·29	2·42 ± 1·16
			31	0·87	1·22 ± 0·53
Fructose bisphosphate aldolase (glycolysis)	GT617672	67785087	3	0·47	1·16 ± 0·58
			15	1·44	1·23 ± 0·60
			31	0·88	1·02 ± 0·53
Glyceraldeyhde-3-phosphate dehydrogenase (glycolysis)	GT617674	67785089	3	0·42	0·76 ± 0·18
			15	0·90	1·13 ± 0·27
			31	0·72	0·88 ± 0·43

*Difference at $\alpha = 0{\cdot}05$ level between treatment and control samples (Mann–Whitney U-test with false discovery rate correction).

†Ratio of treatment, control for microarray = (mean value of treatment pool or reference pool) divided by (mean value of control pool divided by reference pool); for qrt-PCR = (normalized treatment sample) divided by (normalized control sample).

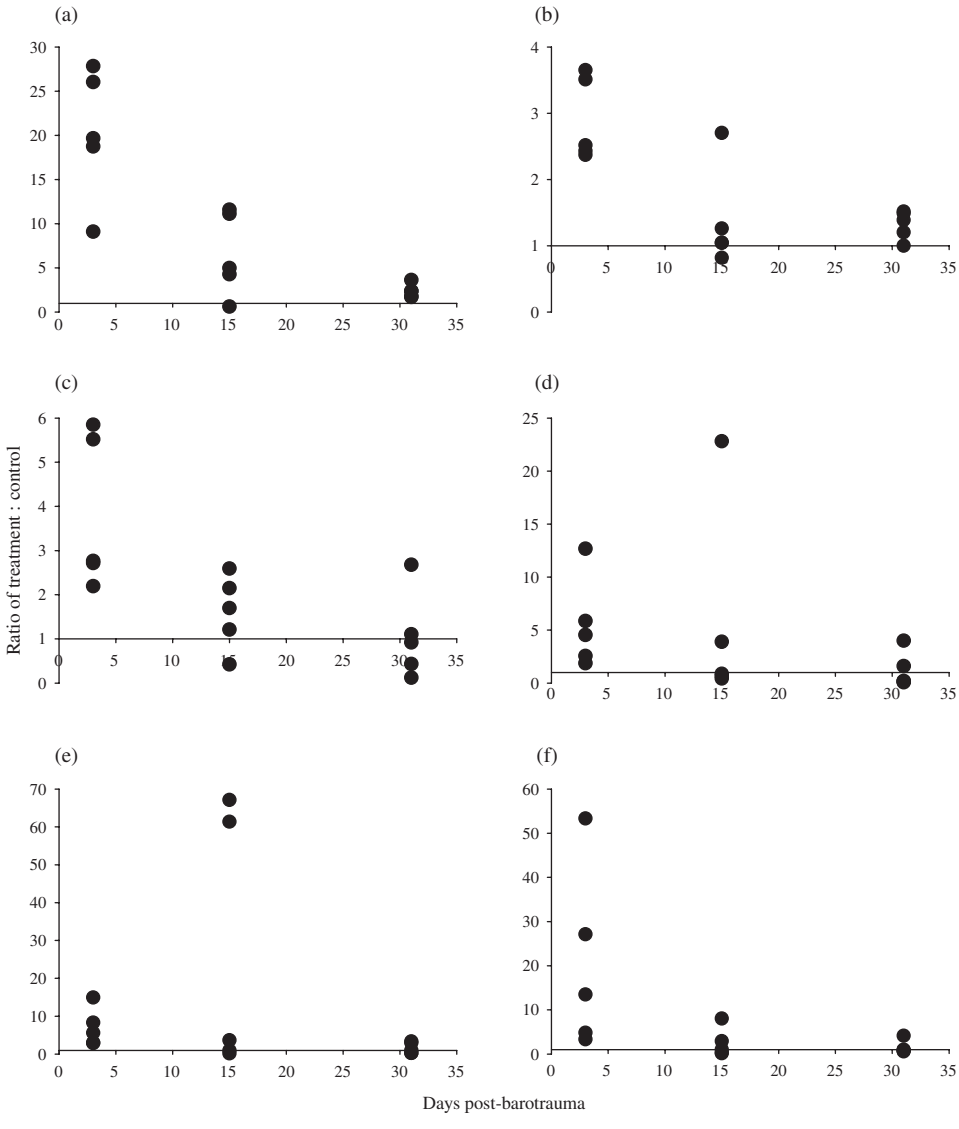

FIG. 1. Gene expression of biological replicates at days 3, 15 and 31 post-barotrauma for liver tissue: (a) *complement Clq-like protein 2*, (b) *complement regulatory plasma protein*, (c) *complement component 3,* (d) *c-type lysozyme*, (e) *serum amyloid A-5* and (f) *hepcidin precursor type I.* Each point (●) represents averaged pseudoreplicates from two *Sebastes melanops* ($n = 5$ for each sample day (some points overlap and thus are difficult to differentiate).

larval nematodes. No differences in gene expression level of the six up-regulated immune genes were found when compared among the three levels of parasite abundance (1 = no parasites, 2 = light parasite load and 3 = heavy parasite load) in the heart (Kruskal–Wallis test, d.f. = 2; $P > 0{\cdot}05$ for all six tests), head kidney (Kruskal–Wallis test, d.f. = 2; $P > 0{\cdot}05$ for all six tests) or liver (Kruskal–Wallis test; d.f. = 2; $P > 0{\cdot}05$ for all six tests).

DISCUSSION

Six potential biomarkers for assessing recovery from barotrauma in *S. melanops* were identified utilizing the *S. melanops*-specific microarray: *complement C1q-like protein 2*, *complement component C3*, *complement regulatory plasma protein*, *serum amyloid A-5*, *c-type lysozyme* and *hepcidin precursor type I*. All of these genes were significantly elevated in treatment *S. melanops* compared to control *S. melanops* at day 3 post-barotrauma, even after accounting for the handling stress both treatment and control *S. melanops* experienced during the experiments. These genes were no longer elevated at days 15 and day 31 post-barotrauma, indicating probable recovery in treatment *S. melanops* over time.

Each of the six potential biomarkers discovered are associated with the innate immune system, indicating this is probably the predominant pathway affected by barotrauma. The innate immune system is non-specific and the first line of defence against pathogens. In mammals, the complement system is one of the central immune responses initiated by the innate immune system. The complement system consists of *c*. 20 proteins that are part of a biochemical cascade when activated, and can recruit inflammatory cells, opsonize pathogens and kill pathogens (Janeway *et al*., 2005). Three of the biomarkers are part of the complement system: *complement C1q*, *complement component C3* and *complement regulatory plasma protein*. Complement C1q initiates the classical pathway of complement activation by binding to antigen:antibody complexes and to pathogen surfaces. Complement component C3 initiates the activation of the alternative pathway of complement activation by binding to pathogen surfaces. In addition, all three complement pathways produce C3 convertase, which cleaves complement component C3 into C3a and C3b. C3a recruits inflammatory cells to the site of infection and C3b binds to bacterial cell membranes and opsonizes the bacteria (Janeway *et al*., 2005). Thus complement component C3 plays a primary role in the activation of the complement system. Complement regulatory plasma proteins regulate complement activation and protect the host against cell damage from the complement system (Meri & Jarva, 2008). Regulation of the complement system in fishes in response to pathogen and toxicant stress appears to be variable, depending on the stressor and species. The complement system was down-regulated in *O. mykiss* exposed to *Yersinia ruckeri* (Raida & Buchmann, 2009), however, it was up-regulated in *O. mykiss* exposed to *Vibrio anguillarum* (Bayne *et al*., 2001) and in *C. carpio* exposed to cadmium (Reynders *et al*., 2006). Raida & Buchmann (2009) suggested that one possibility for the down-regulation of the complement response was that the level of infection was insufficient to initiate activation of the complement system. In *S. melanops*, it appears barotrauma is a severe enough stressor to activate the complement system.

Serum amyloid A-5 is part of the serum amyloid A (SAA) superfamily of acute phase proteins which are primarily produced by the liver [although other organs have also been reported as producing SAAs in response to inflammation (Villarroel *et al*., 2008)]. In fishes, SAA is a common acute-phase protein that is activated in response to a variety of stressors (Jensen *et al*., 1997; Jensen & Whitehead, 1998; Raida & Buchmann, 2009; Talbot *et al*., 2009). Levels of *saa* mRNA showed over a 40 fold increase in Arctic charr *Salvelinus alpinus* (L. 1758), challenged with *Aeromonas salmonicida* after 5 days (Jensen *et al*., 1997). Levels of *saa* mRNA peaked at a 3000 fold increase in *O. mykiss* challenged with *Y. ruckeri* after 3 days, remained

elevated after 14 days, but returned close to control levels after 28 days (Raida & Buchmann, 2009). Expression levels of *saa* in *O. mykiss* subjected to confinement stress was highly variable, with three *O. mykiss* showing elevated levels (18–221 fold increases) of *saa* after 7 days of confinement and two *O. mykiss* showing no increase (Talbot *et al*., 2009). These studies suggest *saa* levels do not peak immediately after a stressor, but take a few days to reach peak levels. Thus, the peak in *saa* expression levels in two of the replicates 15 days post-barotrauma may not be unusual; it is also possible *saa* levels peaked earlier than 15 days post-barotrauma and most *S. melanops* had already returned to neutral regulation levels, while other *S. melanops* were taking longer to return to neutral regulation. The decline towards neutral expression at day 31 in all treatment *S. melanops* is a good indicator that they were recovering from barotrauma.

Lysozymes are important antimicrobial agents of the innate immune system and are produced in the liver as well as other tissues. Besides having antimicrobial properties, lysozymes can also activate the complement system or act as opsonins (molecules that enhance the binding of a phagocyte to the antigen). The c-type lysozyme is thus named because the lysozyme was originally obtained from chickens *Gallus domesticus* (chicken-type lysozyme) (Saurabh & Sahoo, 2008). Lysozyme activity in fishes, similar to complement, also appears to vary depending on the stressor. Stressors such as transport (Möck & Peters, 1990), water pollution (Möck & Peters, 1990) and subordination stress (Caruso & Lazard, 1999) caused a reduction in lysozyme levels whereas other stressors such as handling stress (Caruso *et al*., 2002) and disease infection (Demers & Bayne, 1997) caused an increase in lysozyme levels. Again, barotrauma appears to be a sufficient stressor to cause an increase in lysozyme levels. After 31 days of recovery, however, lysozyme levels declined, again indicating possible recovery.

The final gene confirmed with qrt-PCR was *hepcidin*. Hepcidin is a recently discovered protein (Park *et al*., 2001) and is responsible for regulating plasma iron concentrations and for controlling the distribution of iron to tissues in order to maintain homeostasis (Nemeth & Ganz, 2006). Hepcidin is also associated with the innate immune system as an antimicrobial peptide because it becomes elevated during infection and inflammation. It is likely that hepcidin plays a role in limiting iron availability to foreign invaders (Nemeth & Ganz, 2006). *Oncorhynchus mykiss* exposed to *V. anguillarum* showed up-regulation of *hepcidin* by 22 fold (Gerwick *et al*., 2007) and *P. flesus* exposed to *A. salmonicida* showed up-regulation of *hepcidin* by six-fold (Diab *et al*., 2008). Korean black rockfish *Sebastes schlegelii* Hilgendorf 1880 also showed up-regulation of two different types of *hepcidin* when infected with *Streptococcus iniae* (Kim *et al*., 2008).

The six up-regulated genes identified from the microarray were from the innate immune system; however, it is possible other systems responded to barotrauma as well. Due to financial constraints, only about half of the differentially regulated genes identified from the microarray were sequenced. Thus, it is likely there were other genes responding to barotrauma that were not sequenced. It is also important to acknowledge that this study measured levels of mRNA, and not proteins; levels of gene expression do not always correlate to the levels of protein expression and protein levels should be measured to confirm the gene expression results.

Modelling of the six up-regulated genes against the presence and absence of injury and feeding showed *complement C1q* had higher expression levels in *S. melanops* with a ruptured swimbladder compared to *S. melanops* without a ruptured swimbladder. Elevation of *complement C1q* in fish with a ruptured swimbladder suggests that a return to neutral regulation in *complement C1q* may be a good indicator of recovery as well as injury in *S. melanops*. Another reason immune genes such as complement may remain elevated is as a result of the tertiary stress response, or when an organism has been experiencing a chronic stressor. Cortisol, the primary corticosteroid produced in teleosts, is often used as an indicator of the stress response. This is because cortisol levels typically increase dramatically from baseline levels during the primary stress response, and may not return to baseline levels if the stressor is chronic (Wendelaar Bonga, 1997). Modelling of the six up-regulated genes against plasma cortisol, however, did not yield any significant relationships. Although it is possible there were no relationships between immune gene expression and cortisol, another possibility is that elevated cortisol levels from barotrauma were masked by already elevated cortisol level from the stress response to capture (Pribyl *et al*., 2012).

Recovery of *S. melanops* in this study is defined as a return to control conditions, however, it is important to acknowledge that the control *S. melanops* were under laboratory conditions for several months and experienced netting, transport, confinement and a two-week fasting period during the course of the experiments. A better evaluation of recovery would be to compare treatment *S. melanops* to a negative control from the wild, however it is very difficult to obtain a true negative control sample because *S. melanops* would still need to be brought to the surface for sampling, and it would be unknown from what depth the *S. melanops* were neutrally buoyant and if some genes were already responding as a result of the barotrauma and capture stress. In addition, variability in ocean conditions could affect the gene profile of the negative control, making it difficult to determine a normal profile. By keeping all *S. melanops* at the same conditions one month prior to experiments, and utilizing hyperbaric pressure chambers to ensure all *S. melanops* were acclimated to the same depth, the only difference between treatment and control *S. melanops* was the actual treatment of simulated decompression resulting in barotrauma and subsequent recompression.

The up-regulation of six genes from the innate immune system in treatment *S. melanops* strongly suggests that the innate immune system was activated in response to barotrauma and not as a result of handling stress. It is also possible these immune genes may be part of a general response to physical trauma in *S. melanops*. This is a novel discovery, because the physiological analysis of these *S. melanops* (Pribyl *et al*., 2012) indicated that handling stress outweighed the additional stress of barotrauma for many typical measures of stress (*i.e.* cortisol and glucose). These genes could thus serve as good biomarkers to assess the ability of different *Sebastes* species to recover from barotrauma and help fishery managers assess if assisted release should be used as a management tool in these species. From this study, it appears assisted release will work for the majority of *S. melanops* captured at depths $\leq$35 m. Further studies assessing the expression of these genes over time in other *Sebastes* species are needed to determine if they can also recover from barotrauma at a genomic level.

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