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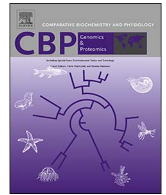

RNA-seq analysis of the head-kidney transcriptome response to handling-stress in the red cusk-eel (*Genypterus chilensis*)

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ABSTRACT

Stress is a primary contributing factor of fish disease and mortality in aquaculture. We have previously reported that the red cusk-eel (*Genypterus chilensis*), an important farmed marine fish, demonstrates a handling-stress response that results in increased juvenile mortality, which is mainly associated with skeletal muscle atrophy and liver steatosis.

To better understand the systemic effects of stress on red cusk-eel immune-related gene expression, the present study assessed the transcriptomic head-kidney response to handling-stress. The RNA sequencing generated a total of 61,655,525 paired-end reads from control and stressed conditions. *De novo* assembly using the CLC Genomic Workbench produced 86,840 transcripts and created a reference transcriptome with a N50 of 1426 bp. Reads mapped onto the assembled reference transcriptome resulted in the identification of 569 up-regulated and 513 down-regulated transcripts. Gene ontology enrichment analysis revealed a significant up-regulation of the biological processes, like response to stress, response to biotic stimulus, and immune response. Conversely, a significant down-regulation of biological processes is associated with metabolic processes. These results were validated by RT-qPCR analysis for nine candidate genes involved in the immune response. The present data demonstrated that short term stress promotes the immune innate response in the marine teleost *G. chilensis*. This study is an important step towards understanding the immune adaptive response to stress in non-model teleost species.

1. Introduction

Stress is defined as physical, chemical, or biological factors that induce systemic physiological responses and may lead to disease and death (Barton and Iwama, 1991). When fishes suffer intensive farming or environmental alterations that fall outside normal ranges, the consequence may be disastrous (Barton, 2002; Ellis et al., 2012). At the organismal level, a series of physiological responses occur following stressful challenges, with the aim of adapting to the new conditions (Harper and Wolf, 2009). The physiological response known as GAS (general adaptation syndrome) explains the three predictable stages that the body uses to respond to stressors (Selye, 1950). The first stage consists of an alarm reaction, in which stress hormones are released, providing a burst of energy. The second stage of resistance, where the body attempts to adapt to the stressor, is characterized by elevated glucose levels, immunological response, and a change in other metabolic parameters. Finally, the third stage of exhaustion is where energy is depleted and adaptation is lost, because the stress was long-lasting (Selye, 1950).

At the level of immunological response, the response pattern is stimulatory and the fish response shows and activating phase that specifically enhances innate response when the stressor is acute. If the stressors are chronic, the immune response shows suppressive effects, and the chance of an infection may thus be enhanced (Tort, 2011;

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Nardocci et al., 2014). The contribution of cortisol, the main stress hormone, *in vitro* have made it possible to clarify its participation as a potent suppressor of cellular immunity by affecting the expression of genes related to inflammatory signaling pathways. Treatment with cortisol in rainbow trout macrophage primary culture significantly suppressed LPS-induced changes in its transcriptome, suppressing the expression of proinflamatory molecules like TNFα and IL1β (MacKenzie et al., 2006). These observations have also been replicated in other cellular models, such as trout head-kidney leukocytes, carp head-kidney phagocytes and gilthead seabream head-kidney cells (Holland et al., 2003; Saeij et al., 2003; Castillo et al., 2009).

While this general rule is widely accepted, there are certain particularities in the adaptive response of different reared fish species. For example, low handling-stress tolerance has recently been described in the red cusk-eel (*Genypterus chilensis*), an important marine species for the Chilean aquaculture industry. This low tolerance to stress is reflected in high mortality rates during juvenile stages (Vega et al., 2015), and is associated with increased skeletal muscle atrophy and liver oxidative damage (Aedo et al., 2015; Naour et al., 2017). Progress in the characterization of the molecular response to stress in this species has been possible due the rapid advances in next generation sequencing technologies (NGS). This technology has permitted studying transcriptomes of non-model species at low cost in short periods of time (Qian et al., 2014). The data obtained with this technology have also provided important molecular information regarding the immune adaptive response in a range of commercially important teleost species (Xiang et al., 2010; Sun et al., 2012; Polinski et al., 2016; Zhao et al., 2016). In this context, the aim of this study was to evaluate in head-kidney, the major lymphoid tissues in teleost fishes, its response to handling-stress. For this, Illumina reads obtained from control and stressed fish were mapped onto an assembled head-kidney reference transcriptome to identify differentially expressed transcripts. Gene ontology enrichment analysis revealed a significant up-regulation of immune-related genes, with expression validated by RT-qPCR.

2. Material and methods

2.1. Ethics statement

The study adhered to animal welfare procedures, and was approved by the bioethical committees of the Universidad Andres Bello and the National Commission for Scientific and Technological Research (CONICYT) of the Chilean government.

2.2. Animals and experimental design

Juvenile red cusk-eels (*Genypterus chilensis*) with an average weight of 900 ± 50 g and length of 55 ± 5 cm were collected from the Centro de Investigación Marina de Quintay (CIMARQ) (33°13′S 71°38′W, Valparaíso Region, Chile). Fish were kept under natural temperature and light:dark photoperiod conditions (13 °C ± 1 °C and L:D 12:12). The animals were then split into control and stressed groups, placed in separate 90 L tanks (stocking density 6.8 ± 1.3 kg m^{-3}), and acclimated for two weeks before the handling-stress protocol. Fish were fed once daily with 6-mm commercial pellets, containing 60% protein, 6% lipids, 11% carbohydrates, 15% ashes, and 8% humidity (Skretting, Puerto Montt, Chile). The stressed group was then subjected to a standardized daily handling-stress protocol (Aedo et al., 2015; Naour et al., 2017). On the fifth day of the trial, the control and stressed groups were anaesthetized (3-aminobenzoic acid ethyl ester, 100 mg/L). Blood was collected from the caudal vein with heparinized syringes and centrifuged at 5000 × *g* for 10 min at 4 °C to obtain plasma. The fish were then euthanized through an overdose of anesthetic (3-aminobenzoic acid ethyl ester, 300 mg/L). Head-kidneys were collected and immediately frozen in liquid nitrogen and stored at − 80 °C.

2.3. Head–kidney RNA extraction and sequencing

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, TX, USA). RNA was quantified spectrophotometrically using NanoDrop and the Epoch Multi-Volume Spectrophotometer System (BioTek, VT, USA). Total RNA isolated from the head-kidney was treated with DNase I to remove genomic DNA. RNA concentration was measured using a Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA), and RNA integrity was determined using the Fragment Analyzer™ Automated CE System (Analytical Advanced Technologies, Ames, IA, USA). Equal quantities of total RNA from four fish were pooled by condition, and the total RNA was used to prepare mRNA libraries. Complementary DNA (cDNA) libraries were constructed using the TruSeq RNA Sample Preparation kit v2 (Illumina®, USA) following previously described procedures (Aedo et al., 2015). Libraries were sequenced (2 × 250 bp) using the MiSeq (Illumina®) platform.

2.4. Reads processing, transcriptome de novo assembly and annotation

Raw sequencing reads were trimmed by removing adaptor sequences, low quality sequences (quality scores < 10), and sequences with lengths < 30 bp. *De novo* assembly was conducted using the CLC Genomics Workbench version 8.5.1 (www.clcbio.com) using *de novo* assembly tool, following previously described procedures (Aedo et al., 2014). Chimeric transcripts were checked using an exploratory blast (BLASTX) to search against *Danio rerio* uniprot database to identify and split incorrectly assembled contigs. To validate the completeness and integrity of this *de novo* assembly, a comparison between the head-kidney assembled transcriptome was conducted by BUSCO (Benchmarking Universal Single-Copy Orthologs) against OrthoDBv9 database (actinopterygii), to identify highly conserved orthologous genes (Simão et al., 2015). The annotation of head-kidney *G. chilensis* transcripts was carried out by BLASTX searches against the Uniprot and NR databases following previously described procedures (Aedo et al., 2014). The Blast2GO application was used for the functional annotation of transcripts, applying the mapping function of GO terms to transcripts with BLAST hits obtained from blast searches against NR. Ontologies hits with e-value < $1E^{-6}$, annotation cut-off > 55, and a GO weight > 5 were used for annotation.

2.5. Differentially expressed transcripts and GO enrichment analysis

To identify differentially expressed transcripts, the reads from control and stressed conditions were mapped to the head-kidney assembled transcriptome, using CLC Genomics Workbench, v.8.5.1 (http://www.clcbio.com), with the following parameters: mismatches = 2; minimum fraction length = 0.9; minimum fraction similarity = 0.8, and maximum hits per read = 5. Gene expressions were based on reads per kilobase of exon model per million mapped read (RPKM) values. Transcripts with absolute fold-change values > 2.0 and FDR corrected P-values < 0.05 were included in the GO and KEGG enrichment analyses. The differential regulated genes were analyzed for gene ontology (GO) using the DAVID database (Huang et al., 2008) and categorized based on GO terms (level 2) for biological processes, molecular functions, and cellular components, following previously described procedures (Aedo et al., 2015).

2.6. RNA-seq validation by real-time qPCR

Total RNA was extracted from liver tissue using the RNeasy Mini Kit (Qiagen, TX, USA). RNA was quantified spectrophotometrically using NanoDrop and the Epoch Multi-Volume Spectrophotometer System (BioTek, VT, USA). RNA with an A260/280 ratio between 1.9 and 2.1 were used for cDNA synthesis. Reverse transcription was performed using 1 μg of total RNA with the Quantitect Reverse Transcription Kit (Qiagen, TX, USA) and following the manufacturer's recommendations

to generate cDNA. All quantitative real-time polymerase chain reaction (qPCR) assays were carried out in compliance with MIQE guidelines (Bustin et al., 2009). The qPCR assessments were performed using a Stratagene MX3000P qPCR system (Stratagene, La Jolla, CA, USA), following the previously described procedure (Naour et al., 2017). Briefly, each reaction mixture contained 7.5 μl of 2× Brilliant® II SYBR® master mix (Stratagene, La Jolla, CA, USA), 6 μl of cDNA (40-fold diluted) and 250 nM of each primer in 20 μl final volume. The list of primers and amplification efficiencies are described in Supplementary file 1. The reference gene, 40S ribosomal protein S30 (*fau*), was used for gene expression normalization, and control reactions included a no-template control and a no-reverse-transcriptase control. QGene was used for analyzing gene expression (Simon, 2003); graphs are expressed as fold-changes over basal levels.

2.7. Statistical analysis

Significant differences in gene expression between control and stress groups were determined by one-way ANOVA followed by Tukey's test. Correlations between RNA-seq and qPCR data were assessed through multiple linear regressions, using coefficients of determination (R^2) and P-values. All statistical analyses were performed using GraphPad Prism, v.5.00 (GraphPad Software, CA, USA).

3. Results

3.1. Sequence data, de novo head-kidney transcriptome assembly, and annotation

We previously reported that plasmatic glucose and cortisol levels significantly increased (5.3 fold and 1.7 fold, respectively) following 5 days of the repeated handling-stress protocol (Aedo et al., 2015). To understand how stress modulates the immune response in a global and comprehensive manner, RNA sequencing was performed on head-kidney samples from control and stressed groups. To examine sequencing variations, cDNA library replicates of each condition were constructed. We obtained a total of 25,215,248 and 37,905,168 paired-ends reads from control and stressed conditions, respectively. Raw reads are available at the NCBI Sequence Read Archive under study accession number SRX1056881. After sequence trimming for adapter and filtering of low quality base pairs, the data sets were reduced to 24,767,366 and 36,888,159 high-quality reads, respectively. A total of 61,655,525 reads were used for the *de novo* assembly (Table 1).

The *de novo* transcriptome assembly generated a total of 133,646 transcripts. After a filtering step to exclude transcripts smaller than 300 bp and average coverage < 5, the transcripts number was reduced to 85,623 transcripts. Additionally, through an exploratory BLAST against *Danio rerio* uniprot database, we identified 1081 incorrectly assembled contigs which were splitted adjusting to a final set of 86,840 sequences. The average length was 840 bp and the N50 of the assembly was 1426 bp (Table 1). Our transcriptome assembly was compared against the BUSCO database, which contains information about highly conserved orthologous genes. Of the 3698 BUSCO genes of the class actinopterygii, 2826 complete (76.4%), 392 fragmented (10.6%), and 480 missing genes (13%) were found in the assembly.

Annotation was performed through homology search with BLASTx across all sequences against the NR and Uniprot databases. After BLASTx comparison, we detected significant similarity with a total of 39,164 (45.1%) transcripts among which 36,647 (42.2%) were also annotated with Gene Ontology (GO) terms. The BLAST2GO annotation was carried out to assign GO terms to each sequence (Fig. 1). The analysis generated 16,255 GO annotation results for biological process (33.5%), 15,809 GO annotation results for cellular components (32.6%) and 16,706 GO annotation results for molecular function (34.5%). Among the annotated biological processes, a large percentage of annotated transcripts were assigned to biological regulation, cellular process, and metabolic process but it was also possible to identify 4091 (3.5%) transcripts associated with the immune system, including transcripts associated with chemokine signaling pathways and toll- and NOD-like receptor signaling pathways (Fig. 2). Finally, among cellular components and molecular function, the majority of transcripts were assigned to cell and binding GO terms, respectively.

Table 1
Summary of *Genypterus chilensis* head-kidney transcriptome sequencing and assembly statistics. bp: base pair. R: replicate.

Read sequencing	Control	Control (R)	Stress	Stress (R)
Read number before trimming	20,383,632	17,521,536	14,549,890	10,665,358
Read average length before trimming (bp)	200.2	195.5	187.2	182.4
Read number after trimming	20,077,804	16,810,355	14,439,699	10,327,667
Read average length after trimming (bp)	187.0	186.0	183.1	181.9
Transcriptome assembly statistics				
Total Transcripts	133,464			
Transcripts after filtering - exploratory blast	86,840			
Average length (bp)	840.3			
N50 (bp)	1426			
Average coverage	101 x			
Shortest contig (bp)	300			
Longest contig (bp)	18,156			

3.2. Differentially expressed transcripts, GO enrichment analysis, and RNA-seq validation

Differentially expressed transcripts (DETs) were estimated by mapping obtained reads to the head-kidney assembled transcriptome, resulting in a mapping of 94.3% of total reads. The expression level of each gene was represented as RPKM, with 569 transcripts up-regulated under stressed conditions and 513 transcripts down-regulated under stressed conditions (Supplementary file 2).

The differentially expressed transcripts were analyzed using the DAVID database and categorized as biological process, molecular function, cellular component, or KEGG pathways. The up-regulated transcripts under stressed conditions were significantly enriched in biological processes, such as response to stress (GO: 0006950), response to biotic stimulus (GO: 0009607), and immune response (GO: 0006955), among others (Table 2). As for molecular function and cellular component pathways, the GO terms for up-regulated transcripts were assigned to protein binding (GO: 0005515) and contractile fiber part (GO: 0044449), respectively (Supplementary files 3 and 4). Conversely, the down-regulated transcripts under stress conditions were significantly enriched in terms of biological processes, including single-organism metabolic process (GO: 0044710), cellular metabolic process (GO: 0044237), and organic substance metabolic process (GO: 0071704), among others (Table 2). As for the molecular function and cellular component pathways, the GO terms for down-regulated transcripts were assigned to oxidoreductase activity (GO: 0016491) and extracellular exosome (GO: 0070062), respectively (Supplementary files 3 and 4).

KEGG Pathway analysis revealed that several transcripts associated with cytokine-cytokine interaction, TNF signaling, and NOD signaling were up-regulated (Table 3), namely: C-C motif chemokine ligand 2 (*ccl2*), C-C motif chemokine ligand 20 (*ccl20*), C-X-C motif chemokine ligand 10 (*cxcl10*), C-X-C motif chemokine receptor 1 (*cxcr1*), C-X-C motif chemokine receptor 2 (*cxcr2*), CD27 molecule (*cd27*), interleukin 1 beta (*il1b*), interleukin 1 receptor type 2 (*il1r2*), interleukin 6 receptor (*il6r*), interleukin 7 receptor (*il7r*), PYD and CARD domain-containing proteins (*pycard*), and heat shock protein 90 alpha family class B

Fig. 1. GO functional classification of annotated transcripts, according to their assigned A) biological process, B) molecular function, and C) cellular component. Analyses were carried out with the Blast2Go program for the red cusk-eel head-kidney transcriptome.

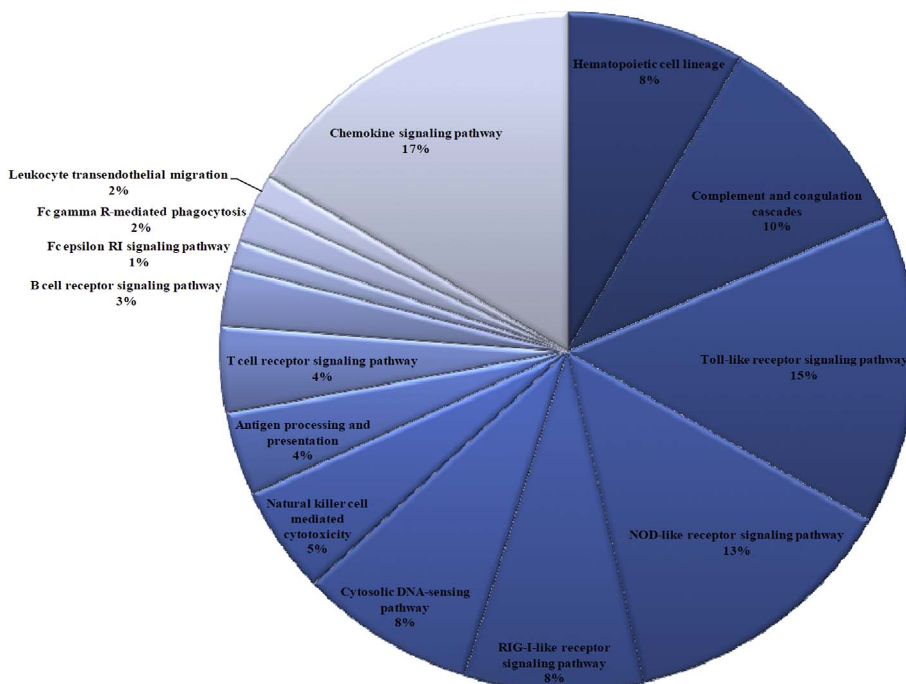

Fig. 2. Distribution of immune system KEGG pathways in the *G. chilensis* head-kidney transcriptome. The chart shows the percentages of transcripts that are assigned within different pathway categories.

Table 2
Enrichent of up-regulated and down-regulated transcripts in response to handling-stress associated to biological processes.

GO ID	GO term	Genes counts	p-Value
Enrichment of up regulated transcripts in head-kidney under stress			
GO: 0006950	Response to stress	80	5.90E-10
GO: 0009607	Response to biotic stimulus	31	4.50E-08
GO: 0006955	Immune response	43	6.20E-08
GO: 0032879	Regulation of localization	57	1.10E-07
GO: 0002252	Immune effector process	27	2.70E-07
GO: 0009605	Response to external stimulus	49	6.70E-07
GO: 0098602	Single organism cell adhesion	27	6.90E-07
GO: 0051239	Regulation of multicellular organismal process	57	1.30E-06
GO: 0044763	Single-organism cellular process	166	1.60E-06
GO: 1,902,578	Single-organism localization	69	3.50E-06
GO: 0007155	Cell adhesion	41	4.90E-06
GO: 0044710	Single-organism metabolic process	73	1.40E-05
GO: 0042221	Response to chemical	73	2.30E-05
GO: 0050900	Leukocyte migration	16	2.40E-05
GO: 0002682	Regulation of immune system process	34	2.80E-05
Enrichment of down regulated transcripts in Head-Kidney under stress			
GO:0044710	Single-organism metabolic process	76	4.10E-14
GO:0044237	Cellular metabolic process	111	3.00E-06
GO:0071704	Organic substance metabolic process	114	5.10E-06
GO:0009056	Catabolic process	36	1.30E-05
GO:0044238	Primary metabolic process	104	8.30E-04
GO:0006807	Nitrogen compound metabolic process	75	3.20E-03
GO:0044763	Single-organism cellular process	115	9.20E-03
GO:0019882	Antigen processing and presentation	7	1.20E-02
GO:0051234	Establishment of localization	53	1.50E-02
GO:0044764	Multi-organism cellular process	16	1.50E-02
GO:0044419	Interspecies interaction between organisms	16	1.80E-02
GO:0051707	Response to other organism	14	2.00E-02
GO:0051641	Cellular localization	31	2.60E-02
GO:0009607	Response to biotic stimulus	14	2.90E-02
GO:0042221	Response to chemical	45	3.40E-02

member 1 (*hsp90ab1*). A general down-regulation was observed in metabolic pathways, including 2-oxocarboxylic acid metabolism, carbon metabolism, glutathione metabolism, oxidative phosphorylation, glyoxylate and dicarboxylate metabolism, citrate cycle (TCA cycle), alanine-aspartate-glutamate metabolism, cysteine and methionine metabolism, metabolism of xenobiotics by cytochrome P450, and biosynthesis of amino acids (Supplementary file).

Nine up-regulated transcripts related to immune response were selected for RT-qPCR analysis: interferon regulatory factor 1 (*irf1*), C-C motif chemokine ligand 44 (*ccl44*), CCAAT enhancer-binding protein delta (*cebpd*), C-C motif chemokine ligand 20 (*ccl20*), C-C motif chemokine ligand 2 (*ccl2*), C-X-C motif chemokine ligand 10 (*cxcl10*), C-X-C chemokine receptor type 1 (*cxcr1*), C-X-C chemokine receptor type 2 (*cxcr2*), and tumor necrosis factor alpha (*tnfa*). The fold-changes in transcript expression, as measured using qPCR and RNA-seq, had a high statistical correlation ($R^2 = 0.84$, P-value < 0.001) (Fig. 3).

4. Discussion

In the present study, we investigated the effects of handling-stress on the global gene expression in the head-kidney of the red cusk-eel (*G. chilensis*), a marine teleost. The head-kidney, equivalent to the mammalian adrenal gland, is an exceptional tissue for teleost physiology (Geven and Klaren, 2017). This organ plays a central role in organizing the stress response involving a close communication between neural, immune and endocrine systems (Tort, 2011). It comprises cytokine-producing lymphoid cells from the immune system and endocrine cells secreting stress hormones (cortisol and catecholamines) (Takahashi et al., 2013). By using next generation sequencing (NGS), we have been able to sequence and annotate a head-kidney reference transcriptome for the red cusk-eel, important specie for Chilean aquaculture diversification. With Illumina MiSeq technology, we generated around 61,655,525 reads from head-kidney tissue, which were *de novo* assembled in one transcriptome consisting of 86,840 transcripts. Our transcriptome compared to Actinopterygii BUSCO database revealed a residual incompleteness of the *de novo* assembled transcriptome evidenced by the missing BUSCOs (14%). This percentage can be explained by the fact that some genes were not expressed in the head kidney tissue, similar to the percentage reported in spiny dogfish shark (*Squalus acanthias*) kidney transcriptome (Chana-Munoz et al., 2017). The functional annotation and gene ontology analysis revealed that 3.51% of the annotated transcripts were related immune system process, allowing the identification of Cytokines, Complement system, Immunoglobulins, Toll and Nod receptors, among others immune-relevant genes. Recently, we reported a hybrid reference transcriptome for this species based on liver and muscle samples, however only a very low number of sequences related to immune activity were detected (Aedo et al., 2014). Similar approximation has been reported in recent years to characterize the head-kidney transcriptome from different non-model fish as a source for immune relevant genes. For example in a recent study performed in the Antarctic notothenioid fish (*Trematomus bernacchii*), the *de novo* assembly of the head-kidney transcriptome allowed the identification of a subset of genes associated with immune response, representing 3.33% of the genes contained in the transcriptome (Gerdol et al., 2015), similar to our finding.

Additionally in this study, RNA-seq analysis was used to determine

Table 3
List of up-regulated transcripts related to cytokine–cytokine interaction, TNF signaling, and NOD signaling.

Accession	Gene description	Fold change	Gene ID	KEGG pathways involved
Kidney-contig506	CD27 molecule	15.66	*cd27*	Cytokine cytokine interaction
Kidney-contig12054	Interleukin 1 receptor type 2	11.35	*il1r2*	Cytokine cytokine interaction
Kidney-contig5779	PGAM family member 5, mitochondrial serine/threonine protein phosphatase	8.80	*pgam5*	TNF signaling
Kidney-contig31440	PYD and CARD domain containing	8.64	*pycard*	NOD signaling
Kidney-contig15944	Interleukin 1 beta	7.45	*il1b*	Cytokine cytokine interaction; NOD signaling; TNF signaling
Kidney-contig20870-e1	C-X-C motif chemokine receptor 2	6.72	*cxcr2*	Cytokine cytokine interaction
Kidney-contig29782	Heat shock protein 90 alpha family class B member 1	3.85	*hsp90ab1*	NOD signaling
Kidney-contig20871	C-X-C motif chemokine receptor 1	2.64	*cxcr1*	Cytokine cytokine interaction
Kidney-contig5410	Interleukin 7 receptor	2.54	*il7r*	Cytokine cytokine interaction
Kidney-contig11544	C-C motif chemokine ligand 2	2.29	*ccl2*	Cytokine cytokine interaction; NOD signaling; TNF signaling
Kidney-contig3216	C-X-C motif chemokine ligand 10	2.22	*cxcl10*	Cytokine cytokine interaction; TNF signaling
Kidney-contig4043-e2	C-C motif chemokine ligand 20	2.08	*ccl20*	Cytokine cytokine interaction; TNF signaling
Kidney-contig5819	Interleukin 6 receptor	2.02	*il6r*	Cytokine cytokine interaction
Kidney-contig26477	Tumor necrosis factor alpha	2.01	*tnfa*	TNF signaling

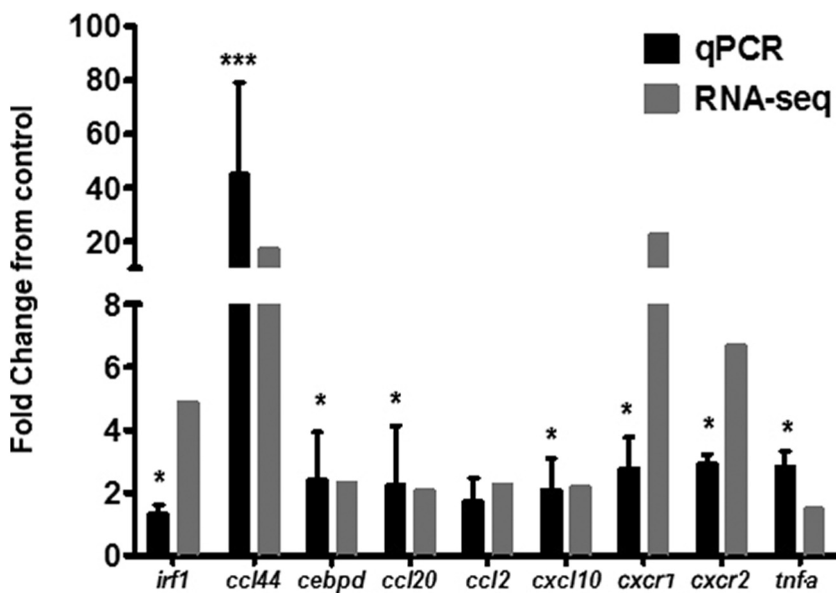

Fig. 3. Quantitative real time PCR validation of differentially expressed transcripts. Expression of fold-changes measured by RNA-seq and qPCR are indicated in the grey and black columns, respectively. *fau*: 40S ribosomal protein. Significant differences between the control and stress groups are shown as * ($P < 0.05$) and ** ($P < 0.01$).

the effects of handling-stress on head-kidney transcriptome. We determined that up-regulated genes were mainly associated with biological processes, such as stress response and immune response, and the down-regulated genes were associated with metabolic processes. Numerous studies have demonstrated a direct relationship between the intensity, frequency, and duration of stress with the immune response (Yada and Tort, 2016). For example, short term acute stressors may lead to enhanced immune response, while long term chronic stressors may result in decreased immune competence (Aluru and Vijayan, 2009). Although this rule is widely accepted, it is also important to consider the species receiving the stimulus, since each species implements different adaptive mechanisms and molecular responses against stress (Pankhurst, 2011). The comparison of head-kidney transcriptomic profiles of rainbow trout (*Oncorhynchus mykiss*) and Maraena whitefish (*Coregonus maraena*), both salmonids, exposed to high stocking densities over 9 days revealed different expression patterns. Maraena whitefish exposed to high stocking densities induced the up-regulation of 369 genes and the down-regulation of 507 genes (Korytář et al., 2016). Remarkably, 80 out of 396 upregulated genes in the head-kidney were related to immunological processes; similar to our findings. Conversely, rainbow trout showed a poor head-kidney response, with only a single differentially expressed gene corresponding to glycogen phosphorylase, involved in glycogen metabolism (Rebl et al., 2017). Another study performed in rainbow trout determined that handling-stress induced a minor transcriptomic response in the kidney compared to brain (Krasnov et al., 2005). In the same study, handling-stress was shown to induce repression in the expression of genes associated with carboxylic acid metabolism, amine metabolism and oxidative phosphorylation after the fifth day of the application of the stimulus, which is in agreement with our observations.

Among the genes associated with immune response that showed an increased expression due to stress, several stand out, namely inflammatory cytokine (*il1b*, *tnfa*), receptors (*il1r2*, *il6r*, *il7r*), and mediating molecules (*ifi35*, *irf1*). Our results are consistent with those obtained in other species using similar acute stress protocols. In common carp (*Cyprinus carpio* L.), acute restraint-stress induced an up-regulation of *il1b* and its receptor in head-kidneys 24 h following stimulus (Metz et al., 2006). In Atlantic cod (*Gadus morhua*), short term overcrowding induced an up-regulation of pro-inflamatory cytokines *il1b* and *il8* (Caipang et al., 2008). Similarly, in channel catfish (*Ictalurus punctatus*) and Atlantic salmon (*Salmo salar* Linnaeus), the over expression of *il1b* in head-kidneys was also observed after handling-stress (Fast et al., 2008; Yang et al., 2015). Interestingly, a similar up-regulation of pro-inflamatory cytokines and NOD signaling were observed in the Japanese pufferfish (*Takifugu rubripes*) head kidney cells stimulated with nigericin (Bilen et al., 2014). However, extensive periods of chronic stress have been shown to induce a decrease in the expression of the proinflammatory cytokines *tnfa*, *il1b*, *il8* and *inf* in rainbow trout (Yarahmadi et al., 2016). Finally, cortisol has been shown to preferentially modulate immunosuppressive processes. In rainbow trout, cortisol dispensed through an intraperitoneal slow release implant induced a down-regulation of *il1b* and *tnfa* after 5 days (Cortés et al., 2013). Moreover, *in vitro* studies performed on rainbow trout macrophages and leukocytes revealed that cortisol inhibited the gene expression of proinflammatory cytokines (Holland et al., 2003; MacKenzie et al., 2006). Undoubtedly, this fine balance between immunostimulation and immunosuppression is conditioned by the periods and type of stressors, as well as the species-specific immune response.

In summary, handling-stress induced major changes in the red cusk-eel head-kidney response. Under stress, 569 transcripts, mostly associated with response to stress and immune response, were up-regulated. Conversely, 513 transcripts, associated with metabolic processes were down-regulated. The present data demonstrate that short term handling-stress promotes an immune response in the marine teleost *G. chilensis*, through the component expression of inflammatory cytokines and NOD signaling pathways.

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