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# Stocking density induces differential expression of immune-related genes in skeletal muscle and head kidney of fine flounder (*Paralichthys adspersus*)

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ABSTRACT

Immunity can be modulated by different internal and external factors, being stress one of the most important. However, the stress effects on the immunocompetence of the skeletal muscle has not been studied in detail in earlier vertebrates. Here, we examine the effect of chronic (4 and 7 weeks) crowding stress on the immunocompetence of skeletal muscle and head kidney in the fine flounder (*Paralichthys adspersus*). Corticosteroid receptor transcript levels and their target genes; pro-inflammatory cytokines, and Toll-, NOD-, and RIG-like receptors were quantified by qPCR. The results indicate that chronic stress down-regulates the expression of these genes in muscle, compromising skeletal muscle immunocompetence, while the expression of these genes is upregulated in head kidney after seven weeks of crowding stress. The data suggests that chronic stress modulates the expression of these immune-related genes in a tissue-specific manner.

## 1. Introduction

In vertebrates, stress and immunity are important physiological processes that mediate the ability to respond to harmful elements such as pathogens. In early vertebrates such as teleosts, research has primarily focused on such immunity-associated organs as spleen, gut-associated lymphoid tissue, thymus, and head kidney (Zapata and Amemiya, 2000), while skeletal muscle has received little attention (Valenzuela et al., 2017). However, in mammals, skeletal muscle is known to play a protective role when animals become infected, and this is likely to be of biological significance as skeletal muscle is the largest organ of the body with extensive immunological reaction sites (Marino et al., 2011; Wiendl et al., 2005). Thus, skeletal muscle expresses several immune-related molecules such as Toll, NOD, and RIG-like receptors, as well as pro-inflammatory cytokines (IL-1β, TNFα, and IL-8) (Frost and Lang, 2007; Keller et al., 2011; Rigamonti et al., 2013).

The stress generated by rearing fish under high-density conditions can leads to immunosuppression, which increases pathogen susceptibility, negatively affecting fish welfare and health (Salas-Leiton et al., 2010; Yarahmadi et al., 2016). Short periods of strong stressor exposure, i.e. acute stress, enhances immune responses in fish, whereas low-level stress over longer time, i.e. chronic stress, decreases it (Tort, 2011). Both in acute or chronic stress, the primary response is a release of cortisol, a hormone modulating several physiological processes, including the ability to develop an immune response (Cortés et al. 2013; Wunderink et al., 2011). Cortisol increases the gene expression of its receptors, GR1, GR2 and MR, which have been studied in several tissues under stress responses (Philip and Vijayan, 2015; Teles et al., 2013a, b). Previously, we have shown that high stocking-density induces a chronic stress response in the fine flounder, by releasing cortisol, activating the glucocorticoid pathway in the muscle, and decreasing growth (Valenzuela et al., 2018). However, the effect of chronic stress response on skeletal muscle immunocompetence in teleosts has not been studied.

In this study, we examine the molecular effect of high stocking density stress over the expression of immune-related genes in the skeletal muscle and compare it with that of the head kidney in fine

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**Table 1**
Primer sequences for qPCR assay, amplicon size, PCR efficiencies, and GeneBank accession number for the genes used in this study.

| Gene | Primer | Sequence (5´-3´) | Size (bp) | Annealing T° (°C) | Efficiency (%) | Accesion Number |
|---|---|---|---|---|---|---|
| *il-1β* | Foward | AGGGAGGCAGCAACAA | 215 | 58 | 103.6 | KJ605419.1 |
| | Reverse | AATCGCACCATCTCACTTTC | | | | |
| *il-8* | Foward | CGAGGGTTCAGACAGACCTT | 194 | 60 | 103.1 | SRX612429* |
| | Reverse | TTTGCTCTCAGTGACGGGAT | | | | |
| *tnfα* | Foward | AGCGGAGAAACACCTCACAT | 144 | 56 | 102.3 | KJ605417.1 |
| | Reverse | TACCAGCCCTGTCCGTCTC | | | | |
| *tlr2* | Foward | GTTTTCAAGTTTGTCTCATTTCT | 155 | 57 | 104.2 | SRX612429* |
| | Reverse | GTCAGCAGGTTGTCGGA | | | | |
| *tlr9* | Foward | TTAGTGAGGACCCAAAATGTC | 198 | 58 | 98.5 | SRX612429* |
| | Reverse | CAGAGTCTGAAGGTTGAGAACA | | | | |
| *tlr14* | Foward | AGACGAGGCAGATGGGT | 102 | 60 | 99.5 | SRX612429* |
| | Reverse | ACTGGATGACGCTGAATGT | | | | |
| *tlr21* | Foward | GTTTTCAAGTTTGTCTCATTTCT | 192 | 58 | 103.4 | SRX612429* |
| | Reverse | GTCAGCAGGTTGTCGGA | | | | |
| *gr1* | Foward | ACCTGCCTTTGCCCAACCTT | 130 | 64 | 97.4 | SRX612429* |
| | Reverse | CGACCGCTGTTCCCATCCA | | | | |
| *gr2* | Foward | CCAGAGTTACTGCCAAATGA | 162 | 59 | 99.5 | SRX612429* |
| | Reverse | GACACTGACTTCGGTTCCTT | | | | |
| *mr* | Foward | TACAGAACGACACCAACAGCC | 96 | 60 | 100.4 | SRX612429* |
| | Reverse | CCCCGTCAACCATCTCTACTT | | | | |
| *klf15* | Foward | GGCGAAAGGTTCAAGGACA | 246 | 60 | 100.2 | SRX612429* |
| | Reverse | ACACTGGAGGCACTGGTATC | | | | |
| *redd1* | Foward | GACTCAGGCTCCGACATCTCC | 126 | 63 | 99.8 | SRX612429* |
| | Reverse | TTTGGCTTCTCGCAGGCTT | | | | |
| *hsp60* | Foward | CACTGTGTTCGGGGATGA | 207 | 58 | 101.1 | SRX612429* |
| | Reverse | TTGAGTTTCTCCTTCTCGTAGT | | | | |
| *hsp70* | Foward | ATCGTCCTGGTTGGTGG | 95 | 60 | 103.1 | SRX612429* |
| | Reverse | GGGTTGATACTCTTGTTCAGTT | | | | |
| *hsp90b* | Foward | CACTCTCAGTTCATCGGC | 194 | 58 | 99.8 | SRX612429* |
| | Reverse | TTCTTCTTCTTGTCCTTGTCT | | | | |
| *nlrc3* | Foward | GCCAACAAACAACCTCCCTGACG | 192 | 60 | 101.6 | SRX612429* |
| | Reverse | TTCCTGTCTGCGTCAGC | | | | |
| *nod1* | Foward | CCTGAGGCTGCTGTTCTC | 170 | 60 | 102.1 | SRX612429* |
| | Reverse | TTGATGTTGGGATGTGGGG | | | | |
| *mda5* | Foward | CCGCAAGAAAATGATGAACA | 166 | 60 | 99.4 | SRX612429* |
| | Reverse | CACTTTAGATGGACACTCACTC | | | | |
| *lpg2* | Foward | GCGGTGTAGCGACTCCT | 226 | 60 | 98.9 | SRX612429* |
| | Reverse | CGCAAGACAGCCAGTATTCAG | | | | |
| *fau* | Foward | CATTTAGGAGTTGGCGTTGG | 134 | 60 | 100 | JN635279.1 |
| | Reverse | CCAAGGTTGAAAAGCAGGAG | | | | |

* Sequences derived from the fine flounder transcriptome raw data (SRX612429).

flounder (*Paralichthys adspersus*), a flatfish species endemic to the southeast Pacific with a great aquaculture potential in the region (Fuentes et al., 2008), subjected to 4 and 7 weeks of crowding stress.

## 2. Materials and methods

### 2.1. Ethics statement

The study 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 and has adhered to accepted animal welfare procedures.

### 2.2. Fish husbandry, experimental design and sampling

One-year old, sexually immature fine flounders, 105 ± 10 g body weight and 19 ± 2 cm body length, were obtained from the Centro de Investigación Marina de Quintay (CIMARQ), Valparaíso, Chile. This experimental design of stress model has been recently validated (Valenzuela et al., 2018). Briefly, fish were randomly divided in four different tanks. Two "low stocking-density" (LD 6.8 ± 1.3 kg $m^{-3}$), and two "high stocking-density" tanks (HD, 17.3 ± 1.8 kg $m^{-3}$). Fish from LD and HD tanks were sampled at four and seven weeks. Six individuals were collected from each group at four and seven weeks (sampling points) and immediately euthanized under anesthesia overdose (3-aminobenzoic acid ethyl ester, 300 mg $L^{-1}$). White myotomal muscle and head kidney were sampled, immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Previously, plasma cortisol levels and growth-related parameters (weight, length and condition factor) have been reported from this experiment (Valenzuela et al., 2018), demonstrating that the HD treatment causes chronic stress. The data obtained for the HD group after four weeks of treatment can be then interpreted as the physiological transition from acute to chronic stress response characterized by no weight gain and a decrease in condition factor. For this reason, from now on the four weeks treatment will be mentioned as the transition period (TP) throughout this article.

### 2.3. Quantitative real time PCR (qPCR)

Total RNA was isolated from skeletal muscle and head kidney using the RNeasy® Mini Kit (Qiagen, Austin, TX, USA), following the manufacturer's recommendations. RNA was quantified using the Epoch Multi-Volume Spectrophotometer System (BioTek, Winooski, VT, USA). RNA quality was assessed via electrophoresis in a 1.2% formaldehyde agarose gel containing ethidium bromide. Subsequently, residual genomic DNA was removed and 1 μg of RNA was reverse transcribed onto cDNA for 30 min at 42 °C, following manufacturer recommendations of Quantitect® reverse transcription kit (Qiagen, Austin, TX, USA).

To obtain sequences of stress, and immune-related genes, we searched in the raw transcriptome data for fine flounder available on the NCBI database (SRX612429) by the BioEdit Sequence Alignment Editor

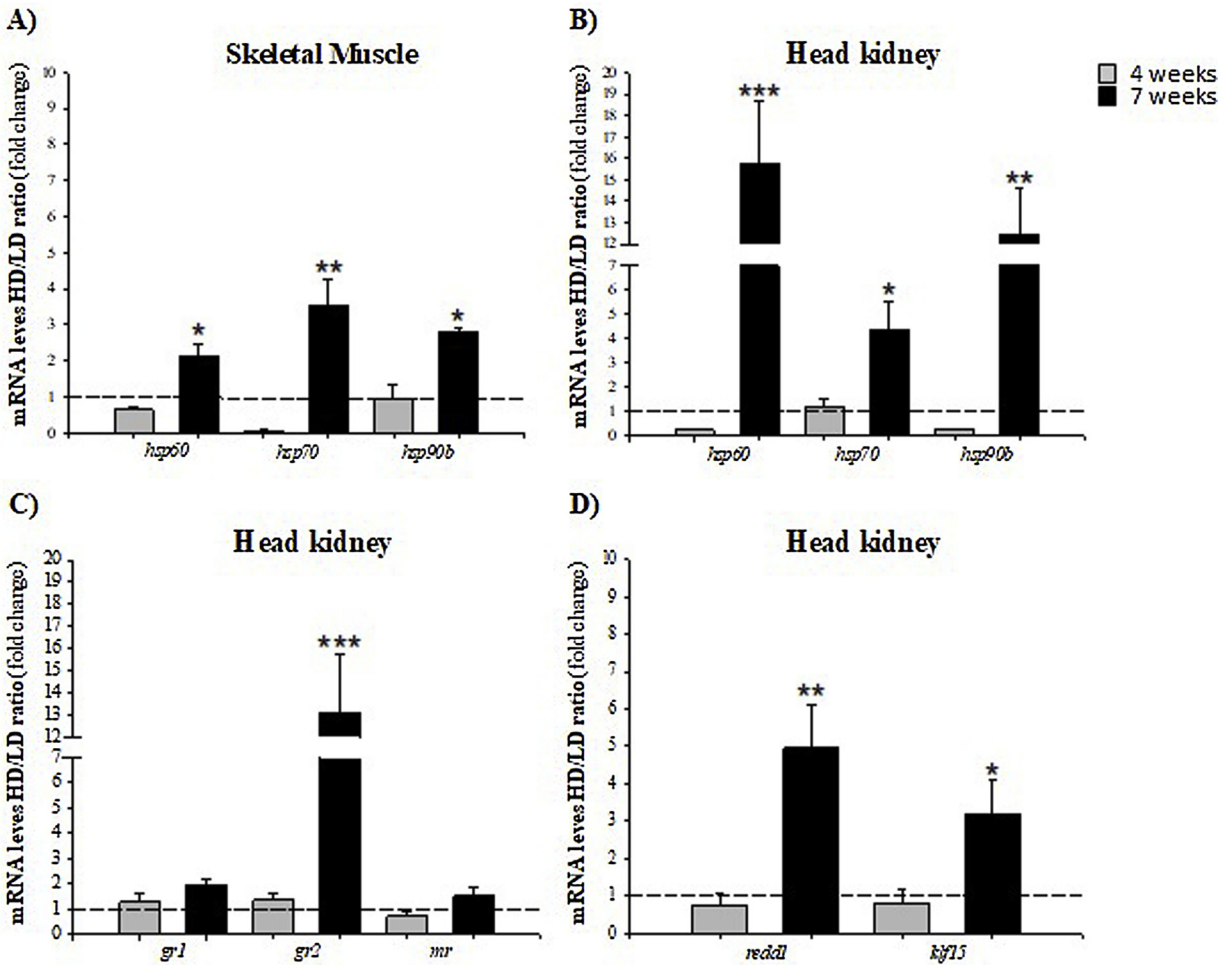


**Fig. 1.** Real-time qPCR analysis of stress related genes *gr1*, *gr2*, *mr*, *redd1*, *klf15*, *hsp60*, *hsp70*, and *hsp90b* in the skeletal muscle (A) and in head kidney (B–D) of fine founder following four (grey bar) and seven (black bar) weeks of high stocking-density stress protocol. Values shown as the mean ± SEM for control and experimental groups (n = 6 per group). Grey and black bars represent, respectively, the 4- and 7-week sampling points in the high stocking-density treatment. Expression values were normalized to *fau* as housekeeping gene. Significant differences are highlighted as * ($P \leq 0.05$), ** ($P \leq 0.01$) and *** ($P \leq 0.001$). The horizontal line originating at $y = 1$ denotes the control group against which expressions were compared.

Tool (Ibis Biosciences, Carlsbad, CA, USA). All information on the fine flounder transcriptome has been made available (Mendez et al., 2018).

To ensure high-quality primer design, we used Primer3web (https://primer3plus.com/primer3web/primer3web_input.htm) and NetPrimer (www.premierbiosoft.com/netprimer) software. The list of primers and efficiencies used in this study are listed in Table 1.

All qPCR assessments were performed by MX3000P system (Stratagene, La Jolla, CA, USA) and described in detail in Valenzuela et al., 2018 with minor modifications. Amplifications were performed in triplicate using the following thermal cycling conditions: initial activation 95 °C for 10 min, 40 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 54–60 °C, and 30 s of elongation at 72 °C. The QGene program was used for analyzing gene expression (Simon, 2003 DOI: https://doi.org/10.1093/bioinformatics/btg157) and results were expressed as HD/LD ratio in fold change using *fau* as stable reference gene.

### 2.4. Statistical analysis

All data were analyzed using a two-way ANOVA and a Tukey's honestly significant difference (HSD) as a post-hoc test, using STATISTICA 7 (Tulsa, OK, USA). A probability level of $P \leq 0.05$ was used as minimum to indicate statistical significance.

## 3. Results and discussion

In a previous report, we demonstrated that crowding stress (high stocking density (HD) results in an increase of plasmatic cortisol levels, a well know indicator of stress in fish. These findings were associated to the skeletal muscle response in fine flounder, where the stress condition led to an activation of the proteolytic mechanisms after 4-weeks and subsequently triggering autophagy processes and body weight loss after 7-weeks in this tissue (Valenzuela et al., 2018). In this context, the present study shows that crowding stress can regulate different pathways associated with health effects (stress and immunity) in fine flounder, particularly the differential expression of immune-related genes between skeletal muscle and head kidney.

### 3.1. Up-regulation of stress-related genes in the skeletal muscle and head kidney of fine flounder during transition and chronic stress

The mRNA levels of the stress-related genes *hsp60, hsp70, hsp90, gr1, gr2, mr, klf15* and *redd1* were assessed. After 7-week HD, mRNA levels of the *hsp* genes were upregulated in skeletal muscle (Fig. 1A), while, head kidney exhibited increased mRNA levels of all stress-related genes throughout the study, except for *gr1* and *mr* that were not affected by stress (Fig. 1B–D). Similar effects have been observed in rainbow trout (*Oncorhynchus mykiss*) liver stimulated with cortisol, where an increased expression of the stress markers *gr2* and *hsp90* was observed

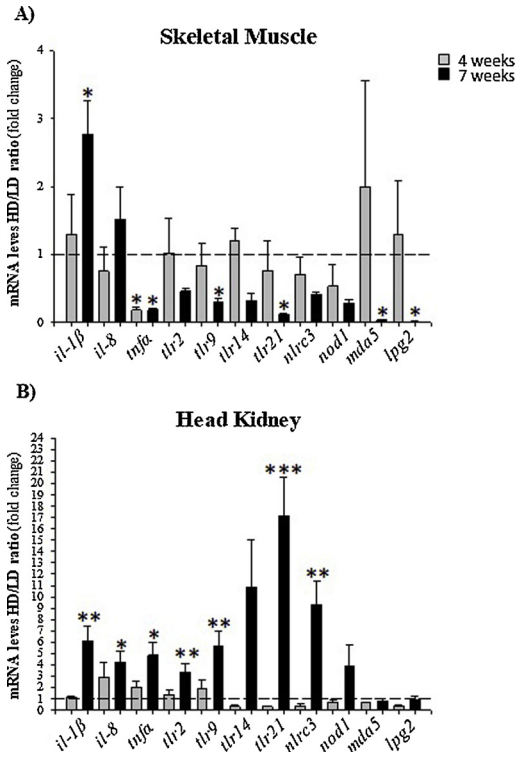


**Fig. 2.** Real-time qPCR analysis of immune-related genes *il-1β*, *il-8*, *tnfα*, tlr*2*, *tlr9*, *tlr14*, *tlr21*, *nlrc3*, *nod1*, *mda5*, and *lpg2* in the skeletal muscle (A) and in the head kidney (B) and of fine founder maintained under high stocking-density stress protocol after 4 (grey bars) and 7 (black bars) weeks, respectively). Values shown as the mean ± SEM for control and experimental group (n = 6 per group). Expression values were normalized to *fau* as housekeeping gene. Significant differences are highlighted as * ($P \leq 0.05$), ** ($P \leq 0.01$) and *** ($P \leq 0.001$). The horizontal line originating at $y = 1$ denotes the control group against which the expression was compared.

(Vijayan et al., 2003). Moreover, the mRNA levels of *hsp70* increased and the pro-inflammatory cytokines decreased in head kidney of rainbow trout maintained under different stocking densities, suggesting that immune system down-regulation was induced by chronic stress (Yarahmadi et al., 2016).

### 3.2. Contrasting mRNA levels of pattern recognition receptors and pro-inflammatory cytokines between skeletal muscle and head kidney during transition and chronic stress

The mRNA levels of several receptors associated with the immune response (PRRs; pattern recognition receptors: *tlr2*, *tlr9*, *tlr14*, *tlr21*, *nlrc3*, *nod*1, *mda5*, *and lpg2)* and pro-inflammatory cytokines (*il-1β*, *tnfα*, and *il-8)* were assessed. *il-1β* and *il-8* remained unchanged in the skeletal muscle, while *tnfα* expression significantly decreased during 4-week stress (Fig. 2A). A similar response was observed in the skin of turbot (*Psetta maxima*) subjected to long-term overcrowding, where the mRNA levels of *il-1β* and *tnfα* were down-regulated and the levels of plasma cortisol and glucose as well as the *hsp70* expression were increased (Jia et al., 2016). In addition, the PRRs gene expression remained unchanged after this transition period (Fig. 2A). However, after 7-week HD, only *il-1β* was up-regulated, while the PRRs were down-regulated (Fig. 2A). The correlation between these immune-related genes and stress markers was previously reported in fish species under different nutritional status (Valenzuela et al., 2015), implicating the participation of *il-1β* in the activation of catabolic processes under stress conditions in muscle. Further, in mammals, increased mRNA levels of *il-1β* have been described as an atrophy promoter through its activation of inhibitors of the kappa B alpha (IkBa)/nuclear factor kappa B (NFkB) and the mitogen-activated protein kinase P38-MAPK signaling pathways (Glass, 2005; Ladner et al., 2003).

In contrast, the expression levels of the PRRs remained unchanged following 4-weeks of treatment (transition period) in head kidney (Fig. 2B). Also, the expression levels of *il-1β*, *tnfα*, *il-8, tlr2*, *tlr9*, *tlr21*, and *nlrc3* were up-regulated following 7-weeks HD, as were the expression levels of the pro-inflammatory cytokines (Fig. 2B). A few reports have shown effects of stress on PRR expression, where thermal and hypoxic-induced stress increases mRNA levels of several toll like-receptors and nod like-receptors (Basu et al., 2015, 2016).The present study adds to the state-of-the-art by showing a tissue-specific effect of the stress response, where the skeletal muscle tissue is strongly affected, which can suggests an increased pathogen susceptibility of stressed fish reared in high stocking-density conditions, as found in the aquaculture industry. In a recent article, we described the skeletal muscle as an active site of immune reactions in fish challenged with *V. ordalii*, where the mRNA level of pro-inflammatory cytokines, PRRs and effector factors were up-regulated (Valenzuela et al., 2017). However, functional experiments are still required to demonstrate the skeletal muscle immunocompetence to respond against pathogens.

## 4. Conclusion

In conclusion, high stocking-density triggers a strong stress response at the molecular level, with an up-regulation of immune-related genes in head kidney while the muscle becomes down-regulated, which may adversely affect the normal muscle function. The strong effect of stress on skeletal muscle could explain why this tissue is one of the most affected by different pathologies in aquaculture. In addition, these results suggest IL-1β is a good stress-response marker in fish muscle, which could be associated to the activation of catabolic mechanisms, such as autophagy. Our results indicate that skeletal muscle could be an important focus of study, where the immuno-modulation of this tissue can benefit the aquaculture industry. In this context, our research group is conducting different approaches (in vitro and in vivo) to demonstrate the importance of skeletal muscle as a focus of immune reactions.

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## Author contributions

CAV, BThB, JAV and AM conceived and designed the experiment. CAV, SE, RZ, and TVT performed the experiments. CAV, SE, RZ, TVT, and LM analyzed and interpreted the results. CVA, SE, RZ, and LM wrote the first draft. All authors read, commented, and approved the final version of the manuscript.

## Competing interests

The authors declare no conflicts of interest, financial or otherwise.

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