Cellular stress responses of *Eleginops maclovinus* fish injected with *Piscirickettsia salmonis* and submitted to thermal stress

D. Martínez^{1,2,3} · C. Vargas-Lagos^{1,3,4,5} · J. Saravia^{1,2,3} · R. Oyarzún^{1,2,3} · C. Loncoman¹ · J. P. Pontigo¹ · L. Vargas-Chacoff^{1,3}

Received: 8 August 2019 / Revised: 25 October 2019 / Accepted: 31 October 2019 / Published online: 13 December 2019
© Cell Stress Society International 2019

Abstract

Fluctuations in ambient temperature along with the presence of pathogenic microorganisms can induce important cellular changes that alter the homeostasis of ectothermic fish. The aim of this study was to evaluate how sudden or gradual changes in environmental temperature together with the administration of *Piscirickettsia salmonis* modulate the transcription of genes involved in cellular stress response in the liver of *Eleginops maclovinus*. Fish were subjected to the following experimental conditions in duplicate: C– 12 °C: Injection only with culture medium, C+ 12 °C: Injection with *P. salmonis*, AM 18 °C: Injection only with culture medium under acclimation at 18 °C, AB 18 °C: Injection with *P. salmonis* under acclimation at 18 °C, SM 18 °C: Injection only with culture medium and thermal shock at 18 °C and SB 18 °C: Injection with *P. salmonis* and thermal shock at 18 °C and sampling at 4-, 8-, 12-, 16- and 20-day post injection (dpi). The genes implied in the heat shock response (HSP70, HSC70, HSP90, and GRP78), apoptosis pathway (BAX and SMAC/Diablo), ubiquitination (E2, E3, ubiquitin, and CHIP), and 26 proteasome complex (PSMB7, PSMC1, and PSMA2) showed expression profiles dependent on time and type of injection applied. All the genes greatly increased their expression levels at day 16 and showed moderate increases at day 20, except for PSMA2 which showed a higher increase between 4- and 12-day post challenges. Our results suggest that the changes observed at the final days of the experiment are due to temperature more than *P. salmonis*.

Keywords *Eleginops maclovinus* · Heat shock proteins · Sub-Antarctic Notothenioid · Gene expression · Thermal stress · *Piscirickettsia salmonis*

Introduction

Fish are the first vertebrates to develop a stress response that involves the activation of the Hypothalamus-Pituitary-Interrenal axis and the Hypothalamus-Sympathetic-Chromaffin axis (Wendelaar Bonga 1997; Weyts et al. 1999). Both axes can be activated by sudden or extreme

changes in stress stimulators; such as changes in temperature, salinity, turbidity, predation, parasites, microorganisms, pH, heavy metals, and organic chemicals (Wendelaar Bonga 1997). All these stimuli can be perceived by the central nervous system, specifically in the hypothalamus, where they trigger physiological changes at a primary (release of catecholamine's and cortisol), secondary (cellular changes,

✉ D. Martínez
danixapamela@gmail.com

✉ L. Vargas-Chacoff
luis.vargas@uach.cl

¹ Instituto de Ciencias Marinas y Limnológicas, Laboratorio de Fisiología de Peces, Universidad Austral de Chile, Valdivia, Chile

² Escuela de Graduados, Programa de Doctorado en Ciencias de la Acuicultura, Universidad Austral de Chile, Av. Los Pinos s/n Balneario Pelluco, Puerto Montt, Chile

³ Centro Fondap de Investigación de Altas Latitudes (IDEAL), Universidad Austral de Chile, casilla, 567 Valdivia, Chile

⁴ Escuela de Graduados, Programa de Magister en Microbiología, Universidad Austral de Chile, Valdivia, Chile

⁵ Interdisciplinary Center for Aquaculture Research (INCAR), Concepción, Chile

osmotic, hematological, and immunological), and tertiary (changes at the level of development and behavior) level, which are intended to preserve homeostasis (Barton 2002).

Environmental temperature is considered a crucial extrinsic factor on the regulation of fish physiology, determining its performance (fecundity, growth, metabolic rate, and swimming speed), its distribution, and its abundance (Schulte 2011). Specifically, the temperature can induce important cellular changes in the tissue of fish, including an increase in HSPs, activation of the core ubiquitin system, and induction of the apoptotic pathway (Cheng et al. 2015; Dalvi et al. 2017; Huang et al. 2017; Todgham et al. 2017). HSPs participate in the remodelling of damaged or misfolded proteins to conserve their native state. However, if these proteins cannot be repaired, they will be degraded by proteinases via the Ub-proteasome or lysosomal route (Ciechanover 2005). This ubiquitination process is a mechanism of post-translational modification that through the action of E1 (activation), E2 (conjugation), and E3 (ligation) marks the damaged or misfolded protein with multiple ubiquitin molecules which can then be degraded by the 26S complex proteasome (Goldberg 2003).

Many bacterial pathogens interfere with the ubiquitination pathway of their host cell as a mechanism of survival and replication (Perrett et al. 2011). This type of strategy remains unexplored in the tissues of fish challenged with *Piscirickettsia salmonis*, a facultative intracellular bacterium (Mauel et al. 2008; Mikalsen et al. 2008; Gómez et al. 2009; Yañez et al. 2012a, b) that causes high mortalities in the Chilean salmon industry (Rozas and Enríquez 2014). This microorganism was originally reported as a salmonids pathogen; however, there are reports in non-salmonid fish such as *Atractoscion nobilis* (Arkush et al. 2005), *Dicentrarchus labrax* (Athanasopoulou et al. 2004), *Sarotherodon melanotheron*, and *Oreochromis mossambicus* (Mauel et al. 2003). Even the DNA of *P. salmonis* can be detected in native Chilean fish such as *Eleginops maclovinus*, *Odontesthes regia*, *Sebastes capensis*, and *Salilota australis* (Contreras-Lynch et al. 2015), and it is unknown if the latter can act as a reservoir of this pathogen.

Infection with *P. salmonis* in salmonids causes positive regulation of genes associated with both cellular stress and protein synthesis/degradation processes, meanwhile the genes involved in the apoptotic pathway are downregulated 48-h post bacterial challenge (Tacchi et al. 2011). However, Rozas-Serri et al. (2018) indicate that *P. salmonis* can cause a downregulation in the expression of genes involved in the ubiquitin-proteasome response in the head kidney 5-day post bacterial challenge. To date, there are no studies that evaluate the

temporal regulation of genes associated with the cellular stress response in salmonids when they are exposed to *P. salmonis*, much less in the native fish species with which they cohabit and those with less commercial importance such as *E. maclovinus*. Fortt et al. (2007) indicate that *E. maclovinus* can be captured in sectors near and far from salmon farming centers, where they feed off the pellets not consumed by the farmed fish. The latter suggests an interaction in the natural environment between native fish and farmed fish (salmonids), and therefore a possible horizontal transmission of *P. salmonis*.

E. maclovinus is a Notothenioid fish that in its natural environment survives in a wide temperature range, which is why it is considered an eurythermic species (Pequeño 1989). As an ectotherm, many of this species physiological processes are influenced by environmental temperature, such as growth; feeding, metabolism, and osmoregulation (Vanella et al. 2012; Lattuca et al. 2018; Oyarzún et al. 2018); meanwhile, its connection with *P. salmonis* has been investigated in previous studies which suggest an activation of iron metabolism (Martínez et al. 2017a, b) and the upregulation of the immune response (Vargas-Chacoff et al. 2014a; Martínez et al. 2018a,b). However, it is still unknown how sudden or gradual changes in temperature together with the injection of *P. salmonis* modulate the temporal transcription of genes involved in the cellular stress response in this native species which cohabitates with *S. salar* and *P. salmonis*. This research is the first molecular approximation of the expression patterns of these genes in a native fish whose availability of genetic information is extremely scarce.

Materials and methods

Samples

Assessments were conducted with the same specimens and experimental procedures as used in Martínez et al. (2018b). The acclimatization of *E. maclovinus* (4 weeks) and the experimental challenge were performed in the Laboratory of Aquatic Pathology and Biotechnology, Faculty of Veterinary Sciences, Universidad Austral de Chile. Fish were acclimated (4 weeks) in 500-L tanks with seawater (32 psu, 1085 mOsm kg⁻¹) and at a density of 3.1 kg m⁻³, following indications given by Vargas-Chacoff et al. (2014b).

The experiment was performed following the standards of the Guide for the Care and Use of Laboratory Animals of the National Commission of Science and Technology (CONICYT, Chile) and the Universidad Austral de Chile. The Ethics protocol was approved by the Committee on the Ethics for Animal Experimentation of Universidad Austral de Chile Memorandum No. 261/2016.

Primer design

All steps involving massive cDNA sequencing were performed at the Austral-omics Laboratory on a GS Junior Titanium Series (Roche) following the manufacturer's protocols. In detail, total RNA was extracted from one tissue (head kidney of a healthy fish) with the commercial RNA NucleoSpin® RNA kit (Macherey-Nagel). RNA was selected with optimal integrity (RIN > 7). Subsequently, mRNA isolation was performed with the PolyATtract III kit® in an mRNA Isolation System (Promega). The quantity and quality of total mRNA was evaluated using an A260 NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. The cDNA library was constructed following the "Rapid cDNA library preparation" protocol recommended by Roche. Bioinformatics analysis of the obtained data included the formation of transcripts (contigs), specifically by using a combination of specialized transcriptomic analysis software followed by transcript annotation against the non-redundant NCBI database. After this process, mRNA sequences of the E2, E3, HSC70, and HSP90 were obtained and analyzed using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/>), and the deduced amino acid sequences were obtained by the Expert Protein Analysis System (ExPASy) (<http://www.expasy.org/>). Raw sequencing data have been deposited in the NCBI SRA database (Sequence Read Archive) with the accession ID SRP129851 and were published by Martínez et al. (2018c). The other genes selected for this investigation were amplified using heterologous primers. Partial cDNA coding sequences were obtained and loaded into GenBank with the accession numbers indicated in Table 1.

P. salmonis LF-89

Piscirickettsia salmonis was donated by the Metabolism and Biotechnology Laboratory, Faculty of Science, Universidad Austral de Chile (Valdivia, Chile). The growth protocol of this bacteria (ATCC® VR-1361™) was reported in Martínez et al. (2018c).

Infection assays with *P. salmonis* LF-89

Assessments were conducted with the same specimens and experimental procedures as used in Martínez et al. (2018b). Briefly, after the acclimatization period of 4 weeks (12 °C), the fish were randomly distributed in 100-L tanks for the application of the different experimental treatments in duplicate ($n = 180$ total). **C–12 °C:** Injection only with culture medium, **C+ 12 °C:** Injection with *P. salmonis*, **AM 18 °C:** Injection only with culture medium under acclimation at 18 °C, **AB 18 °C:** Injection with *P. salmonis* under acclimation at 18 °C, **SM 18 °C:** Injection only with culture medium and thermal shock at 18 °C, and **SB 18 °C:**

Injection with *P. salmonis* and thermal shock at 18 °C. The acclimation was achieved by increasing 1 °C/day from 12 to 18 °C, once acclimatized the fish were injected with culture medium or *P. salmonis*. The heat shock was achieved by the sudden transfer of the fish from 12 to 18 °C and before the thermal transfer the fish were injected with culture medium or *P. salmonis*. Each injection consisted to 100 µL of *P. salmonis* at a concentration of 1×10^8 bact/µL or 100 µL of culture medium, sampling six fish per group at 4-, 8-, 12-, 16-, and 20-day post injection (dpi). During the experiment, the fish were maintained in seawater (32 psu) at a density of 3.1 kg m^{-3} with a recirculating regime, and under a natural photoperiod, following indications given by Vargas-Chacoff et al. (2014b). The dissolved oxygen range was 9.2–10.4 and the oxygen saturation was over 80%. Fish were fed once daily at a ration of 1% of total body weight, using commercial dry pellets (Skretting Nutreco 100). This ration produces the highest growth and metabolism following the recommendation given by Oyarzún et al. (2019). The bacterial dose was the same as that used in previous studies (Martínez et al., 2017a, b, 2018a, b). The environmental temperature during the months (acclimatization + challenge) that the experiment was performed was 12 °C; therefore, it was selected as temperature control of our experiment. In addition, this temperature was the same used by Oyarzún et al. (2019) in *E. maclovinus* injected with different doses of *Francisella noatunensis*. 18 °C was the maximum thermal used by Oyarzún et al. (2018) in this same species and is within the optimal thermal range of *P. salmonis* replication (Fryer et al. 1990).

Sampling procedure

Twenty hours before each day of sampling, the fish were not fed. Subsequently, the fish were anesthetized with a lethal dose of 2-phenoxyethanol (1 mL L^{-1}), weighed, measured, and sampled. The liver of each fish was extracted aseptically and stored at -80 °C .

Total RNA extraction

Total RNA extraction was from 50 mg of liver via homogenization in TRIzol Reagent (Ambion), according to reported by Martínez et al. (2018c). Reverse transcription used 2 µg of total RNA as template, MMLV-RT reverse transcriptase (Promega), and oligo-dT primer (Invitrogen), according to standard procedures published by Martínez et al. (2018c).

qPCR gene expression analysis

Reactions were carried out using an AriaMx Real-Time PCR System (Agilent). cDNA was diluted to 100 ng and used as a template for qPCR analysis using the Brilliant SYBRGreen

Table 1 Primer sequences used in the experiments

Primer	Nucleotide sequences (5'→3')	PCR product size	Efficiency liver (%)	Accession number
Ubiquitin Fw	CACTGGCAAGACCATCACCC	157	99.7	—
Ubiquitin Rv	GTTGTAGTCCGAGAAGGTGCG			
BAX Fw	GGGGCAGGGTGGTTGCACTGTT	148 pb	102.9	—
BAX Rv	CCTTGCTCCCTGATCCAGTTGA			
GRP78 Fw	TACTGAGAAGAAGAGCAAGCCT	143 pb	104.7	Clark et al. [2008]
GRP78 Rv	GTGTGACCTTCTTGCCCAGAT			
HSP70 Fw	GGAGATGGAGGGAAACCCAAAA	119 pb	103.9	—
HSP70 Rv	AGGTACGCCTCAGCAATTCCTTC			
SMAC/Diablo Fw	ACATCAGGAGCTGAACATGCGT	149 pb	99.8	—
SMAC/Diablo Rv	TGTTGCGCCATCCTTTCAACCT			
CHIP Fw	AAAGCGGGAGATCCCAGATTACCT	110 pb	102.9	—
CHIP Rv	GCTCTTCAATGTCCTTGCGATCA			
PSMC1 Fw	AGGTCCATGCTGTGATTGGTGT	101 pb	105.7	—
PSMC1 Rv	TCCGATGTCAGCGTAGGTTTCTTG			
PSMB7 Fw	GGTGTGTTGAGGATCGCTACAAG	100 pb	102.2	—
PSMB7 Rv	GGAGCCCAGGTCGTAAAGATTC			
PSMA2 Fw	GAATGGGTCCTGACTACAGGGTTT	134 pb	97.1	—
PSMA2 Rv	TGTGTACTCCTGCATTACAGAGGC			
HSC70 Fw	ACCCAGGCCAGCATAGAAATTGAC	147 bp	103	MG799548
HSC70 Rv	CATCTTGGCATCACGGAGTGACTT			
HSP90 Fw	GATACCCAGTCCGAGCTTGATCAT	137 bp	102.6	MG799547
HSP90 Rv	ACAAGAACGACAAGGCCGTGAA			
E3-5066 Fw	AGACGCTGGAAGAGTTCTCCAA	112 bp	105.5	MG799549
E3-5066 Rv	TGATCTGAAACCCCTCTGCGAGATG			
E2-2994 Fw	TGCTGTAGGAGTTCTGCCACTT	126 bp	99.6	MG799543
E2-2994 Rv	TACCCAGAATCCCTCCGTTTGTC			
18s het Fw	GTCCGGGAAACCAAAGTC	116 bp	102.11	—
18s het Rv	TTGAGTCAAATTAAGCCGCA			

qPCR reagent (Stratagene) and specific primers. qPCR reactions were performed according previously reported for Martínez et al. (2018b) and the expression levels were analyzed using the comparative Ct method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen 2001). Data were expressed as the fold change in gene expression normalized to an endogenous reference gene (housekeeping 18 s) and relative to the untreated control. The primers used are listed in Table 1. All data provided are given in terms of relative expression and are expressed as the mean $\pm$ standard error (SE) of the mean. PCR efficiencies were calculated using the equation: $E = 10^{-1/\text{slope}}$ (Rasmussen 2001)

Statistical analyses

Assumptions of both normality and homogeneity of the variances were tested. Logarithmic transformations of the data were performed when necessary to fulfil conditions for parametric analysis of variance. Three-way ANOVA was used to assess temperatures, injections, and time as factors of variance. ANOVA were followed by a Tukey's post-hoc test to identify different groups in qPCR analysis. Statistically

significant differences were determined using a $P < 0.05$. The p values resulting from three-way ANOVA of the assessed parameters are shown in Table 2. Different letters indicate statistical differences in the same treatment in the time. Symbols indicate statistical differences among different treatments (C– 12 °C, C+ 12 °C, AM 18 °C, AB 18 °C, SM 18 °C, and SB 18 °C) in the same time.

Results

Mortality

No mortality was observed in any of the groups during the experimental period.

Genes implicated in heat shock response

Gene expression of HSP70 in the control positive (C+ 12 °C) showed significant differences compared with group C– at day 4 post bacterial injection. The “AM 18 °C” treatment

exhibited an increase in the number of transcripts at days 16 and 20 after the challenge; meanwhile, the acclimation treatment with *P. salmonis* (AB 18 °C) showed a significant increase at days 8, 16, and 20. The groups without thermal acclimation also caused an elevation in the amount of mRNA of HSP70 at days 8, 12, and 16 for “SM 18 °C” and at days 16–20 for “SB 18 °C.” At day 16, there was an excessive increase in the gene expression of HSP70, which was 109 to 146 times higher for all treatments applied at 18 °C compared with the C– condition (Fig. 1a).

The transcription of HSC70 showed no significant differences compared with the C– condition between days 4 and 12 for all the applied treatments. However, at day 16, there was an evident increase in transcription levels, up to 27 times more, compared with the C– in all treatments at 18 °C. This same pattern was observed at day 20, but the differences were not significant for the “AM 18 °C” and “SM 18 °C” conditions. The treatment with bacterial injection at 12 °C (C+) did not show variations in the expression of this gene during the experiment (Fig. 1b).

Fish liver submitted to the C+ condition showed a statistically significant increase in HSP90 expression, from day 8 until day 20. This same tendency was observed in the treatments without acclimation (“SM 18 °C” and “SB 18 °C”) and with acclimation (“AM 18 °C” and “AB 18 °C”), although the differences were not always significant compared with the C– condition. HSP90 expression brusquely increased at day 16 in the liver of fish exposed to 18 °C compared those exposed to 12 °C (Fig. 1c).

The number of GRP78 transcripts increased in the C+ condition at days 4 and 20 compared with the C– condition. Additionally, the treatments with/without acclimation at 18 °C showed variations in the expression of this gene during the experiment, with increases of up to 68 and 79 times more than the C– condition at day 16 and up to 8.5 to 24 times more than the C– at day 20 in all treatments at 18 °C (Fig. 1d).

Genes implicated in the apoptosis pathway

SMAC/Diablo showed increases in mRNA levels during the experiment, which were dependent on the applied treatment (injections and time). Specifically, the C+ condition showed statistical differences compared with the C– at days 4, 8, and 20 post injection. Meanwhile, the treatments with/without acclimation showed a tendency to increase the number of transcripts at days 8, 12, 16, and 20. However, these increases were not always significant compared with the C–. At day 16 post injection, the same pattern was observed with most of the previously mentioned genes, which were increased by up to 31 times more than C– treatment in the liver of fish submitted to treatment at 18 °C (Fig. 2a).

BAX did not present statistical differences at day 4 post injection in any of the applied treatments, compared with day 8, where there were statistically significant increases in all the applied treatments. BAX transcription generally increased between days 8 and 20 post injection with highly elevated expression peaks in the liver of fish exposed to 18 °C, which can be up to 91 times higher than the C– condition (Fig. 2b).

Genes implicated in ubiquitination

The gene expression profile obtained for ubiquitin was similar to that obtained for E2 and E3, with increases in all the evaluated experimental conditions and dependent on the analyzed experimental time. The statistical differences between the conditions at 18 °C compared with the C– condition at 12 °C were evident at days 12, 16, and 20 post challenge, with day 16 presenting the highest increase in the transcription of these genes, with increases of up to 35, 56, and 80 times more than the C– for ubiquitin (Fig. 3a), E2 (Fig. 3b), and E3 (Fig. 3c), respectively.

CHIP increased its transcription at days 4 and 20 post injection in the C+ treatment condition and showed no statistically significant differences on the other evaluated experimental time. However, on day 16, treatments at 18 °C showed an increase of up to 20, 22, 25, and 33 times more for the conditions “AB 18 °C,” “AM 18 °C,” “SB 18 °C,” and “SM 18 °C,” compared with the C– group, respectively. The same treatments previously mentioned together with the C+ treatment statistically increase the transcription of CHIP at day 20 (Fig. 3d).

Genes implicated in 26S proteasome complex

The transcription genes whose proteins form part of the proteasome, such as PSMA2, PSMB7, and SMC1, also show variations during the experiment. PSMA2 mRNAs tends to increase in the first few days post challenge and specifically days 4 and 12. Contrary to that observed in the other genes mentioned above, PSMA2 transcription did not present statistical differences compared with the control negative at day 16 (except in the “AB 18 °C” treatment where there was a decrease) (Fig. 4a).

PSMB7 increased its transcription at days 4, 8, and 20 in the liver of *E. maclovinus* submitted to the “C+ 12 °C” treatment. The conditions with acclimation (AM 18 °C and AB 18 °C) showed statistically significant increases at days 8, 16, and 20; meanwhile, the treatments without thermal acclimation (SM 18 °C–SB 18 °C) also increased the transcription of this gene on days 8, 12, and 16 post challenge. Similar to that observed in the previously mentioned genes (except PSMA2), transcription of PSMB7 increased between 23 to 44 times more than the C– condition at day 16 in all treatments applied at 18 °C (Fig. 4b).

Table 2 p values from Factorial ANOVA of genes implicated in cellular stress response in liver of *E. maclovinus*

Gene	Time	Temperature	Bacteria	Time × Temperature	Time × Bacteria	Temperature × Bacteria	Time × Temperature × Bacteria
HSC70	> 0.001	>0.001	N.S.	> 0.001	N.S.	0.026	N.S.
Ubiquitin	> 0.001	> 0.001	0.009	> 0.001	N.S.	0.038	N.S.
E2	> 0.001	> 0.001	N.S.	> 0.001	N.S.	0.005	N.S.
E3	> 0.001	> 0.001	N.S.	> 0.001	N.S.	N.S.	N.S.
BAX	> 0.001	> 0.001	N.S.	> 0.001	0.006	0.012	N.S.
GRP78	> 0.001	> 0.001	N.S.	> 0.001	N.S.	N.S.	N.S.
HSP70	> 0.001	> 0.001	N.S.	> 0.001	N.S.	N.S.	N.S.
SMAC/Diablo	> 0.001	> 0.001	N.S.	> 0.001	N.S.	0.010	N.S.
HSP90	> 0.001	> 0.001	N.S.	> 0.001	N.S.	0.023	N.S.
CHIP	> 0.001	> 0.001	N.S.	> 0.001	N.S.	N.S.	N.S.
PSMC1	> 0.001	> 0.001	N.S.	> 0.001	N.S.	N.S.	N.S.
PSMB7	> 0.001	> 0.001	N.S.	> 0.001	N.S.	0.026	N.S.
PSMA2	> 0.001	N.S.	0.009	N.S.	0.0014	> 0.001	0.006

N.S. not significant

PSMC1 showed a significant increase in the number of transcripts at days 4 and 20 after the application of C+ treatment, without variations on the other experimental days evaluated. Interestingly, there was a decrease in the transcription of this gene at days 4 and 12 in the “AM 18 °C” treatment; while at day 16, a statistically significant increase was observed in all the treatments applied at 18 °C, the same pattern was observed at day 20, although this was not statistically significant for “AM 18 °C” and “SM 18 °C” treatments (Fig. 4c).

Graphic representation of results

The graphic representation of the results obtained suggest that the increase in environmental temperature triggers much more the expression of genes involved in the cellular response to stress compared with the injection to *P. salmonis* (Fig. 5).

Discussion

Fluctuations in environmental temperature along with exposure to pathogenic microorganisms can induce important cellular changes that interfere with the homeostasis of ectothermic animals (He et al. 2006; Cheng et al. 2015; Dalvi et al. 2017; Huang et al. 2017; Todgham et al. 2017; Rozas-Serri et al. 2018). In our study, we can observe what variations in the environmental temperature lead to the cellular appearance of the endoplasmic reticulum (ER) stress with the activation of one of its classic markers such as GRP78 (Holcik and Sonenberg 2005). Once the cell enters this state, can be activated the molecular chaperones, (HSP70, HSC70, HSP90, and CHIP) that favor the folding of proteins that have been damaged by the stimulus. If these proteins cannot be repaired, they will be degraded by proteinases via the Ub-proteasome

or lysosomal route (Ciechanover 2005). If the cellular damage caused by the stimulus is strong enough and the cell fails to recover its homeostasis, it can enter the apoptotic pathway by activating its classic markers (BAX, Diablo/SMAC, caspases 3, 7, and 9) (Liston et al. 2003, Pawlowski and Kraft 2000). In our study, the temperature more than *P. salmonis* was the most important factor in the transcriptional induction of markers involved in the cellular response to liver stress of *E. maclovinus*.

The magnitude of gene expression compared with the negative control was higher in the conditions applied at 18 °C and at day 16 post challenge (except PSMA2 which was downregulated). This is probably due to the fact that the biological processes of ectothermic poikilotherm fish are closely related to temperature (Kurata et al. 1995; Le Morvan et al. 1995, 1997; Engelsma 2003; Vanella et al. 2012; Oyarzún et al. 2018), and also that during the bacterial pathogenesis, there is an alteration to the ubiquitin-proteasome system that could favor survival, persistence, and proliferation of microorganisms (Perrett et al. 2011). In our study, the temperature and time of experiment are the main variables that are affecting the transcription of the genes involved in the cellular stress response, more than the bacterial stimulus (*P. salmonis*). So far yet, it is widely accepted that temperature and time affect the physiological processes of *E. maclovinus*, from the metabolic, omorregulatory and immunological point of view (Martínez et al. 2018b; Oyarzún et al. 2018).

There are no studies that can be compared with the temporal kinetics of this response (4–20 dpi) on other fish species, having also the conditions of temperatures and bacterial pathogen. But it has been reported that in *Salmo salar*, infection with *P. salmonis* trigger an overexpression of proteins

Fig. 1 mRNA of HSP70 (a), HSC70 (b), HSP90 (c) and GRP78 (d) in liver of *Eleginops maclovinus* injected to *Piscirickettsia salmonis* and subjected to different temperatures. **C- 12°C**: Injection only with culture medium, **C+ 12°C**: Injection with *P. salmonis*, **AM 18°C**: Injection only with culture medium under acclimation at 18°C, **AB 18°C**: Injection with *P. salmonis* under acclimation at 18°C, **SM 18°C**: Injection only with

culture medium and thermal shock at 18°C and **SB 18°C**: Injection with *P. salmonis* and thermal shock at 18°C. Different letters indicate statistical differences in the same treatment in the time. Symbols indicate statistical differences among different treatments (C- 12 °C, C+ 12 °C, AM 18 °C, AB 18 °C, SM 18 °C, and SB 18 °C) in the same time (three-way ANOVA, $P < 0.05$)

Fig. 2 mRNA of SMAC/Diablo (a) and BAX (b) liver of *Eleginops maclovinus* injected to *Piscirickettsia salmonis* and subjected to different temperatures. **C- 12°C**: Injection only with culture medium, **C+ 12°C**: Injection with *P. salmonis*, **AM 18°C**: Injection only with culture medium under acclimation at 18°C, **AB 18°C**: Injection with *P. salmonis* under acclimation at 18°C, **SM 18°C**: Injection only with culture medium and

thermal shock at 18°C and **SB 18°C**: Injection with *P. salmonis* and thermal shock at 18°C. Different letters indicate statistical differences in the same treatment in the time. Symbols indicate statistical differences among different treatments (C- 12 °C, C+ 12 °C, AM 18 °C, AB 18 °C, SM 18 °C, and SB 18 °C) in the same time (three-way ANOVA, $P < 0.05$)

Fig. 3 mRNA of Ubiquitin (a), E2 (b), E3 (c) and CHIP (d) in liver of *Eleginops maclovinus* injected to *Piscirickettsia salmonis* and subjected to different temperatures. **C- 12°C**: Injection only with culture medium, **C+ 12°C**: Injection with *P. salmonis*, **AM 18°C**: Injection only with culture medium under acclimation at 18°C, **AB 18°C**: Injection with *P. salmonis* under acclimation at 18°C, **SM 18°C**: Injection only with

culture medium and thermal shock at 18°C and **SB 18°C**: Injection with *P. salmonis* and thermal shock at 18°C. Different letters indicate statistical differences in the same treatment in the time. Symbols indicate statistical differences among different treatments (C- 12°C, C+ 12°C, AM 18°C, AB 18°C, SM 18°C, and SB 18°C) in the same time (three-way ANOVA, $P < 0.05$)

involved in the synthesis/degradation processes at 48 h after the bacterial challenge as a final time (Tacchi et al. 2011). However, on day 5, in this same species, with the same bacteria, there is a decrease in the expression of genes that participate in the ubiquitin-proteasome response (Rozas-Serri et al. 2018). We can suggest that temperature and time are factors that would be activating the processes of protein synthesis and degradation in the cell, affecting the turnover protein in liver of *E. maclovinus*. Probably, at day 16 after challenge, the machinery of turnover protein is working hard to compensate for adaptation to a higher temperature, although this species is eurythermic.

In our study, an increase from 12 to 18 °C caused differences in the transcription of genes involved in cellular stress response, mainly at day 16 post inoculation, indicating that the temperature more than the injection with *P. salmonis* can interfere with the transcription of heat shock proteins, Ub-proteasome system, and apoptotic pathways. Temperature, as reported by Oyarzún et al. (2018), also affects the intermediary metabolism of *E. maclovinus*, which rearrange their

metabolic machinery to compensate for environmental temperatures of 10 °C, 14 °C, and 18 °C. Other authors have reported differences in growth, feeding, and swimming among the fish maintained at 4 °C and 18 °C (Vanella et al. 2012). Fulton's condition factor (K) is decreased when this species is acclimatized to 16 °C, but not when it is acclimatized to 2 °C, 4 °C, and 10 °C (Lattuca et al. 2018). All these observations suggest that fish, like all living organisms, have an optimal body temperature where their growth, reproduction, foraging, and behavior are maximized, even though some species are biologically capable of living in a wide thermal range.

In this investigation, the HSPs had differential expression profiles dependent on temperature, time, and type of injection applied. HSC70 expression was only upregulated at day 16 and in all treatments at 18 °C. Meanwhile, the transcriptions of HSP70, HSP90, and GRP78 were induced in both experimental temperatures, with 18 °C at 16 dpi presenting the greatest increase. Our results suggest that the temperature and time influence the transcription of heat shock proteins in liver of

Fig. 4 mRNA of PSMA2 (a), PSMB7 (b) and PSMC1 (c) in liver of *Eleginops maclovinus* injected with *Piscirickettsia salmonis* and subjected to different temperatures. **C- 12°C**: Injection only with culture medium, **C+ 12°C**: Injection with *P. salmonis*, **AM 18°C**: Injection only with culture medium under acclimation at 18°C, **AB 18°C**: Injection with *P. salmonis* under acclimation at 18°C, **SM 18°C**: Injection only with

culture medium and thermal shock at 18°C and **SB 18°C**: Injection with *P. salmonis* and thermal shock at 18°C. Different letters indicate statistical differences in the same treatment in the time. Symbols indicate statistical differences among different treatments (C- 12°C, C+ 12°C, AM 18°C, AB 18°C, SM 18°C, and SB 18°C) in the same time (three-way ANOVA, $P < 0.05$)

E. maclovinus, in accordance with what was previously reported for *Takifugu obscurus* (Cheng et al. 2015) and *Horabagrus brachysoma* (Dalvi et al. 2017). However, in *Salmo salar*, the expression of HSP10 and HSP70 is down-regulated 5-day post inoculation with *P. salmonis* (Rozas-Serri et al. 2018). Meanwhile, the transcription of HSP40 and HSP90 is upregulated in specimens of the same species at 2- and 14-day post infection with this same pathogen (Rise et al. 2004; Tacchi et al. 2011).

This is the first report of transcriptional monitoring of GRP78, Diablo/SMAC, and BAX in fish subjected to thermal stress and *P. salmonis*. GRP78 can inhibit caspase activity and therefore inhibit apoptosis during stress conditions (Clark et al. 2008). Clark et al. (2008) reported that if Diablo/SMAC is upregulated, GRP78 should be downregulated; however, in our study, the transcription of both genes was not antagonistic. Probably, transcript levels are not exactly reflecting active protein levels in liver cells. Additionally, BAX is a proapoptotic molecule of the Bcl-2 family that regulates the release of cytochrome C from the

mitochondria to the cytoplasm via the intrinsic apoptosis pathway (Pawlowski and Kraft 2000). Meanwhile, Diablo/SMAC binds to apoptosis inhibitory proteins (AIPs) promoting the activation of caspases 3, 7, and 9 (Liston et al. 2003). Our results show that both temperature and time can trigger the cellular stress response at the transcriptional level, leading to upregulation of the expression of genes involved in the apoptotic pathway. These results are similar to other found in the literature, where BAX mRNAs increase in the blood cells of *Takifugu obscurus* after an acute increase in water temperature (Cheng et al. 2015).

CHIP not only participates in the ubiquitination of damaged proteins that are linked to HSC70 and HSP90 (Connell et al. 2001), but can also tag HSP70 to be degraded by the proteasome (Shu-Bing et al. 2006). In our study, CHIP expression was similar to that obtained for HSC70, increasing at day 4 in condition C+ and at day 16 in all conditions applied at 18°C. If we assume that all mRNAs are being translated, the temperature of 18°C would favor ubiquitination of damaged proteins, mainly at day 16, which is where the highest

Fig. 5 Generalized graphical representation of results obtained. Size of the blue and red arrows indicate the increase in gene expression caused by the temperature or by *P. salmonis*, respectively. Figure was did for D. Martinez, the first author

expressions of HSP70 and HSP90 are observed. Other authors have reported that the expression of CHIP in gill of Antarctic fish (*Trematomus bernacchii*-*Pagothenia borchgrevinkii*) and non-Antarctic fish (*Notothenia angustata*-*Bovichtus variegatus*), indicating that its expression is species-specific with differences between the fish kept 2 °C (Antarctic) and 10 °C (not Antarctic) (Todgham et al. 2017).

Other molecules that are also involved in the ubiquitination process are E2 (conjugation), E3 (ligation), and ubiquitin, which have practically identical expression patterns, with an increased number of transcripts in fish exposed to conditions at 12 °C and 18 °C, suggesting that both the challenge with *P. salmonis* (C +, AB 18 °C and SB 18 °C) and the thermal challenge (AM 18 °C and SM 18 °C) positively regulate the transcription of these genes. Our results differ from those reported by Rozas-Serri et al. (2018) who indicate that the head kidney of *Salmo salar* infected with *P. salmonis* decreases the expression of the ubiquitin-proteasome system as a survival mechanism within the hosts' cells at day 5 post bacterial challenge. Meanwhile, Tacchi et al. (2011) suggest that *Salmo salar* liver infected with *P. salmonis* increases the expression of genes related to synthesis as well as protein degradation 48-h post bacterial challenge (Tacchi et al. 2011).

The 26S proteasome complex participates in the degradation of damaged or misfolded proteins, once they have been ubiquitinated and marked for degradation after the action of E1, E2, and E3 (Goldberg 2003). In our study, the three genes involved in the formation of the proteasome showed variations in their expression during the experiment and in all the applied treatments, indicating that both the temperature (12–

18 °C) and *P. salmonis* could act as stress stimuli, generating an alteration in transcript levels and, consequently, in the levels of proteins that make up the subunits (20S–19S) of the 26S proteasome. Specifically, PSMA2 and PSMB7 are part of the α or β subunit of the 20S core, while PSMC1 is part of the 19S regulatory subunit (Wang and Maldonado 2006). PSMA2 was the only one that was preferentially up-regulated during the first days of the experiment (4–12 dpi) and the only one that did not have a statistically significant increase in transcription at days 16 and 20 post challenge. There are no studies that have previously been related the transcription of these genes in fish infected with *P. salmonis*; however, there are records that indicate that their expression is lower in the gills of non-Antarctic Notothenioid compared with Antarctic Notothenioid, revealing that their expressions is dependent on ambient temperature (Todgham et al. 2017).

Conclusion

The *E. maclovinus* transcriptome published by Martínez et al. (2018c) is a significant advancement in the genomic data available for this species and deepest that research previously described (Papetti et al. 2015). The expression patterns obtained allow us to suggest that the time, temperature, and *P. salmonis* activate the cellular stress response by increasing the transcription of genes involved in this response. No differences were observed in the magnitude of the expression between the treatments applied at 18 °C with or without acclimation, indicating that the animal manages to compensate this

thermal shock and respond in the same way as those that were acclimated. The expression of the genes evaluated in this study was generally higher in the liver of fish exposed to 18 °C compared with fish subjected to 12 °C, and there were no large differences between those injected with medium culture (AM 18 °C and SM 18 °C) or with *Piscirickettsia salmonis* (AB 18 °C and SB 18 °C). The most evident cell damage was observed at 16 dpi and 20 dpi, suggesting that during the final days of the experiment, the cellular stress response is caused mainly by the temperature more than by injection of *P. salmonis*.

Acknowledgments Authors would like to thank to Dr. Alejandro Yáñez, Science Faculty, Universidad Austral de Chile, for providing the *Piscirickettsia salmonis* isolate.

Authors' contributions D.M. and L.V.-C.: Conception and design of research.

D.M., C.V.-L., J.S., R.O., J.P.: performed the experiments.

D.M., C.V.-L., J.S., R.O., and J.P.: analyzed the samples.

D.M., J.P., C.L. and L.V.-C: prepared the figures and interpreted the results of experiments.

D.M., C.V.-L., J.S., R.O., C.L., J.P., and L. V.-C: drafted the manuscript.

D.M., C.V.-L., J.S., R.O., C.L., J.P., and L. V.-C: edited, revised, and approved the manuscript.

D.M. and LV.-C: approved the final version of manuscript, include to send files as it.

Funding information This investigation was financed by Fondap-Ideal Grant no. 15150003 and Fondecyt Regular Grant no. 1160877, and DID UACH. Danixa Martínez was financed by doctoral scholarship of CONICYT. Carolina Vargas-Lagos was funded by Fondap-INCAR No. 15110027.

Compliance with ethical standards The Ethics protocol was approved by the Committee on the Ethics for Animal Experimentation of Universidad Austral de Chile Memorandum No. 261/2016.

Conflict of interest The authors declare that they have no conflicts of interest.

References

- Arkush KD, McBride AM, Mendonca HL, Okihiro MS, Andree KB, Marshall S, Henriquez V, Hedrick RP (2005) Genetic characterization and experimental pathogenesis of *Piscirickettsia salmonis* isolated from white seabass *Atractoscion nobilis*. Dis Aquat Org 63: 139–149
- Athanassopoulou F, Groman D, Prapas T, Sabatakou O (2004) Pathological and epidemiological observations on rickettsiosis in cultured sea bass (*Dicentrarchus labrax* L.) from Greece. J Appl Ichthyol 20:525–529
- Barton A (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr Comp Biol 42:517–525
- Cheng CH, Yang FF, Liao SA et al (2015) High temperature induces apoptosis and oxidative stress in pufferfish (*Takifugu obscurus*) blood cells. J Therm Biol 53:172–179
- Ciechanover A (2005) Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin – proteasome system and onto human diseases and drug targeting. Angew Chem Int Ed Eng 44:5944–5967
- Clark MS, Fraser KPP, Burns G, Peck LS (2008) The HSP70 heat shock response in the Antarctic fish *Harpagifer antarcticus*. Polar Biol 31: 171–180
- Connell P, Ballinger CA, Jiang J, Wu Y, Thompson LJ, Höhfeld J, Patterson C (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat Cell Biol 3:93–96
- Contreras-Lynch S, Olmos P, Vargas A, Figueroa J, González-Stegmaier R, Enríquez R, Romero A (2015) Identification and genetic characterization of *Piscirickettsia salmonis* in native fish from southern Chile. Dis Aquat Org 115:233–244
- Dalvi R, Das T, Debnath D, Yengkokpam S, Baruah K, Tiwari LR, Pal AK (2017) Metabolic and cellular stress responses of catfish, *Horabagrus brachysoma* (Günther) acclimated to increasing temperatures. J Therm Biol 65:32–40
- Engelsma M (2003) Multiple acute temperature stress affects leucocyte populations and antibody responses in common carp, *Cyprinus carpio* L. Fish Shellfish Immunol 15:397–410
- Fortt A, Cabello F, Buschmann A (2007) Residuos de tetraciclina y quinolonas en peces silvestres en una zona costera donde se desarrolla la acuicultura del salmón en Chile. Rev Chil Infectol 24: 14–18
- Fryer JL, Lannan CN, Garces LH et al (1990) Isolation of a rickettsiales-like organism from diseased coho salmon (*Oncorhynchus kisutch*) in Chile. Fish Pathol 25:107–114
- Goldberg AL (2003) Protein degradation and protection against misfolded proteins. Nature 426:895–899
- Gómez F, Henríquez V, Marshall S (2009) Additional evidence of the facultative intracellular nature of the fish bacterial *Piscirickettsia salmonis*. Arch Med Vet 267:261–267
- He W, Yin ZX, Li Y et al (2006) Differential gene expression profile in spleen of mandarin fish *Siniperca chuatsi* infected with ISKNV, derived from suppression subtractive hybridization. Dis Aquat Org 73:113–122
- Holcik M, Sonenberg N (2005) Translational control in stress and apoptosis. Nat Rev Mol Cell Biol 6:318–327
- Huang R, Zhang J, Zhu G, He J, Xie J (2017) The core ubiquitin system of mandarin fish, *Siniperca chuatsi*, can be utilized by infectious spleen and kidney necrosis virus. Fish Shellfish Immunol 70:293–301
- Kurata O, Okamoto N, Suzumura E et al (1995) Accommodation of carp natural killer-like cells to environmental temperatures. Aquaculture 129:421–424
- Lattuca ME, Boy CC, Vanella FA et al (2018) Thermal responses of three native fishes from estuarine areas of the Beagle Channel, and their implications for climate change. Hydrobiologia 808:235–249
- Le Morvan C, Clerton P, Deschaux P, Troutaud D (1997) Effects of environmental temperature on macrophage activities in carp. Fish Shellfish Immunol 7:209–212
- Le Morvan C, Troutaud D, Deschaux P (1995) Effects of temperature on carp leukocyte mitogen-induced proliferation and nonspecific cytotoxic activity. Dev Comp Immunol 19:87–95
- Liston P, Fong WG, Korneluk RG (2003) The inhibitors of apoptosis: there is more to life than Bcl2. Oncogene 22:8568–8580
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ddct Method. Methods 25:402–408
- Martínez D, Díaz-Ibarrola D, Vargas-Lagos C, Oyarzún R, Pontigo JP, Muñoz JLP, Yáñez AJ, Vargas-Chacoff L (2018a) Immunological response of the Sub-Antarctic Notothenioid fish *Eleginops maclovinus* injected with two strains of *Piscirickettsia salmonis*. Fish Shellfish Immunol 75:139–148

- Martínez D, Oyarzún R, Pontigo J et al (2017a) Nutritional immunity triggers the modulation of iron metabolism genes in the sub-Antarctic Notothenioid *Eleginops maclovinus* in Response to *Piscirickettsia salmonis*. *Front Immunol* 8:1–12
- Martínez D, Oyarzún R, Vargas-Lagos C, Pontigo JP, Soto-Dávila M, Saravia J, Romero A, Núñez JJ, Yáñez AJ, Vargas-Chacoff L (2017b) Identification, characterization and modulation of ferritin-H in the sub-Antarctic Notothenioid *Eleginops maclovinus* challenged with *Piscirickettsia salmonis*. *Dev Comp Immunol* 73:88–96
- Martínez D, Pontigo J, Morera F et al (2018c) Head kidney transcriptome analysis and characterization for the sub-Antarctic Notothenioid Fish *Eleginops maclovinus*. *Fishes* 3:8
- Martínez D, Vargas-Lagos C, Oyarzún R, Loncoman CA, Pontigo JP, Yáñez AJ, Vargas-Chacoff L (2018b) Temperature modulates the immunological response of the sub-antarctic notothenioid fish *Eleginops maclovinus* injected with *Piscirickettsia salmonis*. *Fish Shellfish Immunol* 82:492–503
- Mauel MJ, Miller DL, Frazier K et al (2003) Characterization of a piscirickettsiosis-like disease in Hawaiian tilapia. *Dis Aquat Org* 53:249–255
- Mauel MJ, Ware C, Smith P (2008) Culture of *Piscirickettsia salmonis* on enriched blood agar. *J Vet Diagn Investig* 20:213–214
- Mikalsen J, Skjaervik O, Wiik-Nielsen J, Wasmuth MA, Colquhoun DJ (2008) Agar culture of *Piscirickettsia salmonis*, a serious pathogen of farmed salmonid and marine fish. *FEMS Microbiol Lett* 278:43–47
- Oyarzún R, Martínez D, Muñoz JLP, Dantagnan P (2019) Effect of ration level on growth performance , body composition , intermediary metabolism and serum parameters in juvenile Patagonian blennie *Eleginops maclovinus*. *Comp Biochem Physiol Part A* 230:122–130
- Oyarzún R, Muñoz JLP, Pontigo JP et al (2018) Effects of acclimation to high environmental temperatures on intermediary metabolism and osmoregulation in the sub-Antarctic notothenioid *Eleginops maclovinus*. *Mar Biol* 165:22
- Papetti C, Windisch H, La Mesa M et al (2015) Non-Antarctic notothenioids: past phylogenetic history and contemporary phylogeographic implications in the face of environmental changes. *Mar Genomics* 25:1–9
- Pawlowski J, Kraft AS (2000) Bax-induced apoptotic cell death. *Proc Natl Acad Sci U S A* 97:529–531
- Pequeño G (1989) The geographical distribution and taxonomic arrangement of South American Nototheniid fishes (Osteichthyes, Nototheniidae). *Bol Soc Biol Concepc* 60:183–200
- Perrett CA, Lin DYW, Zhou D (2011) Interactions of bacterial proteins with host eukaryotic ubiquitin pathways. *Front Microbiol* 2:1–18
- Rasmussen R (2001) Rapid Cycle Real-time PCR. *Applications, Methods and*
- Rise ML, Jones SRM, Brown GD, von Schalburg K, Davidson WS, Koop BF (2004) Microarray analyses identify molecular biomarkers of Atlantic salmon macrophage and hematopoietic kidney response to *Piscirickettsia salmonis* infection. *Physiol Genomics* 20:21–35
- Rozas-Serri M, Peña A, Maldonado L (2018) Transcriptomic profiles of post-smolt Atlantic salmon challenged with *Piscirickettsia salmonis* reveal a strategy to evade the adaptive immune response and modify cell-autonomous immunity. *Dev Comp Immunol* 81:348–362
- Rozas M, Enríquez R (2014) Piscirickettsiosis and *Piscirickettsia salmonis* in fish: a review. *J Fish Dis* 37:163–188
- Schulte PM (2011) Temperature: responses and adaptations to the environment. Elsevier Inc.
- Shu-Bing Q, Holly M, Frank B et al (2006) CHIP-mediated stress recovery by sequential ubiquitination of substrates and HSP70. *Nature* 40: 551–555
- Tacchi L, Bron JE, Taggart JB, Secombes CJ, Bickerdike R, Adler MA, Takle H, Martin SA (2011) Multiple tissue transcriptomic responses to *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*). *Physiol Genomics* 43:1241–1254
- Todgham AE, Crombie TA, Hofmann GE (2017) The effect of temperature adaptation on the ubiquitin–proteasome pathway in notothenioid fishes. *J Exp Biol* 220:369–378
- Vanella FA, Boy CC, Fernández DA (2012) Temperature effects on growing, feeding, and swimming energetics in the Patagonian blennie *Eleginops maclovinus* (Pisces: Perciformes). *Polar Biol* 35:1861–1868
- Vargas-Chacoff L, Martínez D, Oyarzún R, Nualart D, Olavarria V, Yáñez A, Bertrán C, Ruiz-Jarabo I, Mancera JM (2014a) Combined effects of high stocking density and *Piscirickettsia salmonis* treatment on the immune system, metabolism and osmoregulatory responses of the Sub-Antarctic Notothenioid fish *Eleginops maclovinus*. *Fish Shellfish Immunol* 40:424–434
- Vargas-Chacoff L, Moneva F, Oyarzún R et al (2014b) Environmental salinity-modified osmoregulatory response in the sub-Antarctic notothenioid fish *Eleginops maclovinus*. *Polar Biol* 37:1235–1245
- Wang J, Maldonado M (2006) The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Mol Immunol* 3:255–261
- Wendelaar Bonga SE (1997) The stress response in fish. *Physiol Rev* 77: 591–625
- Weyts FAA, Cohen N, Flik G, Verburg-van Kemenade BML (1999) Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. *Fish Shellfish Immunol* 9:1–20
- Yáñez AJ, Silva H, Valenzuela K et al (2012a) Two novel blood-free solid media for the culture of the salmonid pathogen *Piscirickettsia salmonis*. *J Fish Dis* 36:587–591
- Yáñez AJ, Valenzuela K, Silva H, Retamales J, Romero A, Enriquez R, Figueroa J, Claude A, Gonzalez J, Avendaño-Herrera R, Carcamo JG (2012b) Broth medium for the successful culture of the fish pathogen *Piscirickettsia salmonis*. *Dis Aquat Org* 97:197–205

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.