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Expression pattern of heat shock proteins during acute thermal stress in the Antarctic sea urchin, *Sterechinus neumayeri*

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Abstract

Background: Antarctic marine organisms have evolved a variety of physiological, life-history and molecular adaptations that allow them to cope with the extreme conditions in one of the coldest and most temperature-stable marine environments on Earth. The increase in temperature of the Southern Ocean, product of climate change, represents a great challenge for the survival of these organisms. It has been documented that some Antarctic marine invertebrates are not capable of generating a thermal stress response by means of an increase in the synthesis of heat shock proteins, which could be related with their low capacity for acclimatization. In order to understand the role of heat shock proteins as a compensatory response in Antarctic marine species to projected scenarios of increased seawater temperatures, we assessed the expression of the genes Hsp90, Grp78, Hyou1 and Hsc70 in the Antarctic sea urchin *Sterechinus neumayeri* under three thermal treatments (1 °C, 3 °C and 5 °C), for a period of exposure of 1, 24 and 48 h.

Results: The results obtained showed that these genes were expressed themselves in all of the tissues analyzed in a constitutive form. During acute thermal stress, an overexpression of the Hsp90, Grp78 and Hyou1 genes was observed in coelomocyte samples at 3 °C after 48 h, while in esophageal samples, an increase in Hsp90 and Grp78 expression was observed after 48 h. Thermal stress at 5 °C, in general, did not produce a significant increase in the expression of the genes that were studied. The expression of Hsp70 did not show modifications in its expression as a result of thermal stress.

Conclusions: *S. neumayeri* is capable of overexpressing stress proteins as a result of thermal stress, however, this response is delayed and to a lesser degree compared to other Antarctic or temperate species. These results indicate that adult individuals could cope with the expected impacts caused by an increase in coastal sea temperatures in the Southern Ocean.

Keywords: HSP, Thermal tolerance, Chaperones, Ocean warming, Sea urchin

Background

During the last 35 million years, Antarctic marine organisms have evolved a variety of physiological, life-history and molecular adaptations that allow them to cope with the extreme conditions in one of the coldest and most temperature-stable marine environments on Earth [1, 2]. Some of the most notable adaptations in these highly stenothermal species are the production of antifreeze peptides in notothenioid fishes [3], the higher accumulation of polyunsaturated fatty acyl chains in microorganisms [4], the slow growth rates, low basal metabolism and reduced annual reproductive effort in ectothermic invertebrates [5], as well as other specific cellular or metabolic processes in marine invertebrates [6]. Even though the evolution of these adaptations has permitted life in an extreme environment, it has also reduced the capacity of the majority of Antarctic species to adapt to new environmental conditions [7, 8]. In fact, an important constraint in thermal niche expansion for Antarctic species is the loss of traits whose function is to increase heat tolerance or to facilitate acclimatization to short-term changes in temperature [9, 10]. This

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functional limitation is suggested to be the result of the absence of positive selection during evolution at stable sub-zero temperatures [10].

From a physiological perspective, chronic exposure to extreme temperatures (e.g. cold or heat) is expected to cause thermal stress and deleterious effects on organismal performance [11]. However, organisms can compensate variations of environmental temperatures by modification of their molecular and cellular structures to maintain performance as their environments change [12, 13]. One of the main molecular responses that is activated in a cell under thermal stress is the production of heat shock proteins (HSP), being the Hsp70 family the most studied in its constitutive (Hsc70) and inducible (Hsp70) forms [14]. As molecular chaperones, HSPs stabilize denaturing proteins and refold those that have already been denatured [15, 16], thus, the HSP response-could be considered an ecologically and evolutionarily important factor in thermal adaptation, setting thermal tolerance limits and improving an animal's tolerance to thermal stress [16–18]. Nevertheless, physiological responses to thermal stress conditions are not uniform among different taxa. For instance, it has been documented that some species of marine organisms are capable of generating a thermal stress response, while others have lost such capacity or simply do not have the necessary physiological mechanisms to generate this response [19]. To date, empirical evidences on a very limited number of genes have shown that the mollucs *Nacella concinna* and *Laternula elliptica* are the only Antarctic marine species with a protein induction response to thermal stress [20, 21], whereas other species such as the Antarctic notothenioids fish, the seastar *Odontaster validus* and the gammarid crustacean *Paraceradocus gibber*, cannot respond with an increase of gene expression after chronic thermal exposure [19, 22, 23]. Understanding the role of physiological plasticity as a compensatory response in Antarctic marine species to projected scenarios of increased seawater temperatures is one of the main challenges in order to predict their capacity to adapt to climate change [24].

In the Antarctic Peninsula, long-term environmental monitoring programs have revealed a rapid increase in mean and extreme temperatures, with an elevation of the surface temperature of the Bellingshausen Sea by 1 °C in the past 50 years [25, 26]. Although there have been pronounced seasonal changes in the surface temperature of the Antarctic ocean, the data obtained during five years (2006–2010) of the surface temperatures in Arthur Harbor (Anvers Island, Antarctica), showed extreme events of maximum temperatures (2.9 °C) during the austral summer in February 2009 [27]. These changes in sea surface temperature (increases of 2–3 °C) represent a great challenge for Antarctic marine species as these

may decrease their essential biological functions [28, 29]. Although Antarctic organisms can functionally operate for short-term in temperature ranges between 5 and 12 °C above the minimum sea temperature of −2 °C [30], most of them are unable to survive for long-term the increase of 2–3 °C [8]. The more tolerant species have shown maintenance of physiological performance for a few months in temperatures surrounding 6 °C [31], which are close to the projected temperatures for 2100 in this region [32].

To explore the physiological capacity of Antarctic organisms to physiologically compensate drastic thermal events such as those predicted to occur in surface oceans at the end of the century; we assessed the HSP response in the Antarctic sea urchin *Sterechinus neumayeri*. Thanks to the advances of Next-Generation Sequencing technologies (NGS), we are now able to search for target genes related to heat stress chaperones from the whole transcriptome of this species [33]. These chaperones proteins have been classified into several families based on their molecular mass and functions. The Hsp90 have key roles in the maturation of signal transduction proteins, like hormone receptors and protein folding [34]. Hsp70 proteins are necessary for protein folding, multimer dissociation and association, translocation of proteins across membranes, and regulation of the heat shock response [35]. The Glucose-regulated protein 78 (Grp78) or HspA5 is required for endoplasmic reticulum integrity and stress-induced autophagy [36]. The Hypoxia up-regulated 1 (Hyou 1 or Grp170) under hypoxia condition is accumulated in the endoplasmic reticulum (ER) and this gene play an important role in protein folding, secretion in the ER and an important cytoprotective role during the hypoxia [37]. In this work, we determined the capacity of adult *S. neumayeri* to induce transcriptional activity for the production of the Hsp90 (heat shock protein 90 kDa), Hsc70 (heat shock cognate 70 kDa), Grp78 (glucose-regulated protein 78 kDa) and Hyou1 (hypoxia up-regulated 1) genes in the coelomocyte and esophagus after exposure to acute thermal stress over a short-term stress period.

Methods
Animal sampling and experimental set-up
Antarctic sea urchins were collected by SCUBA divers during the austral summer at Maxwell Bay (62°12′12.2′′ S - 58°56′41.7′′ W), Fildes Peninsula, King George Island. Subsequently animals were kept in a cold chamber with re-circulating seawater at room temperature (1.0 ± 1.0 °C, 34 % salinity) and constant aeration. Specimens were acclimated for one week before experiments. Thirty-two individuals of *S. neumayeri* were randomly selected and maintained in the three experimental

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treatments (1 °C, 3 °C and 5 °C) for 48 h. Animals were directly transferred from control temperature (1 °C ± 0.5 °C) to the experimental aquariums for acute thermal stress without an acclimation period at 3 °C and 5 °C. Eight animals were assigned to the control group and 12 to each of the thermal stress treatments. Four sea urchins were randomly collected from each group at 1, 24 and 24 h post thermal stress, respectively. Coelomocyte and tissue samples were obtained and stored in RNA later according to manufacturer instructions (Ambion).

Mining target genes
The nucleotide sequences of the genes coding for Hsp90, Hsc70, and Grp78 Hyou1 chaperones were obtained from the transcriptome of *S. neumayeri* larvae (Bioproject ID N° PRJNA252503), which was generated by the 454 technology [33]. The degree of homology of these sequences was confirmed by similarity search using Blastx program NCBI (Table 1), where amino acid sequences were extracted for a multiple alignment using the Clustal program to determine conserved areas. Subsequently, from the conserved areas the primers were designed using the software Primer 3.0.

RNA extraction and synthesis of cDNA
Total RNA was extracted from *S. neumayeri* from coelomocytes, digestive tracts, esophagus and gonads using an E.Z.N.A.® Total RNA Kit and treated after with DNAse according to the manufacturer's instructions. The quantity and the integrity of the total RNA was then assessed by spectrophotometric and agarose gel electrophoresis, respectively. Synthesis of the cDNA from approximately 1 μg of the total RNA was carried out using Random Hexamers 50uM (Roche), 1 mM dNTPs (Invitrogen), 20 U RnaseOUT (Invitrogen) and 50 U M-MLV reverse transcriptase in reverse transcriptase buffer (Invitrogen) according to the manufacturers' instructions in a final volume of 20 μl. The cDNA was then diluted ten times and 2 μl were used for PCR reactions. The cDNA was amplified by PCR, using 1 U of Taq polymerase (Invitrogen) and 1 μM of each primer (see Table 2), at a final volume of 25 μl. An amplification program for PCR consisted in 5 min at 94 °C, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, and finally an elongation step at 72 °C for 10 min.

Amplified products were analyzed on 1 % agarose gels and sequenced from both directions using the same set of primers.

mRNA expression analysis by real time PCR
Quantitative PCR analysis was carried out to determine whether there were acute changes in gene expression, sampled at 1, 24 and 48 h during the acute thermal stress. These qPCR reactions were composed by 2.0 μl of cDNA diluted at 1:10 ratio, 0.5 μl of both primers (5 μM) adding 7 μl of a ready to use solution of Brilliant II Sybr Green qPCR Master (Stratagene) into a final volume of 20 μl complete with water. Amplification conditions, were performed in Agilent Mx3005P Thermocycler, consisted in 40 cycles, 95 °C for 10 min; 95 ° C for 15 s; 52 ° C for s; 72 °C for 15 s with a single fluorescence measurement; melting curve program and finally a cooling step. Primer pair efficiencies (E) were calculated from six serial dilutions of pooled cDNA for each primer pair. Primer pair efficiencies were calculated from the given slopes in the software MxProTM (Agilent Technologies) software according to the equation: $E = 10[-1/slope]$. Specificity of a qPCR product was determined by agarose gel electrophoresis and melting curve analysis.

For further analysis of the expression level, the crossing points (CP) were determined for each transcript, using the MxProTM software QPCR System (Agilent Technologies). Four separate individuals were used for each gene tested in real time quantitative PCR reactions. The copy ratio of each analyzed cDNA was determined as the mean of two technical replicates. The relative expression level of chaperone genes was calculated based on the 2-ΔΔCP method using the average of GAPDH/18 S as the reference gene [38]. Calculations of means, standard deviations and statistical analysis using the Kruskal–Wallis test for expression analysis were carried out using GraphPad Prism software version 5.0. A value of $p < 0.05$ indicated statistical significant difference.

Results
The primers, designed to amplify the selected sequences, specifically amplified PCR products between 150 and 218 base pairs. The efficiency of the primers used had

Table 1 Identity between amino acid sequences of heat shock proteins obtained from *Sterechinus neumayeri* and *Strongylocentrotus purpuratus*

Gen	Closest database match	Score	% Identity	Probability (e-value)	Genbank accesión number
Hsp90	*Strongylocentrotus purpuratus*	1221	94	0.0	XP_003725294.1
Hsc70	*Strongylocentrotus purpuratus*	1130	89	0.0	XP_802129.1
Hyou1	*Strongylocentrotus purpuratus*	634	78	0.0	XP_011661677.1
Grp78	*Strongylocentrotus purpuratus*	977	91	0.0	XP_003728714.1

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Table 2 Primers used for qPCR

Gene target	GenBank accession	Direction	Primer DNA sequence	Size of the amplicon (pb)	RSq	PCR Efficiency (%)
Hsp90	isotig08469	Fw	CCGATGCTTTGGACAAGATTC	151	0.989	122.4
		Rv	ATGTCAGCCTTGGTCATTCC			
Hsc70	isotig04537	Fw	ACAAGAGGGCAGTCAGGAGA	218	0.982	121.6
		Rv	TTTGCCCAACTTGGAGTCAC			
Hyou1	isotig05161	Fw	ATGACATGGGTGCTGGTAGT	170	0.992	123.4
		Rv	TGCAAGGTGCTTCTGAAGAC			
Grp78	isotig04367	Fw	CGACTTGGGCACAACCTATT	203	0.980	116.5
		Rv	TTCCGATCAACCTCTTGGCAT			
Gapdh		Fw	AAGGAGGAGCCAAGAAGGTC	93	0.999	109.5
		Rv	TCCCTGCATCGTATTCCTTC			
18S		Fw	GAGCCTGCGCTTAATTTGAC	186	0.998	109.2
		Rv	GGCGCAACTATTTAGCAAGC			

values over 100 % and acceptable for qPCR applications (Table 2).

Transcriptomic profiles of adult animals under control conditions (1 °C), showed signals of expression of the Hsp90, Hsc70, Grp78 and Hyou1 genes in the different tissues (coelomocytes, gonads, esophagus and digestive tract). The overall response of Hyou1 and Grp78 genes showed a lower level of expression in coelomocytes in comparison to other tissues in a control condition (Fig. 1). Nevertheless, significant differences were not found in the expression of HSP genes among the four tissues ($p > 0.05$).
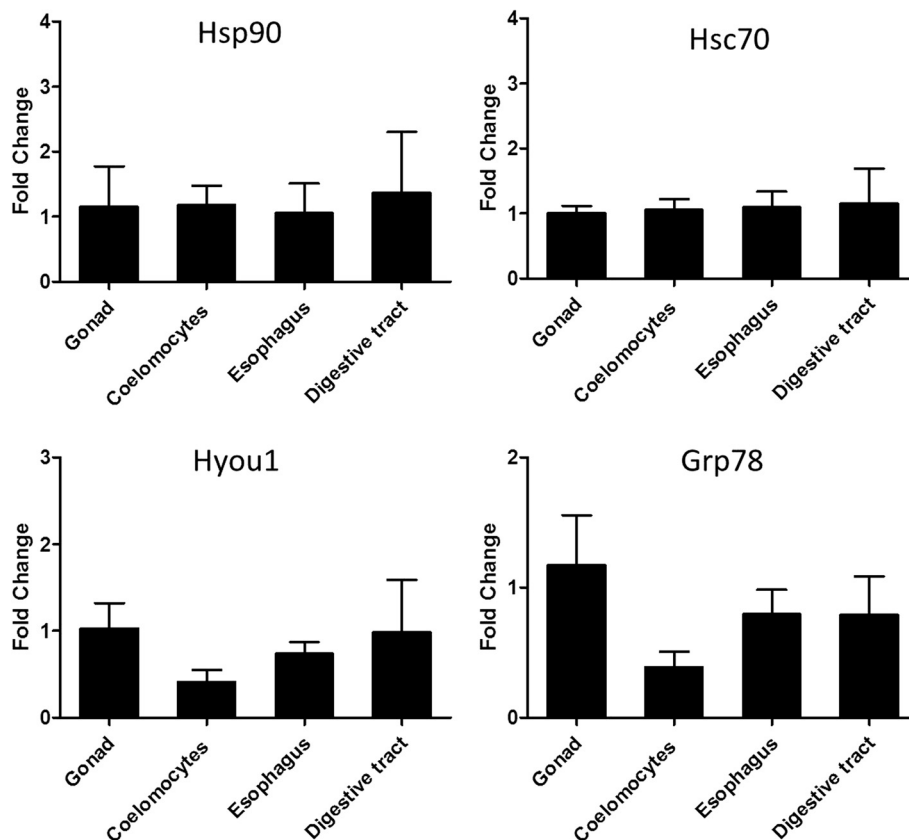
Fig. 1 Tissues expression of chaperones mRNA under control condition. The genes were measured in four different tissues. Endogenous control genes for quantification was the mean of Gapdh and18s. The relative mRNA expression of each tissue was compared to gonad expression to determine the tissue specific expression. No significant differences were found between treatments ($p > 0.05$)

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On the other hand, the analysis of the level of expression of the Hsp90 gene showed a significant increase in coelomocytes at 3 °C only after 48 h ($p < 0.05$) (Fig. 2). Interestingly, at the higher experimental temperature (5 °C), the expression of this gene was not significantly different when compared to the control conditions or the lower thermal stress treatment. Similarly to the coelomocytes profile, the expression of the Hsp90 in the esophagus showed significantly differences at 3 °C after 48 h, while at the 5 °C treatment, a significant increase was observed only after the first hour (Fig. 3).

The expression during thermal stress in three members of the Hsp70 gene family was analyzed in coelomocytes and esophagus. The expression of Hsc70 did not show a significant increase for any temperature and time evaluated in both tissues. The expression of the Hsc70 in the tissues sampled tended to have a lower expression compared to the control condition, however it was not significant. In contrast, the expression of gene Hyou1 and Grp78 showed a significant increase ($p < 0.05$) in coelomocytes at 48 h at 3 °C (Fig. 2). During thermal stress at 5 °C, the expression of both genes was not increased compared to the control condition. In the esophagus, both genes presented a different expression profile. Hyou1 showed an increase at 1 and 24 h, however it was not significant. On the other hand, the gene

Grp78 showed a similar profile to that observed in coelomocytes, being significant at 48 h at 3 °C (Fig. 3). Thermal stress at 5 °C, in the esophagus, did not generate an increase in the expression of these two genes.

Discussion

This investigation is the first description of the expression of chaperones proteins in different tissues of the Antarctic sea urchin *S. neumayeri* during acute thermal stress. To date, few investigations in Antarctic marine invertebrates have evaluated the thermal stress response associated with the expression of different chaperones in different tissues [19]. In Antarctic echinoderms the expression of chaperones type Hsp70 has been assessed only in of the starfish *O.validus* has been evaluated [22]. Therefore, generating new information and molecular resources in other echinoderms species is mandatory in order to understand how these highly vulnerable Antarctic marine invertebrates have faced the current and futures changes in sea water temperature in the Antarctic Peninsula as well as to predict the ecophysiological implications under future climate change scenarios [39]. Although, experimental temperatures used in this investigation matched some of those used with previous studies [19], these temperatures do not correspond to the current environmental temperatures found in the
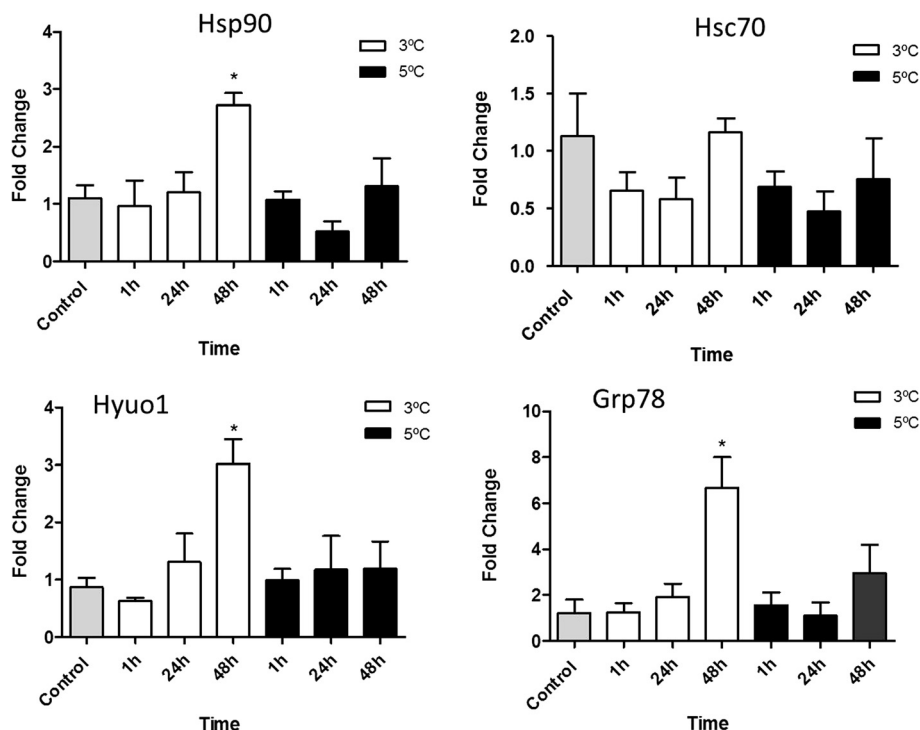
Fig. 2 Variation of gene expression during acute thermal stress in coelomocytes of *S. neumayeri*. Antarctic sea urchins were exposed to 3 °C and 5 °C for 1, 24 and 48 h. Grey bars: control group; white bars: 3 °C and black bars: 5 °C. Bars represent the mean of four individuals and error bars are standard error. *$p < 0.05$

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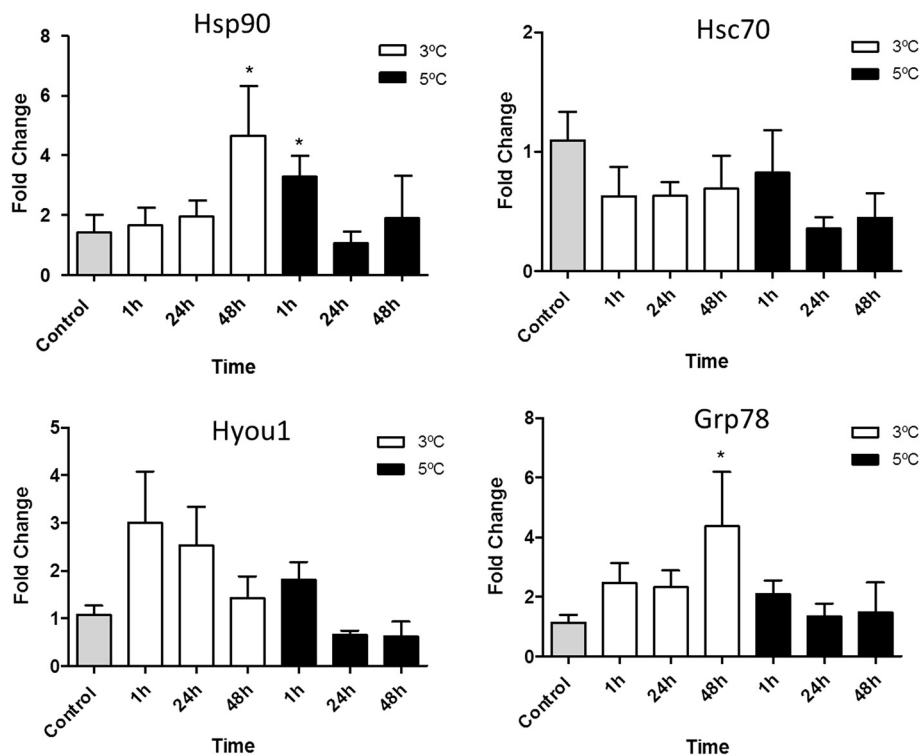
Fig. 3 Variation of gene expression during acute thermal stress in esophagus tissue of *S. neumayeri*. Antarctic sea urchins were exposed to 3 °C and 5 °C for 1, 24 and 48 h. Grey bars: control group; white bars: 3 °C and black bars: 5 °C. Bars represent the mean fold of four individuals and error bars are standard error. *$p < 0.05$

Antarctica. However, it is already possible to find temperatures close to 3 °C in some areas of the Antarctic Peninsula [27], therefore, the information obtained by us at 3 °C is ecologically relevant and may indicate the real capacity of these species to respond to extreme thermal events in their natural environments.

The analysis of the expression of the Hsp90, Hsc70, Hyou1 and Grp78 genes of *S.neumayeri* during thermal stress, showed that these genes expressed themselves in a constitutive form in the four tissues evaluated. Since low temperatures can be responsible for protein damage, constant vigilance is required by the chaperones [20, 40]. This type of expression would be required to maintain cellular homeostasis in *S. neumayeri* at low temperatures to avoid protein damage. While thermal stress responses could be quantifiable due to increased expression of some of these genes, especially in coelomocytes, the same expression was not necessarily observed for the different tissues evaluated.

The expression found for *S.neumayeri* showed a different tendency to what has been determined for other marine invertebrates such as *O. validus* and *P. gibber* that lack a thermal stress response, [22]. In these invertebrates, acute temperatures of 10–15 °C, for 2 h and then return to temperatures below zero and 2 and 6 °C for longer time periods, do not produce a significant increase in the expression of Hsp70. This latter condition, is similar to the one used by us for *S. neumayeri*, however, for some of the genes analyzed (Hsp90, Hyou1 and Grp78), we observed a delayed induction at 48 h over the control group. The expression of the Hsc70 gene in *S. neumayeri* did not vary probably because this gene is constitutive and exhibited a high identity sequence (89 %) compared to the not inducible Hsc70's from *S. purpuratus*. A recent study of two sister species of Euphausiids (Antarctic krills) *Euphausia superba* and *Euphausia crystallorophias* determined the presence of heat shock response (in terms of up-regulation of HSP70 family members), although the response is weak compared with the fold changes observed in temperate species [9]. Surprisingly some of the up-regulated Hsp70 at 3 and 6 °C have the signatures of constitutive chaperones. For this reason, we cannot exclude the presence of inducible forms of Hsp70 in *S. neumayeri*. Thus, it is necessary to continue with further analyses of genomic data to determine the expression of several isoforms of Hsp70.

Other HSP such as the Hsp90 are found in the cytoplasm and their primary role is cellular differentiation, protein regulation, apoptosis control and signal transduction in response to stress [41]. Studies regarding the expression of Hsp90 has been done primarily in marine

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invertebrates, demonstrating that their induction is possible against factors such as thermal stress, hypoxia, osmotic stress and presence of bacteria [42]. Little information exists about the expression of this gene in Echinoderms, where the increase of the Hsp90 expression in thermal stress has been document only in the sea cucumber *Apostichopus japonicus* [43, 44]. The expression of Hsp90 under thermal stress in *S. neumayeri* was similar in the two tissues analyzed, showing a tendency to increase its expression at 3 °C at 48 h, but not at a higher temperature (5 °C). This situation contrasts with that observed in the Antarctic clam *Laternula elliptica* where the relative expression level of Hsp90 messenger RNA at 10 °C was clearly upregulated and peaked at 12 h in the digestive gland and at 24 h in the gills, then dropped progressively [45], evidencing a faster HSP response in this mollusc compared to the sea urchin *S. neumayeri.*

Comparative physiological studies have found that other HSP such as the Grp78, it play an important role in stress mitigation, cellular protection and maintenance of the endoplasmic reticulum functions [36, 46]. For example, Grp78 can protect cells from cellular death by suppressing the accumulation of oxyradicals [47]. However, there is little information about its possible role in Antarctic organisms. The expression of Grp78 has been evaluated in gonad tissue in the Antarctic seastar *O. validus* [20, 22], showing that its expression is not inducible during thermal stress at different temperatures (2, 6 and 10 °C). In contrast, the expression of Grp78 in *L. elliptica* molluscs increased in the gills, mantle and siphon [20, 48]. In *S. neumayeri*, the overexpression of this gene has a delayed response. In *L. elliptica*, temperatures of 8, 10 and 15 °C produce increases in Grp78 expression after 2 h of thermal stress. Therefore, we cannot rule out that higher temperatures could increase expression of Grp78 in *S. neumayeri*. The increase in temperature could also increase the quantity of reactive oxygen species because the concomitant effect on the respiratory activity in *S. neumayeri* (unpublished data), affecting the proteins of the endoplasmic reticulum and therefore Grp78, as well as other chaperones from the ER which could help diminish the oxidative damage [37, 49]. In addition to the Grp78, the endoplasmic reticulum also contains the protein Hyou1 (hypoxia up-regulated 1), which is in charge of the transport of mature proteins from the endoplasmic reticulum to the Golgi apparatus [50]. Under stress conditions by hypoxia, this protein accumulates in the endoplasmic reticulum. Protein suppression is associated with the increase of apoptosis, which suggests an important role in cellular perturbation caused by hypoxia [51]. The gene Hyou1 has not been studied thoroughly in marine invertebrates, however, induction under thermal stress, has

been observed in the gills of the *Crassostrea gigas* molluscs [52]. This study is the first to document gene expression with thermal stress in Antarctic marine organisms. The increased expression in coelomocytes would indicate that these cells could resist better to damage by hypoxia and warming, at least in the short-term.

While coelomocytes showed a greater response capacity to thermal stress associated with an increase in expression of the chaperones Hsp90, Grp78 and Hyou1, the esophagus also showed a significant tendency of increased expression for these genes. This demonstrates the necessity to broaden the number of tissues studied, as the expression of chaperones could be tissue specific and one tissue could respond more than another to a certain thermal stress condition as seen in other models [19, 48].

Recently studies about ocean change stressors have been developed in *S. neumayeri*. These studies have focused on the effect of a single stressor or a combination of temperature and pH on adult sea urchins, during fertilization and early developmental stages [53–57]. *S. neumayeri* has shown that under long term acclimation, adult stages are lest affected by two combined effect of temperature and reduced seawater pH [56]. In the case of fertilization and embryonic development, increased seawater temperature (1.5 and 3.0 °C) was not deleterious to fertilization at pH 7.7–8.0 [54]. These results show that *S. neumayeri* embryos have a relatively robust response in this experimental condition may be due to the expression of heat shock proteins present in the eggs before fertilization or expressed during early developmental stages [54, 55]. Future studies should deepen into the aspects related to the expression of different chaperone isoforms, the capacity of expression during different stages of larval development or the effect of other stress response inductors, such as hypoxia or presence of heavy metals, for example cadmium in *S. neumayeri.*

Conclusions

The sea urchin *S. neumayeri* showed a stress response as a result of an increased expression of three chaperones implicated in response to thermal stress. However, this response is not typical to what has been described for other Antarctic invertebrates such as the molluscs *N.concinna* or *L. elliptica*, that present a rapid increase in the HSP response. Our results, suggest that the increase in the transcription of the Hsp90, Grp78 and Hyou1 genes is associated with the repair and folding of proteins, that would assure cellular and tissue viability. The degree of expression of these chaperones in *S. neumayeri* suggest the existence of specific expression profiles of HSP at different levels of organismal

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organization probably as a result of differences in the thermal sensitivities and tolerances of the tissues, as well of their physical and biological compositions. Nonetheless, further investigation is necessary to elucidate the mechanisms of protection in cellular homeostasis, as well as, how the transcription levels of these genes are correlated with the expression of the proteins they encode. Finally, it is necessary to evaluate other environmental factors that could produce a response the expression of HSPs in laboratory condition and in natural populations.

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