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Differential expression of stress candidate genes for thermal tolerance in the sea urchin *Loxechinus albus*

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ABSTRACT

Marine ectotherms inhabiting intertidal and shallow subtidal environments are continuously exposed to diurnal tidal cycles and seasonal variability in temperature. These organisms have adaptive mechanisms to maintain cellular homeostasis, irrespective of thermal environmental variation. In this study, we describe the molecular responses to thermal stress in the edible sea urchin *Loxechinus albus*. In particular, we determined the differential expression of a set of molecular markers that have been identified as targets of stress-related responses. These include the heat shock proteins (*hsp70* and *hsp90*), cell detoxification proteins (*cytochrome P450*), and osmoregulatory proteins (α and β - Na^+/K^+ *ATPase*). We exposed individuals to different temperatures; a warm treatment (18 ± 1.0 °C), a cold treatment (10 ± 1.0 °C), and a control treatment (average local temperature of 14 ± 1.0 °C) and differential expression was quantified after 2, 6, 12 and 48 h of exposure. Levels of mRNA were quantified by reverse transcription-quantitative polymerase chain reaction, and the relative expression of each gene was calculated using the 18S rRNA gene as a reference, and the control treatment as a calibrator. We found that the expression levels of all studied genes increased during exposure to warmth. The largest increase in expression was observed in *cytochrome p450* genes (ca. sixteen-fold); this was followed by increases in the expression of the Na^+/K^+ *ATPase* (ca. eight-fold) and by the *hsp* (ca. six fold) genes. These results indicate that sea urchin thermal stress responses depend on differential gene-regulation, involving heat-shock, membrane potential, and detoxification genes that generate an integrated adaptive response to acute environmental changes.

1. Introduction

Among marine ectotherms, echinoderms, and in particular sea urchins are especially constrained by their environment because their internal fluids are in constant exchange with their external environment (Willmer et al., 2009), and hence tissues are directly exposed to fluctuations in temperature (Hochachka and Somero, 2016). At the level of cells and tissues, chronic exposure to contrasting temperatures induces periodic thermal stress and deleterious effects which are reflected by the cellular stress response (CSR) (Pörtner, 2002).

The CSR in animals involve a number of conserved molecular changes that cells experience in response to environmental stressors, including temperature, toxins or mechanical damage (see Hochachka and Somero (2016) and Angilletta et al. (2002)). In many species, CRSs and physiological plasticity are achieved by adjustments in gene expression in response to environmental changes (Anestis et al., 2008;

González et al., 2016; Hofmann et al., 2008; Hofmann and Todgham, 2010; O'Donnell et al., 2009; Webster et al., 2013). Then, a straightforward approach to explore these functional responses are transcriptomic studies (i.e., quantifying mRNA changes during experimental exposure; Banerjee et al., 2014; González et al., 2016; Martin et al., 2011; Zhao et al., 2014).

An ubiquitous mechanism for facing stress is the overexpression of heat shock proteins (hsp) which allows for the refolding of denatured proteins and the breakdown and replacement of the proteins that are not repairable (Hofmann and Todgham, 2010; Tomanek, 2008). According to Feder and Hofmann (1999), all species have thermal shock genes but the degree to which these are expressed varies widely. Thus, the maximum capacity to differentially express a given heat-shock protein can be used as a measure of adaptation to environmental fluctuations (Feder and Hofmann, 1999; Osovitz and Hofmann, 2005). However, other molecular processes involved in detoxification could

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indirectly affect thermal stress responses. For instance, Cytochrome P450 (CYP) proteins play an important role in the biotransformation of endobiotic chemical products (e.g. reactive oxygen species) generated during chemical stress (Andersson and Förlin, 1992; Zhang et al., 2012). Similarly, other genes, such as α and β - Na^+/K^+ ATPase, that maintain the membrane potential may also play a role in thermal stress responses. The expression of these genes fluctuates particularly when larvae are exposed to osmotic stress or changes in pH (Dong et al., 2008; Martin et al., 2011). However, no information is available regarding expression patterns of these genes in echinoderms, under thermal stress exposure.

Sea urchins play an important ecological role in coastal ecosystems as a food source for other animals and as a regulator of seagrass and rocky reef communities (Shears and Babcock, 2002). In Chile, the edible species *Loxechinus albus* is one of the most important benthic herbivores in intertidal and shallow subtidal rocky environments (Vásquez, 2007) and is the most economically important species in the littoral system (Cárcamo et al., 2005). *L. albus* is distributed from northern Peru to southern Chile (6°S to 56°S) and is endemic to the Southeastern Pacific coast (Vásquez, 2007). The distribution of *L. albus* is affected by two different current systems: the Humboldt Current System north of 42–45°S and the Cape-Horn Current and Interior Chilean Sea to the south (Thiel et al., 2007). These latter two systems are characterized by great seasonal and daily variation in temperature, salinity, and pH (Aravena et al., 2014; Broitman et al., 2011).

Here we explored the transcriptional response of thermal stress in the sea urchin, *Loxechinus albus*. We were focused on the transient (i.e., in 72 h) differential expression of a set of five molecular markers (i.e., α and β Na^+/K^+ ATPase, cytochrome P450 and *hsp 70* and *90*) that have been identified as targets of the stress response. Although changes in the expression of these genes have been characterized as being influenced by various types of environmental stimuli, to the best of our knowledge, expression of these genes in sea urchins exposed to thermal stress has not been quantified. Given that CSRs are conserved, we predicted that we would find a response similar to that of the classic thermal-stress response; specifically, we expected to see that *hsps* genes will be show a peak in overexpression after a few hours of exposure, followed by a reduction as animals start to acclimate. However, given the short duration of our experimental treatment (see Section 2), acclimation was not complete.

2. Methods

2.1. Study site and animals

One hundred juvenile individuals of *Loxechinus albus* (~30 mm \pm 10 mm average size) were obtained from Los Molinos, Valdivia (39°40'S –73°12'W) by SCUBA diving in spring of 2012. After collection, sea urchins were transported at 14 °C to Calcuco Coastal Laboratory (39°46'44"S –73°23'31"W) and were placed in plastic containers (30 \times 40 cm). Individuals were kept for five days and fed *ad libitum* daily with macroalgae (Gonzalez et al., 2008); the water in the containers was replaced every day before the beginning of the experiments.

2.2. Experimental setup

After an acclimation period to the laboratory, individuals were randomly assigned to one of three treatments: warm treatment with 18 ± 1.0 °C, cold treatment with 10 ± 1.0 °C and a control treatment using the average local temperature (14 ± 1.0 °C). Experimental temperatures were chosen as a measure of the thermal extremes that this species could experience and based on analysis of the variability in sea surface temperature along the Chilean coast. The data analysis included average daily temperatures for summer of 2011–2012 obtained from an Advanced Very High Resolution Radiometer (AVHRR) and an Advanced

Microwave Scanning Radiometer (AMSR) with a spatial resolution of 25 km \times 25 km. These data can be found at <http://www.ncdc.noaa.gov/oa/climate/research/sst/oi-daily.php> (Reynolds et al., 2007).

In order to ensure that there would be at least three biological replicates for the gene expression analysis, 25 individuals were subjected to each treatment. To obtain a temporal expression profile of candidate genes for each treatment, we selected three individuals per treatment and at four different time points: 2, 6, 12 and 48 h. Thus, a total of 12 individuals (with a state of optimal health) were selected per treatment; thus three biological replicates were obtained for each time point, and a total of 36 samples were used for the expression analysis of the five candidate genes. Tissues (from the Aristotle's lantern, see below) were immediately frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

2.3. Phenotypic responses

The phenotypic effect of temperature on *L. albus* was characterized using well-defined methods described for sea urchins exposed to thermal stress (Hernández et al., 2004). Briefly, Hernández et al. (2004) classified the responses as follows. “E1: individuals move actively toward the bottom of the aquarium with the tube feet extended to the maximum; E2: retraction and decrease of tube feet movement also followed by decrease of spine movement; E3: with the tube feet retracted, the urchin increases movement mostly using the large and small spines; E4: individuals stop moving, relax the large spines but activity continues with light movements of small spikes; E5: urchins stop movement with all spines and tube feet relaxed, and in some cases the anus is protruded”.

2.4. Quantitative Reverse Transcription PCR (RT-qPCR)

RNA was extracted from the Aristotle's lantern of each urchin using a commercial RiboPure™Kit (AMBION); total RNA of each stored sample was extracted from all individuals. Integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) with the Agilent RNA 6000 nano commercial kit (Agilent Technologies); only RNA with integrity (RIN) above 7 was selected. After this filtering, the final number of individuals in the analysis was reduced to 30 (we maintained at least two replicates per treatment). RNA quantification was performed using a DQ300 Hoefer fluorometer and the Quant-it RiboGreenRNA Assay Kit (Thermo Fisher Scientific). From approximately 30 ng/ μ L of total RNA (previously treated with the DNA-free™ Ambion kit), cDNA was synthesized using the AffinityScript qPCR cDNA Synthesis kit (Agilent). Then, the cDNA was diluted 1:10, and 1 μ L of this dilution was used for PCR reactions (12.3 μ L final volume). The specific *L. albus* primers (i.e., for *hsp 70* and *90*, cytochrome P450, and α and β Na^+/K^+ ATPase) and two endogenous control genes (*18S rRNA* and *RNA-binding protein 8A*) were designed using AmplifX 1.3.7 (Jullien, 2005) and FastPCR Professional 5.3.103 (Kalendar et al., 2014). These genes were chosen because they have previously been described as being very stable in echinoderms (Runcie et al., 2012; Turbeville et al., 1994). We used the NormFinder algorithm in order to select the most stable of the two control genes; a random sample of 20 individuals dispersed among the treatments were used (Andersen et al., 2004). Given that the results using either endogenous control gene are similar, we only present the results using the 18S gene.

Primer specificity was determined by comparing sequence homology using data from previous studies (see Table 1) and sequences of the annotated *L. albus* transcriptome, deposited in the GenBank public database under the accession number SRP066399 of the bioproject PRJNA302689 (Gaitán-Espitia et al., 2016). All primer pairs were tested for performance and efficiency across a series of cDNA dilutions (1:20; 1:40; 1:100; 1:200; 1:1000; 1:2000) (see Table 2). Each PCR reaction mix contained 1 μ L of each primer at 10 nM, 6.25 μ L SYBR Green PCR Master Mix (Applied Biosystems), and 3.25 μ L ultrapure H₂O. Primer efficiency was calculated as $E = 10^{(-1/S)} \times 100$, with *S* being the slope

Table 1

Candidate genes used in this study. The primary function of these genes, accession number, database, species and source references are presented.

Candidate genes	Primary function	Accession number	Species	Refs.	Database
α -Na ⁺ /K ⁺ /ATPase	sodium potassium pump	AM562805	<i>P. lividus</i>	(Martin et al., 2011)	NCBI
β -Na ⁺ /K ⁺ /ATPase	sodium potassium pump	AM554663.1	<i>P. lividus</i>	(Martin et al., 2011)	NCBI
cytochrome P450	detoxification	SPU_010576	<i>S. purpuratus</i>	(Goldstone et al., 2006)	spbase.org
hsp 70	chaperone	XM_791153.3	<i>S. purpuratus</i>	(Runcie et al., 2012)	NCBI
hsp 90	chaperone	NM_214643.2	<i>S. purpuratus</i>	(Runcie et al., 2012)	NCBI
18S	ribosomal protein	L28056.1	<i>S. purpuratus</i>	(Turbeville et al., 1994)	NCBI
RBM8A	RNA binding protein	XM_782434.3	<i>S. purpuratus</i>	(Runcie et al., 2012)	NCBI

of the linear regression (Pfaffl, 2001). The transcriptional level of the five candidate genes was evaluated by RT-qPCR. The relative expression of the target gene compared to the control endogenous gene was computed by relative quantification using the comparative Ct method (Applied Biosystems User Bulletin No. 2P/N 4303859, 1997) (Schmittgen and Livak, 2008); the gene expression of the control treatment was used as a calibrator. Negative controls were included for detecting foreign contamination, and all PCR reactions were performed in triplicate in a Eco Real-Time PCR System (Illumina) using the following cycling conditions: 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 58 °C and 15 s at 72 °C. To confirm the absence of nonspecific amplifications, a dissociation curve was included immediately after each PCR using a ramp of 58–95 °C. Finally, to test for significant differences in the gene expression levels, we performed a factorial analysis of variance (ANOVA) with gene, time, and treatment as independent factors. All relative expression data were log₂ transformed. Parametric assumptions were checked using the Levene and Wilcoxon tests (Levene, 1960; Wilcoxon, 1945). Every value falling within the range from -1.0 to +1.0 in log₂-relative expression units was considered non-significant (sensu Tapia et al., 2015). These threshold values correspond to duplication or reduction of half of the expression level of a gene.

3. Results

3.1. Phenotypic response to thermal stress

For the initial 12 h, all individuals in our treatments (warm, cold, and control) were in the E1 stage, which was characterized by active movements toward the bottom of the aquarium with their tube feet completely extended. After 48 h of thermal exposure, only 4% of the individuals (all treatments) were observed in the E2 stage; i.e., the tube feet were retracted and movement of the feet and spines decreased. We did not observe any individual at the E5 stage, i.e., without movement of the spines and with relaxed tube feet (only individuals at the E1 stage were used for the transcriptomic analysis).

3.2. Transcriptional levels

3.2.1. Statistical analysis

The statistical analysis indicated that there was a significant effect of treatment ($F_{1,60} = 21.12$; $p < 0.001$); there was a global overexpres-

sion of the genes when individuals were subjected to the warm treatment while the opposite was found for the cold treatment (Fig. 1). In addition, there was a significant effect of time ($F_{3,60} = 11.58$; $p < 0.001$); for all target genes of individuals in the warm treatment, expression increased during the first hours (two and six hours) and was then followed by a decrease (12 and 48 h). Contrary to this, there was no effect of gene analysed ($F_{4,60} = 1.91$; $P = 0.12$). Furthermore, there was no significant effect of the interaction between factors. In the following section we describe the results for each specific gene.

3.2.2. Heat shock protein 70 (hsp70)

Throughout the experiment, this gene was up-regulated in individuals subjected to the warm treatment (Fig. 1a). For individuals in the cold treatment, however, gene expression among the biological replicates was not homogeneous (Fig. 1b).

3.2.3. Heat shock protein 90 (hsp90)

For individuals subjected to the warm treatment, the relative expression of this gene increased after two hours (2.23 ± 1.13) and was then followed by a decrease during the subsequent time points (Fig. 1c). Contrarily, the gene expression of individuals exposed to the cold treatment decreased constantly (Fig. 1d).

3.2.4. Cytochrome P450

For individuals in the warm treatment, this gene was up regulated after two hours. This was followed by a decreased in expression after six and 12 h, but there was a sharp increase in expression at 48 h (Fig. 1e). For individuals in the cold treatment, there was no clear trend (Fig. 1f).

3.2.5. α -Na⁺/K⁺/ATPase

The relative expression of this gene in individuals exposed to warmth increased after 2 h of exposure, and then the expression decreased (Fig. 1g). For individuals in the cold treatment, the expression of this gene increased after six hours of thermal exposure; following this, the gene expression decreased (Fig. 1h).

3.2.6. β -Na⁺/K⁺/ATPase

After 2 h of exposure to the warm treatment, the relative expression of this gene was up-regulated (2.97 ± 1.28); this up-regulation was then followed by a decrease in expression (Fig. 1i). The pattern of gene expression for individuals subjected to the cold treatment was similar

Table 2

Transcripts targeted in the experiment, primer sequences, annealing temperature and efficiency of the primer.

Gene	Forward	Reverse	Tm (°C)	Primer efficiency (%)
α -Na ⁺ /K ⁺ /ATPase	AGC GTA TCC TTG ACC GTT GT	ATC GGC TGG CAT GAA GCA TT	60	93,6
β -Na ⁺ /K ⁺ /ATPase	ACA ACT TTC GGG CAG CGA AT	AGG CCC AGA ATG CAG CTA AA	60	94,6
cytochrome P450	TGG CAT GAT CCG CAC GTA TT	TGC TGG AAG AGG TTG GTG AA	60	93,8
hsp 70	TGC CCG GTT TGA AGA CAT GA	GTT GAG CTC TTT GCC GTT GA	60	93,6
hsp 90	TGA AGA CCA CGG CAA CAG AT	TCA GGA GTC GCT CAA CGA AT	60	100,6
18S	GTG GAG CGA TTT GTC TGG TT	AAG GGC ATC ACA GAC CTG TT	60	96,1
RBM8A	GCG GAC GTT TTG GAC ATT CA	CAT CGT CAG CTG TTC GGA TT	60	93,7

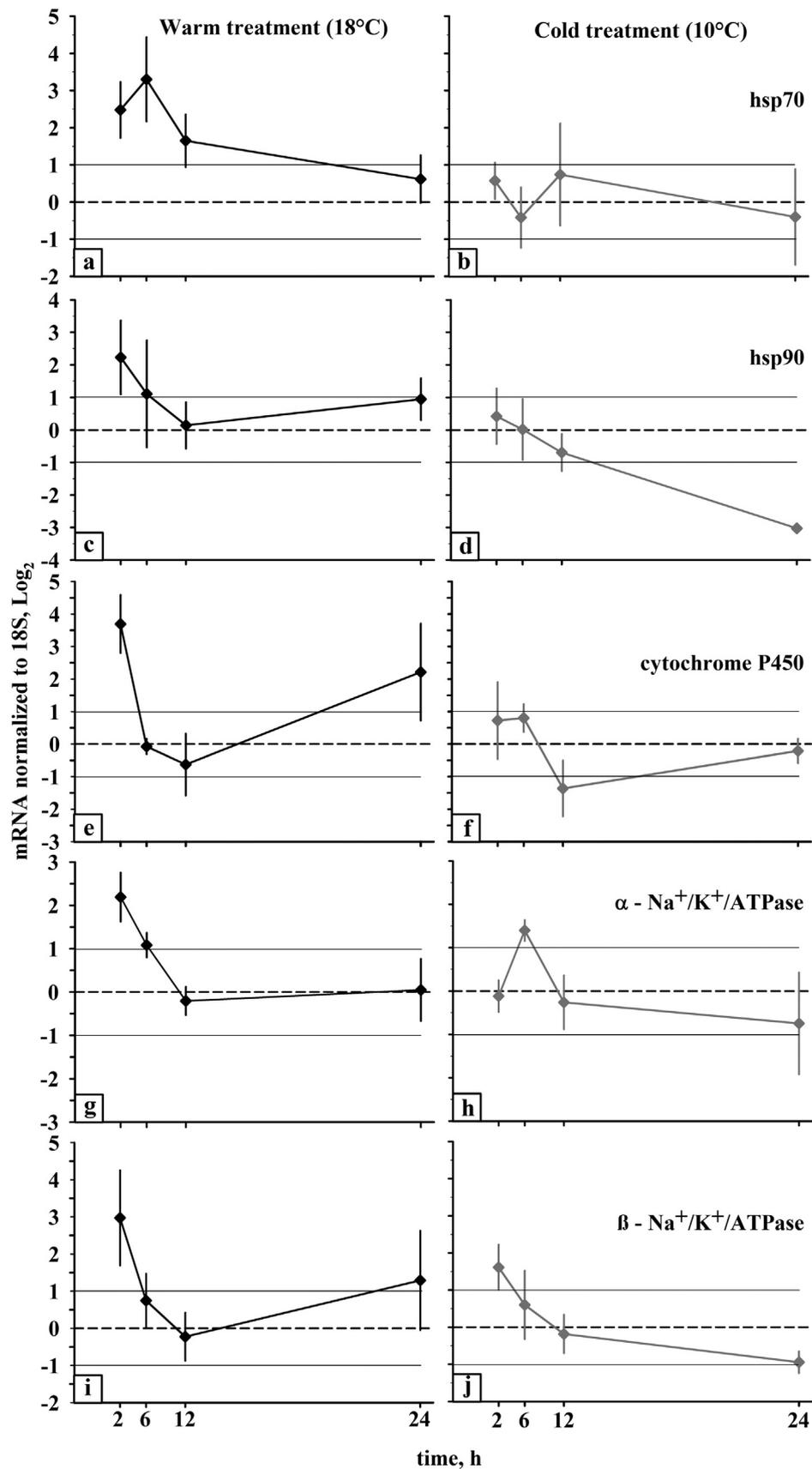


Fig. 1. Expression levels of candidate genes between treatments and control. Log₂ fold change of α and β Na⁺/K⁺ ATPase subunits, cytochrome P450, hsp70 and hsp90 mRNA in sea urchin exposed to 2, 4, 12 and 48 h of heat stress (mean and standard deviation) in relation to the control treatment (dotted lines). Experiments were carried out in warm (left panel) and in cold conditions (right panel). The continuous lines within the plots represent the threshold of +1 and -1 arbitrary values from which we decided that the gene was over- or under-expressed (see Section 2 for details).

but somewhat less pronounced (Fig. 1j).

4. Discussion

Understanding the physiological plasticity of organisms in response to temperature variability has become urgent in light of climate change (Sinervo et al., 2010; Walther et al., 2002). In this context, organisms inhabiting variable environments such as intertidal and subtidal areas are ideal models for elucidating mechanisms of cellular response, as these organisms experience daily and seasonal fluctuations in temperature (Podrabsky and Somero, 2004; Somero, 2002; Tomanek, 2008; Tomanek and Helmuth, 2002). In order to understand the mechanisms for adaptation to these fluctuating environments (Gracey, 2007; Kültz, 2005; Martin et al., 2011), here we monitored the transcriptional response of genes coding for key proteins involved in cellular stress response (CSR) in an ectotherm.

As expected, we found that the expression of heat-shock genes changed in response to stress of increased temperature (warmth); specifically, this expression increased significantly in individuals in the warm treatment after two hours of exposure (six-to-eight fold, compared with the control treatment), but exposure to decreased temperatures (cold) did not elicit the same response (Fig. 1b,d). In sea urchins, overexpression of *hsp70* in response to thermal stress has been detected in species such as *Strongylocentrotus purpuratus* (Hammond and Hofmann, 2010; Osovitz and Hofmann, 2005; Runcie et al., 2012) and *Paracentrotus lividus* (Sconzo et al., 1992). Little information exists about the expression of *hsp90* in echinoderms, but this has been documented for the sea cucumber *Apostichopus japonicus* (Xu et al., 2014; Zhao et al., 2011) and the sea urchins *P. lividus* (Runcie et al., 2012) and *Sterechinus neumayeri* (González et al., 2016).

Given that oxidative damage represents an important metabolic consequence of thermal stress, with the concomitant endobiotic production of toxic compounds (e.g. reactive oxygen species), we expected to find accompanying changes in the cytochrome P450 monooxygenase system (*CYP* genes) (Andersson and Förlin, 1992; Goldstone et al., 2006). In actuality, the transcript levels of p450 were exceedingly high for warm- compared to cold-exposed urchins (transcript levels were over-expressed sixteen-fold after two hours of exposure, see Fig. 1e), suggesting that one of the most compromised functions is detoxification. However, after six-hours of exposure the levels of expression of this gene were reduced enough to not be detectably different from the control (both, in warm and cold-exposed individuals, see Fig. 1e, f), reflecting the transient nature of this response. This result is consistent with what has been found in other invertebrates, such as the reef-building coral endosymbiont *Symbiodinium* (Rosic et al., 2010). Similarly, exacerbated *CYP* expression in oligochaetes of the genus *Thalassodrilides* has been detected for individuals exposed to pollutants and thermal stress (Ito et al., 2016).

By pumping sodium and potassium by active transport, the Na^+/K^+ ATPase enzymes play a crucial role in maintaining the potential of the cell membrane (Cieluch et al., 2005; Dong et al., 2008; Kaplan, 2002). Previous studies have shown that this is one of the most energetically demanding transport processes in invertebrates, especially in energy metabolism regulation (Leong and Manahan, 1997). These results in general coincide with our study, as we found marked differences in gene expression among treatments. That is, the gene expression of the alpha- and beta subunits in individuals subjected to the warm treatment was increased (four-to-eight fold, compared to the control treatment) (Fig. 1g,i). Additionally, individuals exposed to cold showed a less-pronounced response than those exposed to warmth.

Overall, our results suggest that the temporal transcriptomic response of *hsp* genes, *cytochrome P450*, and α and β Na^+/K^+ ATPase is flexible and concerted. Moreover, warm exposure led to similar transcriptomic responses of all genes, and responses to this treatment were drastic in the two first hours and then were followed by more idiosyncratic trends (e.g., *hsp70* expression increased even more).

However, caution is in order when interpreting the detailed trends of these transcriptomic responses. *First*, because of logistical limitations, replication was not complete (urchins were caged together). This problem could have caused some stressed individuals to be affected by the responses of other urchins in the same tank, thus inflating the measured gene-expression. *Second*, we analysed one kind of tissue, which does not represent the full metabolic response of the individual. *Third*, the period of thermal exposure was probably too short for the animals to completely acclimate to the new conditions. These caveats indicate that further and more detailed studies are needed to capture the full molecular response of echinoderms during thermal stress, thermal acclimation, and adaptation.

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