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ORIGINAL PAPER

Effects of thermal stress on the immune and oxidative stress responses of juvenile sea cucumber *Holothuria scabra*

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Abstract *Holothuria scabra* is the most valued and cultured tropical sea cucumber, given the great demand of this species for human consumption. However, despite its ecological and economic relevance, little is known regarding its immune responses under thermal stress. Here, the main goal was to study the response of sea cucumbers to temperature stress, assessing sub-organismal alterations and acclimation capacities of juveniles to temperature changes. After changing temperature (1 °C/day) for 6 days, organisms were exposed to temperature conditions of 21 °C (cold), 27 °C (control), and 33 °C (warm) over a 30 day period. At each 15-day interval (T0, T15, and T30), six replicates per condition were killed for biochemical analysis. Immune responses were addressed by studying the activity of phenoloxidase(PO) and prophenoloxidase (ProPO) in the coelomic fluid. Antioxidant defence responses—catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) enzymatic activities—were measured in the muscle and respiratory tree tissues, whereas oxidative damage was evaluated by measuring levels of superoxide radicals (ROS), DNA-strand breaks and lipid peroxidation (LPO). Juvenile *H. scabra* increased SOD and PO activities when temperature was elevated, and revealed low levels of ROS and damage in both cold and warm treatments throughout the experiment, confirming the organism's moderate thermal stress. After the short acclimation period, the immune and antioxidant responses prevented damage and maintained homeostasis. This multi-biomarker approach highlights its usefulness to monitor the health of *H. scabra* and to gain insight concerning the use of this high-valued species in global-scale aquaculture from different temperature regions.

Keywords Biomarkers · Tropical aquaculture · Climate change · Environmental stress · Antioxidant responses · Acclimation

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Introduction

Water temperature is a crucial factor influencing the physiological status of organisms in terms of growth rates, oxygen consumption and metabolism, or moulting process (e.g. Sierra et al. 1999; Zdanovich 1999; Dong et al. 2006). While it has been shown that changes in water temperature can evoke acute or chronic stress in a variety of organisms (Cheng and Chen 2000; Cheng et al. 2004; Coates et al. 2012), there is still some ambiguity concerning the effects of temperature variations. One common strategy to monitor the effects of temperature stress at lower biological

organizational levels is the use of biomarkers (Peakall 1992; Menezes et al. 2006). Some of the most studied and applied biomarkers are parameters related with oxidative stress defence against reactive free radical production, such as superoxide anions (O_2^{-}), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) (Dröge 2003). The ability to regulate the production of these reactive oxygen species (ROS) and maintain "redox homeostasis" determines the health status of an organism (Ames et al. 1993).

It is generally known that temperature stress induces the generation of ROS (Valavanidis et al. 2006). Disturbing the balance between endogenous and exogenous ROS can cause a consequent incapacity of the antioxidant defences to respond, which may lead to oxidative damage in different target biomolecules and tissues (Sohal et al. 2002; Valavanidis et al. 2006). Superoxide radicals, for example, are known to have negative impacts on antioxidant vitamins (e.g. tocopherol, ascorbate) and enzyme activities [e.g. catalase (CAT), glutathione reductase (GR) and peroxidases], which can in turn result in DNA damage, enzymatic inactivation, or peroxidation in important cellular biomolecules, especially lipids (Kono and Fridovich 1982; Blum and Fridovich 1985; Valavanidis et al. 2006). Thus, antioxidants play a crucial role in the maintenance of cell integrity, homeostasis, and in prevention of oxidative damage (Vigo-Pelfrey 1990; Dix and Aikens 1993). Superoxide dismutase (SOD) and CAT provide the first line of defence in responses to oxidative damage. Initially, SOD converts the superoxide radicals to O_2 and H_2O_2 and CAT in the next step transforms the H_2O_2 into O_2 and H_2O (Howcroft et al. 2009). Another important enzyme to protect the cells is GR, which reduces glutathione disulfide (GSSG) into two molecules of glutathione (GSH), which act as a non-enzymatic antioxidant (Saint-Denis et al. 2001).

Aside from enzymes involved in oxidative stress responses, environmental stress in marine invertebrates can also evoke immune responses through for instance the activity of phenoloxidase (PO) enzyme (Gomez-Jimenez et al. 2000). Phenoloxidase is responsible for the process of melanization, which is involved in wound healing and cellular defence responses (Ratcliffe et al. 1984; Rodriguez and Le Moullac 2000; Cerenius et al. 2008). Due to the cytotoxic nature of PO, this enzyme is usually stored in its inactive precursor form—pro-phenoloxidase (ProPO)—being activated only after external stimuli (Söderhäll and Cerenius 1998; Rodriguez et al. 2014). The ProPO activating system is described for many invertebrates and consists of a cascade of interactions between enzymes and their zymogens, inducing the production of PO as final product. Both PO and ProPO are well studied in arthropods such as crustaceans (Söderhäll and Unestam 1979) and insects (Laughton and Jothy 2011), but many open questions regarding their function and dynamics remain for non-arthropod invertebrates, including sea cucumbers.

Holothuria scabra is economically the most valuable tropical sea cucumber, given the high interest for the food industry (bêche-de-mer), as well as for pharmaceutical purposes (i.e. bioactive compounds) (Battaglene and Bell 1999; Hamel et al. 2001; Venugopal 2009; Bordbar et al. 2011). In addition, concerning their anatomy, sea cucumbers have unique organs/tissues with diverse functions (e.g. cellular aeration, locomotion, metabolism and regenerative processes), suitable for the study of oxidative stress and immune responses (Garcia-Arrarás and Dolmatov 2010), which make them good target tissues in the study of stress responses and oxidative and immune-related analysis. The respiratory tree, for example, is a well-developed structure responsible for cellular aeration and waste excretion (Spirina and Dolmatov 2001). Muscular system and body wall of sea cucumbers are also interesting organs to analyse since they are involved in the organisms' locomotion and in the contraction movements in response to environmental stimuli (Motokawa and Tsuchi 2003).

These organisms also play an important ecological role as bioturbators (Uthicke 2001; Purcell et al. 2012). As shallow, bottom dweller species, they undergo seasonal and daily temperature fluctuations. Some studies demonstrate that *H. scabra* (Wolkenhauer 2008) and *Apostichopus japonicus* (Dong et al. 2006) seem to be adapted to temperature changes in terms of their burying and feeding habits, but very little is known regarding their mechanisms of adaptation and consequences for fitness in the long term.

Therefore, the main objective of the current study was to determine the effects of temperature stress (i.e. cold and warm) on immune and oxidative stress responses of juvenile *H. scabra*, using biochemical biomarkers involved in such processes, in order to understand the capacity of these organisms to cope with thermal stress and to find suitable markers for effect assessment on those levels.

Materials and methods

Test organism

Holothuria scabra (Jaeger, 1833) originated from the hatchery facilities of the Indonesian Research Centre for Oceanography (LIPI) on Lombok, Indonesia, were transported to the Alfred Wegener Institute, Helmholtz-Centre for Polar and Marine Research (AWI) in Bremerhaven, Germany, where they were maintained in recirculation systems for 14 days at 27 °C with a photoperiod of 12:12 h (light:dark) for acclimation. Sea cucumbers were observed and fed every second day with Algamac (Aquafauna—Bio Marine Inc.). To ensure optimal water quality, the aquaria

water was continuously filtered and aerated. The water quality parameters, ammonia, pH and salinity were monitored regularly.

Experimental setup

Experimental design followed previous work from Kühnhold et al. (2016). Briefly, after the acclimation period (14 days), 18 individuals were randomly assigned to each of three water temperature treatments: 21 °C (Cold), 27 °C (Control), and 33 °C (Warm). To achieve such temperatures, seawater temperature was decreased (for cold treatment) or increased (for warm treatment) by one degree per day over 6 days. Once the desired temperatures were reached, six individuals per tank were killed for further analysis, corresponding to day zero of the experiment (T0). Sampling was then performed at 15 days (T15) and 30 days (T30) of exposure to the different temperatures, with six replicates. Before killing the organisms, their coelomic fluid was collected using a 2-ml sterile syringe inserted through the body wall, for the assessment of immune responses. The procedure took no more than 20 s to ensure minimum effects of sampling on the immune responses. Then, muscle, respiratory tree, and body wall tissues were sampled for the oxidative stress-related endpoints (see below for sample processing details). All samples were subsequently stored at −80 °C until further analysis.

Tissue preparations

Immune responses

Following Jiang et al. (2014), two different fractions of coelomic fluid were prepared: Coelomocyte Lysate Supernatant (CLS) and Cell Free Supernatant (CFS). After centrifugation of extracted coelomic fluid at 500*g* for 10 min (4 °C), the supernatant (CFS) was stored at −80 °C, whereas the pellet was suspended with 1× PBS buffer to prepare the CLS fraction. After sonication for 5 min at 30-s intervals (UTR 200, Hielscher, Germany), the re-suspended pellets were centrifuged at 12,000*g* for 10 min (4 °C) and the obtained supernatant (CLS) was stored at −80 °C until further analysis.

Oxidative stress

According to different protocols and procedures, the oxidative stress-related parameters (except ROS) were measured in the muscle and respiratory tree tissues of sea cucumbers adapting the protocols more thoroughly described in Alves et al. (2016) and Silva et al. (2016). Both tissues were homogenized in K-phosphate buffer (0.1 M, pH 7.4) in a 1:4 proportion (w/v). Part of the homogenized tissue (150 µl) was transferred to a microtube containing 4 % BHT solution (2,6-dieter-butyl-4-methylphenol) to prevent tissue oxidation for further determination of lipid peroxidation (LPO), and another portion (50 µl) was separated for quantifying DNA-strand breaks. Samples were then centrifuged at 10,000*g*, for 20 min (4 °C). The resulting post-mitochondrial supernatant (PMS) was stored at −80 °C for further protein quantification and activity measurement of SOD, CAT and GR. For the determination of superoxide free radicals, as a measurement of ROS production, 50 mg of sea cucumber body wall was separated and kept at −80 °C until further analysis.

In all assays, K-phosphate buffer (0.1 M, pH 7.4) was used as blank. The spectrophotometric measurements were done at 25 °C in a synergy H1 Hybrid Multi-Mode microplate reader (Biotek® Instrument, Vermont, USA) and the enzymatic reactions were all previously optimized to ensure zero-order kinetic reactions (substrate in excess).

Biochemical analysis

Immune responses: phenoloxidase and pro-phenoloxidase

PO (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) and ProPO (zymogen form) activities were measured using the method partially described by Söderhäll (1981) with modification made by Laughton and Jothy (2011) and Jiang et al. (2014).

The activities of ProPO and PO were measured in both coelomic fluid fractions, i.e. CLS and CFS. PO activities were measured by adding 5 mM L-DOPA (l l-3,4-dihydroxyphenylalanine; Sigma, USA), dissolved in sodium cacodylate buffer (0.01 M, pH 7.4), to each fraction of the sample (CLS or CFS). For the blank reactions, seawater was used instead of the sample. The procedure for measuring ProPO was similar with the minor difference that chymotrypsin (0.25 mg/ml) was added to the sample, with a 10-min incubation prior to the addition of L-DOPA, to allow the activation of all phenoloxidase. The conversion of L-DOPA into dopachrome was determined spectrophotometrically at 490 nm (25 °C) with readings every 10 s for 5 min, giving an estimation of the enzyme activity. The final PO and ProPO activities were expressed as U/mg of protein, where 1U is defined as the amount of enzyme in the sample that, by converting the substrate, increases the absorbance by 0.001 per min.

Protein quantification

The soluble proteins were quantified according to the Bradford method (Bradford 1976), adapted from BioRad's Bradford microassay set up in a 96-well flat-bottom plate, using bovine γ-globulin as a protein standard. In each well

of the microplate, 10 µl of each sample was added along with 290 µl of Bradford reagent (in quadruplicates). After 15 min of agitation at 150 revs/min, absorbance was read at 600 nm and results were expressed in mg of protein/mL.

Antioxidant defences

The activity of SOD (EC 1.15.1.1) was measured performing an adaptation of the method described by McCord and Fridovich (1969), using the xanthine/xanthine oxidase-mediated reduction of cytochrome *C*. The reduction of cytochrome *C* was followed at 550 nm and SOD activity was expressed in U/mg of protein using an SOD standard of 1.5 U/ml, where 1 U represents the amount of enzyme in the sample that causes 50 % inhibition of cytochrome *C* reduction. CAT (EC 1.11.1.6) activity was estimated following the degradation of H_2O_2 at 240 nm, adapting the method described by Clairborne (1985). CAT activity was expressed in µmol/min/mg of protein, using a molar extinction coefficient of 40 M/cm. The activity of GR (EC 1.8.1.7) was estimated by measuring oxidation of NADPH in the process of reducing GSSG to glutathione (GSH) at 340 nm (Cribb et al. 1989). GR activity was calculated using a molar extinction coefficient of $6.2x10^3$ M/cm and expressed in nmol/min/mg of protein.

Oxidative stress and damage

For the determination of superoxide free radicals production in the body wall of sea cucumber, the method of Drossos et al. (1995) was followed. Briefly, after adding Krebs buffer to the tissue, an incubation with cytochrome *C* (15 µM) was made at 37 °C. The presence of O_2^- was determined by the capacity of the radicals to reduce cytochrome *C*, which was measured at 550 nm. Using a molar extinction coefficient of 19,000 M/cm (Wu et al. 2011), the amount of superoxide radicals produced was calculated and expressed in nmol O_2^-/g wet weight.

Lipid peroxidation levels were assessed by measuring the content of thiobarbituric acid-reactive substances (TBARS), using the method described by Ohkawa et al. (1979) and Bird and Draper (1984), with modifications made by Wilhelm et al. (2001) and Torres et al. (2002). After the reaction with TBA 0.73 % (2-thiobarbituric acid) reagent, the absorbance of the samples was measured at 535 nm. The results were calculated using a molar extinction coefficient of 1.56×10^5 M/cm and expressed as nmol TBARS/mg of wet weight.

The DNA-strand breaks were measured using the DNA alkaline precipitation assay (Olive 1988), adapted from De Lafontaine et al. (2000). After the precipitation of SDS-associated nucleoproteins and genomic DNA, the remaining single and double-stranded DNA in the supernatant was mixed with Hoesch dye (1 µg/mL bisBenzimide, Sigma-Aldrich) and fluorescence was measured using an excitation/emission wavelength of 360/460 nm. Results were expressed as mg of DNA/mg of wet weight, using calf thymus DNA as standard to extrapolate DNA concentration.

Statistical analysis

Statistics was performed using Sigma Plot software for Windows, version 11.0 (SigmaPlot 1997). Data were first tested for normality and homoscedasticity using Kolmogorov–Smirnov and Levene tests, respectively. To determine statistically significant differences between the treatments and between each time point, a two-way analysis of variance (ANOVA) was applied. When significant differences were found, Holm–Sidak post hoc tests were used for multiple comparisons. Correlations between endpoints in different tissues, at each time point, were performed using Pearson correlations. The results are presented as means + standard error (SE). The significance level for all statistical analysis was set at $p \leq 0.05$.

Results

No mortalities were registered at any treatment at any time point.

Immune responses

PO and ProPO activities in cell-free supernatant (CFS)

Although no significant differences were observed in the activity of PO and ProPO in CFS, the activities in both cases were higher in the warm treatment with a tendency for a decrease with the experiment duration (Fig. 1). In this CFS fraction, activities of ProPO and PO were found to be similar (within the same order of magnitude) in every treatment.

PO and ProPO activities in coelomocyte lysate supernatant (CLS)

The PO activity in the CLS fraction followed the same pattern as ProPO, with progressively higher activities in the warm treatment over time of exposure (Fig. 2), which in the case of PO was found to be statistically significant at T30 ($p = 0.005$, Fig. 2a). Significant differences among different temperature treatments were found at the end of the experiment (T30), both for PO and ProPO ($p = 0.004$, Fig. 2a; and $p = 0.011$, Fig. 2b, respectively).

Contrary to the CFS fraction, in CLS ProPO activities were between 1000 and 2000× higher than PO activities.

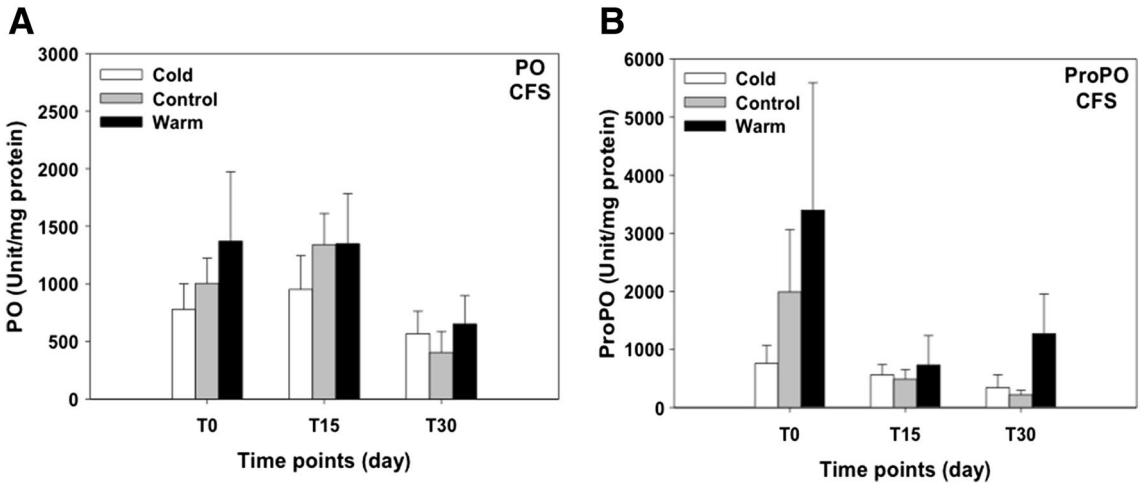

Fig. 1 Immune responses: **a** phenoloxidase (PO) and **b** pro-phenoloxidase (ProPO) activities in the cell-free supernatant (CFS) fraction of *Holothuria scabra* coelomic fluid exposed to cold (21 °C), control (27 °C) and warm (33 °C) temperatures over different time periods (T0, T15, T30 days). Results express average values + standard error

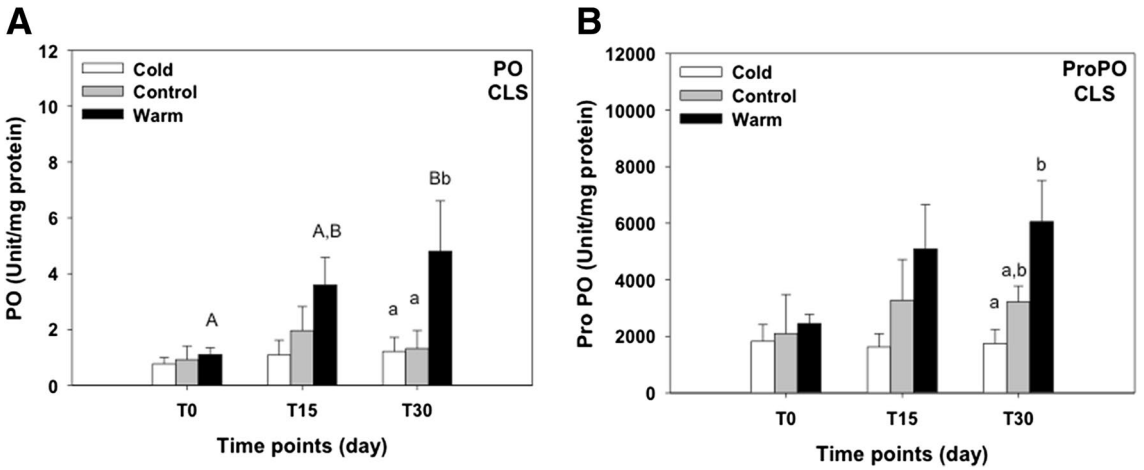

Fig. 2 Immune responses: **a** phenoloxidase (PO) and **b** pro-phenoloxidase (ProPO) activities in the coelomocyte lysate supernatant (CLS) fraction of *Holothuria scabra* coelomic fluid exposed to cold (21 °C), control (27 °C) and warm (33 °C) temperatures over different time periods (T0, T15 and T30 days). Results express average values + standard error. [a,b]Significant differences between cold, control and warm treatments within each time point (two-way ANOVA, Holm–Sidak, $p < 0.05$). [A,B]Significant differences between time points within each temperature treatment (two-way ANOVA, Holm–Sidak, $p < 0.05$)

Despite the increase in the general immune response of the organisms in the warm treatment, the ratio between PO and ProPO remains constant among treatments with no significant differences being observed (Fig. 2c).

Oxidative stress-related endpoints

No significant changes in the activity of the tested antioxidant enzymes (SOD, CAT and GR) were observed, either in the muscle tissue or in the respiratory tree (Figs. 3, 4). However, although the effects were not statistically significant, in the muscle there was a trend for higher SOD activities in the warm treatment, compared to control and cold treatment (Fig. 3a). Antioxidant enzyme activity levels were usually higher in the respiratory tree (Fig. 4) than in the muscle (Fig. 3), independently of the treatments.

In relation to the parameters addressing oxidative damage, no effects of temperature were seen either in peroxidation of lipids or in higher levels of DNA-strand breaks (Figs. 3d, e, 4d, e).

The results of the ROS quantification in the body wall show that in the cold treatment, at T0, the organisms produced significantly less superoxide radicals than in the control treatment ($p = 0.002$—Fig. 3f). However, the levels of

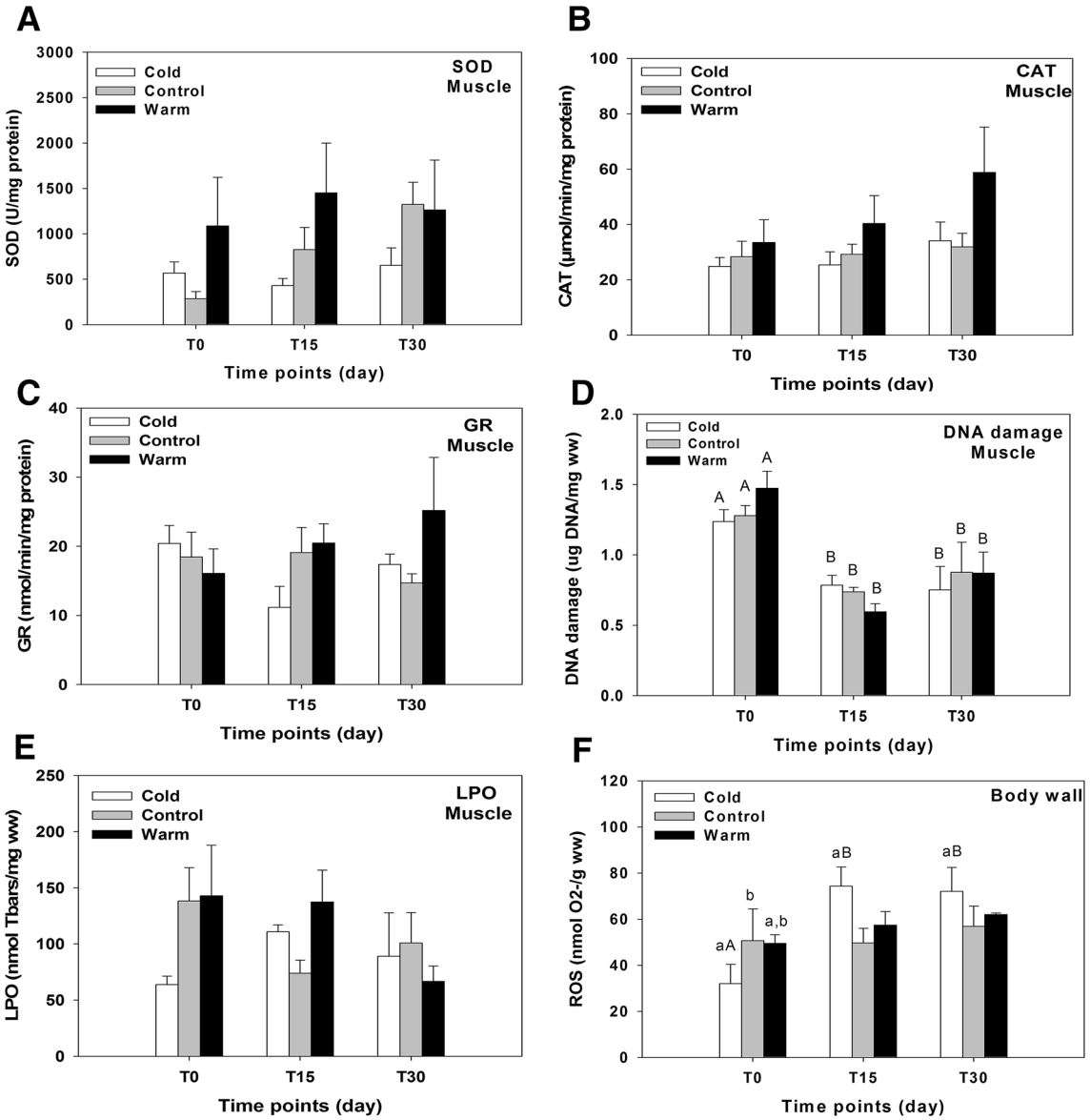

Fig. 3 Oxidative stress-related responses: **a** superoxide dismutase (SOD), **b** catalase (CAT) and **c** glutathione Reductase (GR) enzymatic activities and levels of oxidative damage measured as **d** DNA damage and **e** lipid peroxidation (LPO) in the muscle tissue of *Holothuria scabra* exposed to cold (21 °C), control (27 °C) and warm (33 °C) temperatures over different time periods (T0, T15 and T30 days). **f** Reactive oxygen species (ROS) production in the body wall of *Holothuria scabra* exposed to the same conditions described for muscle. Results express average values + standard error. [a,b]Significant differences between cold, control and warm treatments within each time point (two-way ANOVA, Holm–Sidak, $p < 0.05$). [A,B]Significant differences between time points within each temperature treatment (two-way ANOVA, Holm–Sidak, $p < 0.05$)

ROS in this treatment significantly increased with the duration of the experiment CA ($p = 0.009$), and no further differences were observed with the other temperature treatments.

Correlation analysis between all assessed biomarkers in *H. scabra*, relative to immune and oxidative stress responses, were performed separately for each time point (Tables S1–S3, supplementary material). At all time points, especially in muscle tissue, activities of CAT, SOD and GR correlated with each other positively, indicating that if one of these enzymes is activated or inhibited, the other enzymes follow the same pattern. Similarly, some positive correlations between DNA damage and LPO were observed. Moreover, GR (respiratory tree) and ProPO activating systems (CLS fraction) correlated negatively with ROS, while a positive correlation between DNA damage of the same tissue and ROS is apparent. Furthermore, PO activity of CLS correlated positively with SOD activities from both tissues.

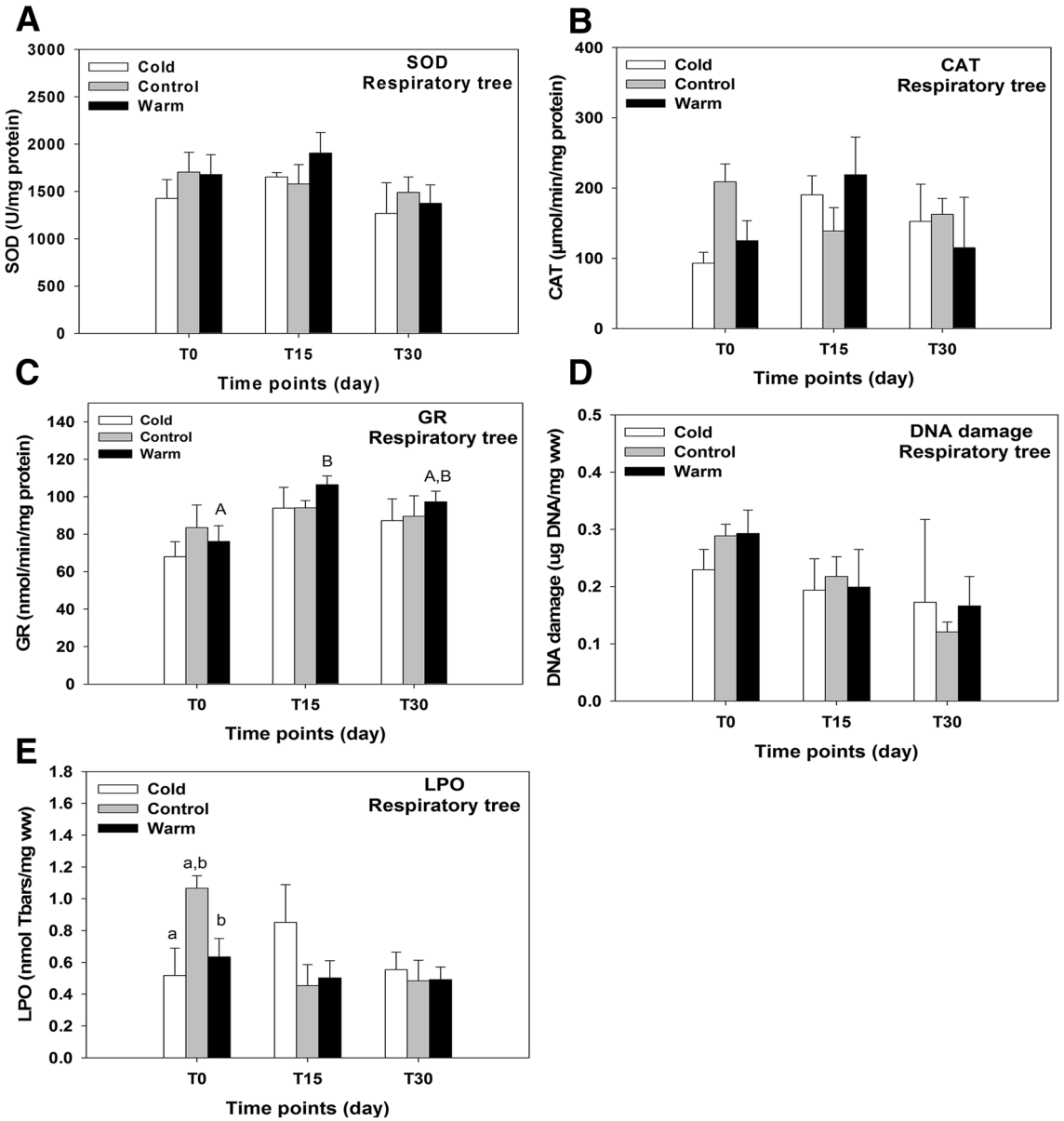

Fig. 4 Oxidative stress-related responses: **a** superoxide dismutase (SOD), **b** catalase (CAT) and **c** glutathione reductase (GR) enzymatic activities and levels of oxidative damage measured as **d** DNA damage and **e** lipid peroxidation (LPO) in the respiratory tree of *Holothuria scabra* exposed to cold (21 °C), control (27 °C) and warm (33 °C) temperatures over different time periods (T0, T15 and T30 days). Results express average values + standard error. [a,b]Significant differences between cold, control and warm treatments within each time point (two-way ANOVA, Holm–Sidak, $p < 0.05$). [A,B]Significant differences between time points within each temperature treatment (two-way ANOVA, Holm–Sidak, $p < 0.05$)

Discussion

Temperature changes influence the growth rate, susceptibility, and the general health status of invertebrates (Hughes et al. 2003; Cheng et al. 2004; Wang et al. 2008; Purcell and Simutoga 2008; Hair 2012). The integrated antioxidant and ProPO activating systems are known as crucial components of invertebrates' self-maintenance (Mathew et al. 2007), but little is known about these responses to different stress levels. The present study is the first to apply combined investigations of immune responses, cellular oxidative damage and antioxidant enzyme activities to assess stress responses in juvenile *H. scabra* at varying temperatures [i.e. cold (21 °C) and warm (33 °C)], and to assess their potential for easy-to-use, fast, and cost-effective multi-biomarker applications in aquaculture.

Immune responses

The nature of sea cucumbers as osmo-conformers (Coteur et al. 2004) indicates that any changes in water temperature may influence their coelomic fluids and particularly affect the activities of coelomocytes (Wang et al. 2008). The ProPO activating system is considered as the first line of defence in the immune response of invertebrates (Sritunyalucksana and Söderhäll 2000), where any reduction in PO activities affects the cellular defence of organisms (Mathew et al. 2007). This study confirmed that, similar to the Pacific oyster (*Crassostrea gigas*) (Hellio et al. 2007), PO activity is detectable in both fractions of the coelomic fluid in *H. scabra*. Most of PO was activated in the CFS fraction, seen by the similar activity between PO and ProPO, in contrast to the CLS fraction, where most PO remained in the inactive form, as expected at least under control conditions, given the cytotoxic nature of the by-products of the PO activating cascade (Tujula et al. 2001; Laughton and Jothy 2011).

In the CFS fraction, which represents the acellular fraction of the coelomic fluid (Gomez-Jimenez et al. 2000), there was a tendency for higher activities of both PO and ProPO activities in the warm treatment, mainly at the beginning of the experiment (Fig. 1). This is in agreement with the findings of a parallel study, where under the same warm temperature condition, the organisms were consuming more energy, possibly indicating the costs of the defence responses by inducing for instance these immune enzymes (Kühnhold et al. 2016). Similarly, Coates et al. (2012) reported that in horseshoe crabs (*Limulus polyphemus*), which have hemocyanin-derived phenoloxidase (Hc-PO), the activity of Hc-PO at the beginning of exposure was initially increased at the warmer treatment, but decreased again over a period of time, suggesting that temperature changes have limited effects on hemocyanin and PO activities. It is important to note that the coelomocytes of sea cucumbers and haemocytes of crustaceans display several common features (Tseng et al. 2009).

In the CLS fraction, this tendency for higher PO activities with increasing temperatures is also seen for both PO and ProPO activities, over the time of exposure. This increase in the total ProPO activity suggests that, along with the PO activation (in a much lower scale), probably more PO is being synthesized (Fig. 2). Although in this study the sea cucumbers responded with an increase in PO in the warmer treatment, the response of this enzyme to temperature changes seems to differ between species. For example, Vargas-Albores et al. (1998) reported that the yellowleg shrimp (*Penaeus californiensis*) had lower PO activity at higher temperature (32 °C) compared to colder treatment (18 °C). Moreover, Cheng and Chen (2000) and Cheng et al. (2004) reported that the PO and phagocytic activities in the giant freshwater prawn (*Macrobrachium rosenbergii*) and the Taiwan abalone (*Haliotis diversicolor supertexta*) at warmer treatments (34 °C) were lower than the ones reared at colder water (27–30 °C).

When comparing the ProPO activities between CFS and CLS factions, it is possible to observe that the activities are higher in the coelomocytes (CLS), indicating a higher potential for PO response in this fraction. These results show the importance of testing both cellular and acellular fractions of the coelomic fluid in order to accurately locate the PO activity. However, the different PO activities found in the two fractions might also indicate different types of PO enzymes (tyrosinase, laccase or catecholase). Although characterization of PO was already done for another holothurian species (Jiang et al. 2014) and this was the base for the methods employed here, it would be important for further studies to understand which specific enzymes are involved in PO activity in each fraction to more precisely target those reactions. Nevertheless, the present study represents already an important indication that PO induction might play an important role in *H. scabra* response to heat stress.

Oxidative stress-related endpoints

Regarding the oxidative stress-related endpoints, results showed that exposure to different temperatures had little impact on *H. scabra*. Various studies demonstrated that free radicals formed by a stressor could enhance the formation of malonaldehyde and therefore increase LPO (Di Pierro et al. 1992). Additionally, the heterogenic DNA molecules are susceptible to breakage and damage inflicted by elevated ROS levels (Cerutti 1985). In the present study, however, no signs of oxidative damage were observed in any temperature manipulation (Figs. 3, 4). In the beginning of the experiment (T0) specimens from the cold treatment exhibited even lower ROS levels, which resulted in lower levels of LPO in the same condition. These lower ROS levels can also be explained by the lower oxygen consumption rates (OCR) verified in a parallel study with the same exposure conditions (Kühnhold et al. 2016).

Increasing temperatures can stimulate oxidative stress and specific antioxidant responses in different classes of invertebrates. Although the antioxidant activities in *H. scabra* did not show a clear treatment response, induction of SOD was a constant trend observed in the warm treatment. Similar patterns of antioxidant response were observed by Ji et al. (2008) and Wang et al. (2008), in studies with *A. japonicus*, where at the beginning of exposure to higher temperatures, SOD and CAT activities measured in both body wall and respiratory tree, increased after a short exposure time (12 h). The tendency for higher activity of SOD is observed in the warm treatment mainly in

the muscle, along with lower activities in the cold treatment (Fig. 3a). A similar pattern was reported for shrimps (Zhou et al. 2010) and the disk abalone *Haliotis discus discus* (Kim et al. 2007), where the activities of manganese superoxide dismutase (MnSOD) and copper superoxide dismutase (CuSOD) increased under heat stress. This SOD induction in the warm treatment in combination with the lack of significant changes in CAT and GR activities over the period of exposure, suggests that this enzyme is the most sensitive antioxidant enzyme among the ones tested, and likely plays an important role in ROS detoxification in juvenile *H. scabra* in response to thermal stress.

However, in the present study, the temperature stress resulted in only marginal increases in the antioxidant activities. This might be explained by the immediate induction of heat shock proteins, which reduce heat stress and oxidative stress in the organism, as reported previously for *Haliotis tuberculata* (Farcy et al. 2007). This cannot, however, be confirmed in the present work, since the expression of heat shock proteins was not studied.

Another observation of the present study was that the respiratory tree in the sea cucumbers had higher antioxidant potential than muscle (higher enzyme activities), similar to the gills in molluscs (Farcy et al. 2007; Box and Sureda 2009). Considering the functionality of the respiratory tree for oxygen circulation and gas exchange at its surface, and also the close contact with water, similarly to the gills in molluscs, this tissue is ought to be more susceptible to environmental changes. However, a clearer pattern of overall response to the induced thermal stress was observed in the muscle tissue. Further studies featuring more levels and higher intensity of treatment (i.e. lower and higher temperature thresholds) are needed in order to create a better understanding on the physiological thresholds and sensitivity of the antioxidant responses in this species.

In sum, immune and oxidative stress responses indicate that temperature manipulation applied in the present study was not severe enough to cause acute stress in juvenile *H. scabra*. This is in accordance with the general assumption that most of the sea cucumbers, reared in intertidal ponds, can tolerate temperature fluctuations from 20 to 30 °C (Dong et al. 2008). Furthermore, with this study it is possible to infer that immune responses through PO activity, and antioxidant activities (particularly SOD) in *H. scabra* seem to be efficient to reduce ROS production and oxidative damage under thermal variations. This is strengthened by the correlation analysis between biochemical responses throughout the duration of the experiment (Tables S1–S3, supplementary material), with positive correlations between SOD and PO activities and negative correlations between the activities of GR or PO enzymes and the levels of ROS. Therefore, this study highlights the importance of combining different endpoints into a multi-biomarker approach in order to gain a holistic picture of the processes and mechanisms underlying stress responses.

Conclusions

Juvenile *H. scabra* displayed sensitivity to thermal stress at the beginning of the experiment, especially in the warm treatment, and after a period of time they acclimated to the higher and lower temperatures. From an immune response-related point of view, PO and ProPO activities in the cell-free coelomic fluid were tendentiously increased in the warm treatment at T0, showing an early immune response with the temperature change. Antioxidant and oxidative damage biomarkers indicated that the temperature manipulations applied in the present study were not severe enough to cause significant oxidative damage to *H. scabra*, seen by the low production of ROS and absence of oxidative damage in either lipids or DNA. SOD seems to be the most sensitive enzymatic antioxidant in *H. scabra* in response to thermal stress.

The present study highlights the benefits of a multi-biomarker analysis to better understand and interpret biochemical responses to stress, and in particular thermal stress. Assessing changes in the immune and antioxidant biomarker endpoints, particularly in juvenile *H. scabra*, are promising tools for monitoring the health status of the organisms. Also, understanding the impacts of temperature stress on these organisms, provide important insight into the possibility of the use of *H. scabra*, a high-valued species, in global-scale aquaculture from different regions with minimum impact concerning thermal stress.

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