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Response of antioxidant defence systems to thermal stress in the Antarctic clam *Laternula elliptica*

HYUN PARK[1]*, IN-YOUNG AHN[1], KYUNG-IL PARK[1,3] and SEUNGHUN HYUN[2]

[1]*Korea Polar Research Institute, Korea Ocean Research and Development Institute, Songdo-dong 7-50, Yeonsu-gu, Incheon 406-840, South Korea*
[2]*Division of Environmental Science and Ecological Engineering, Korea University, Seoul, 136-713, South Korea*
[3]*Present address: Department of Aquatic Life Medicine, Kunsan National University, Kunsan 573-701, South Korea*
**hpark@kopri.re.kr*

Abstract: The effects of thermal stress on antioxidant defences in tissues of the Antarctic clam *Laternula elliptica* were evaluated and the activities of some antioxidant enzymes, and levels of total glutathione (GSH) and protein carbonyl (PC) in digestive gland and gill over 0–4 days under extreme thermal stress (10°C) were measured. Superoxide dismutase activity was slightly higher after one day of thermal stress, although catalase activity was not altered significantly in either digestive gland or gill tissues. Thermal stress was associated with a significant increase in the activity of GSH-related antioxidant enzymes. Glutathione peroxidase and glutathione reductase activities increased up to 1.8- and 2.0-fold, respectively, after two days of thermal stress. Glutathione S-transferase activity drastically increased, to over 3.4- and 4.2-fold in digestive gland and gill, respectively, and remained high on day four. GSH levels also increased in both tissues and remained high on day four. PC content, a marker of protein oxidation, increased after two days of thermal stress. There is evidence that GSH-related antioxidant defence plays a significant role in relation to potential toxicity from reactive oxygen species during thermal stress.

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Key words: bivalves, glutathione, protein carbonyl, Southern Ocean

Introduction

All aerobic organisms are exposed to a wide variety of environmental disturbance. Heat stress is one of the most important external stresses for survival, and it has been found to increase oxidative damage by accelerating the generation of reactive oxygen species (ROS). Although small amounts of ROS are a cellular requirement because they are involved in signalling pathways, regulating a variety of cellular activities and gene expression, excess ROS production increases oxidative damage in the cell, possibly by altering or inactivating proteins, lipid membranes, and DNA (Hensley *et al.* 2000). Although our previous work found that thermal stress on the Antarctic stenothermal clam *Laternula elliptica* elevated expression of the *Hsp70* gene (Park *et al.* 2007), understanding of the mechanism about oxidative stress from heat shock is still lacking. Heise *et al.* (2003) found that ROS formation in mitochondrial isolates from *L. elliptica* is significantly elevated under temperature stress, and also that ROS formation is positively correlated with oxygen consumption. ROS generation by both a temperature increase and enhanced oxygen consumption may promote oxidation of cellular constituents and a response by antioxidant and associated enzyme systems.

Antioxidants help prevent cellular damage caused by metabolically and environmentally produced ROS, such as hydrogen peroxide (H_2O_2) and superoxide anion radicals (O_2^-). Organisms can respond to a high ROS concentration by increasing the expression of the antioxidant system, including enzymatic (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione S-transferase, GST; glutathione reductase, GRd) and nonenzymatic components such as cellular thiol compounds. GRd regenerates reduced glutathione (GSH), which is a direct scavenger of ROS or a substrate for GPx (Halliwell & Gutteridge 1999). GST also plays an important role in the detoxification of ROS in cells (Edwards *et al.* 2000) by protecting lipids from peroxidation. Cellular thiol status is also of great importance in the antioxidant defence system. The tripeptide GSH (γ-glutamyl-cysteinyl-glycine) is one of the most important antioxidant agents, which participates in reducing buffers that maintain the reduced/oxidized ratio (Meister & Anderson 1983, Schafer & Buettner 2001). Additionally, protein modifications caused by direct oxidative attack on Lys, Arg, Pro, or Thr residues by reactive carbonyl compounds can lead to the formation of protein carbonyl (PC) derivatives (Stadtman & Levine 2000). Hence, PCs are considered a broad marker of oxidation, as elevated PC levels are generally signs of oxidative stress.

Previous work on the role of antioxidant defences in thermal stress using *L. elliptica* at the cellular level has reported only the responses of two mitochondrial antioxidant enzymes and lipid peroxidation (Estevez *et al.* 2002, Heise *et al.* 2003). Studies of other antioxidant

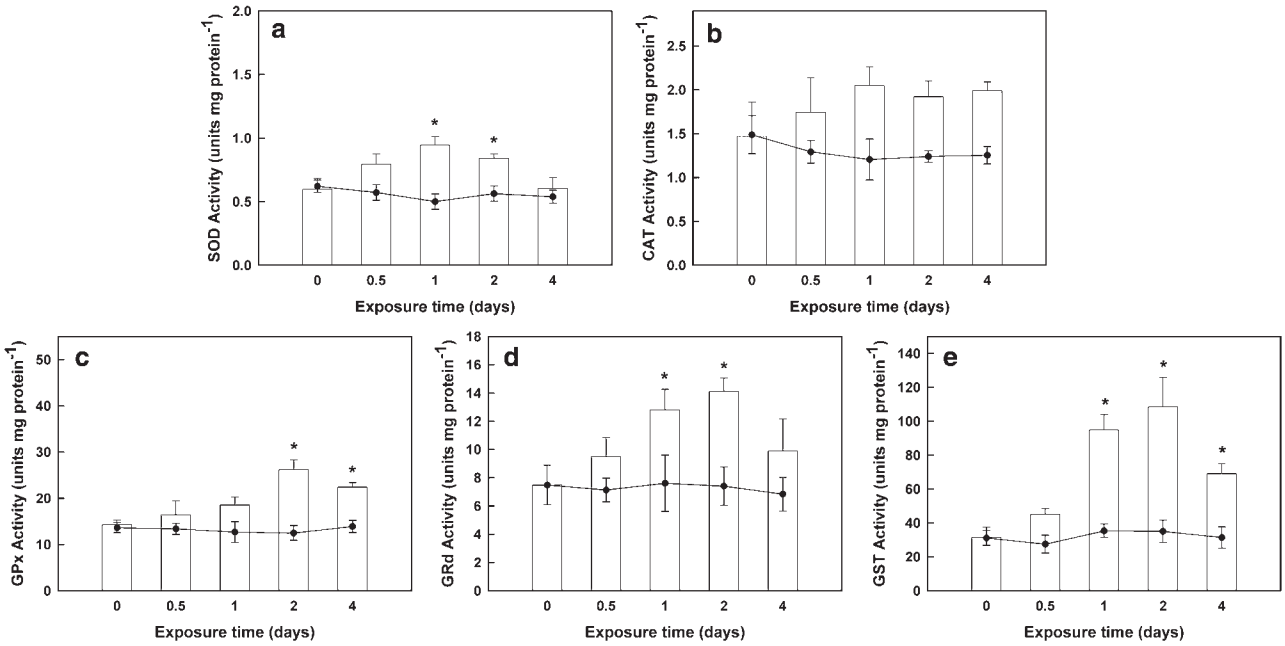

Fig. 1. Antioxidant enzyme activities in digestive gland tissues of control (circles and line) and thermal-exposed (bars) *L. elliptica*. **a.** SOD, **b.** CAT, **c.** GPx, **d.** GRd, **e.** GST. Data are mean ± SD, $n = 3–5$. Asterisks indicate significant differences ($P \leq 0.05$) from the control treatment. Enzymatic activity is expressed in specific units (U) as defined in the materials and methods section.

enzymes and antioxidant components are still lacking. The objective of this study, therefore, was to investigate the change in activity of several oxidative enzymes, including SOD, CAT, GPx, GRD, and GST, in digestive gland and gill tissues of *L. elliptica* when exposed to thermal stress. We also include the GSH and PC contents as markers of oxidative stress.

Materials and methods

Animal collection and heat exposure

Laternula elliptica (shell length ~80 mm) were hand-collected by SCUBA divers at depths of 20–30 m in Marian Cove, near King Sejong Station, King George Island (62°13'S, 58°47'W) in January 2006. The samples were divided into two groups and each was acclimated under conditions equivalent to those in the field at a temperature of *c*. 1.0°C with constant air supply. After acclimation for two days, one group was subjected to 10 ± 1°C as acute heat stress, which is thermal limit for survival (Peck *et al.* 2002), and the other group kept as a control (1.0 ± 1°C) with well-aerated natural sea water and without feeding.

Tissue preparation and enzyme assays

The clams from each group were killed, and digestive gland and gill tissue were dissected immediately after their exposure. The tissues were homogenized in buffer (50 mmol L^{-1} phosphate buffered saline (pH 7.4) and 1 mmol L^{-1} EDTA) containing an antiprotease cocktail (10 mg ml^{-1} antipain, pepstatin A, leupeptin, and soybean trypsin inhibitor, plus 2.5 mmol L^{-1} PMSF). Antioxidant enzymes were assayed at 2°C, similar to the temperature of the clam's habitat in the Southern Ocean, using a thermostatic spectrophotometer (HP8453; Hewlett-Packard, Palo Alto, CA, USA). SOD activity was assayed by the xanthine oxidase/cytochrome *c* method (McCord & Fridovich 1969). The reaction mixtures contained 10 uM cytochrome *c* and 50 uM xanthine in 50 mM sodium carbonate, 0.1 mM EDTA, at pH 10. Reactions were started by adding xanthine oxidase in an amount sufficient to cause A_{550} nm of 0.025/min in the cytochrome *c* assay. One unit of SOD was defined as the amount of sample needed to achieve 50% inhibition. CAT activity was assayed by the method of Beers & Sizer (1952). The decomposition of H_2O_2 ($\epsilon = 0.0394$ mmol L^{-1} cm^{-1}) was followed directly by measuring the decrease in absorbance at 240 nm. One unit of CAT was defined as the amount of enzyme decomposing 1 umol of H_2O_2 in 1 min at pH 7.0, and specific activity was given as U per mg protein. GPx activity was measured in a coupled enzyme system in which the oxidized GSH formed in the GPx reaction was converted to the reduced form GSH by GRd. The consumption of NADPH was monitored at 340 nm and was directly proportional to the GPx activity in the sample, which was expressed as U per mg protein. GRd activity

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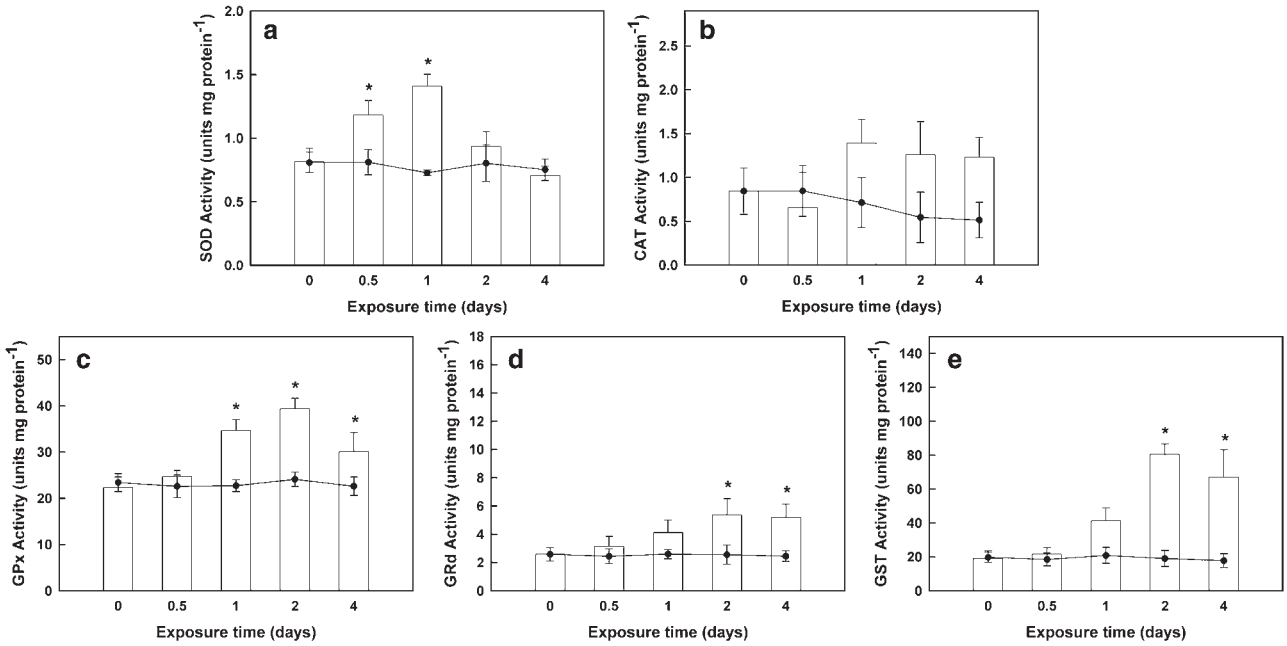

Fig. 2. Antioxidant enzyme activities in gill tissues of control (circle and line) and thermal-exposed (bar) *L. elliptica*. **a.** SOD, **b.** CAT, **c.** GPx, **d.** GRd, **e.** GST. Data are mean ± SD, $n = 3–5$. Asterisks indicate significant differences ($P \leq 0.05$) from the control treatment. Enzymatic activity is expressed in specific units (U) as defined in the materials and methods section.

was determined according to the method of Carlberg & Mannervik (1985), measuring the oxidation of NADPH to $NADP^+$ by GRd in the presence of oxidized glutathione. The reduction of GSSG was determined indirectly by measuring the oxidation rate of NADPH, as visualized by the time-dependent decrease in absorbance at 340 nm. One GRd unit was defined as the reduction of 1 µmol of GSSG min^{-1} at pH 7.6, and specific activity was expressed as U per mg protein. GST activity was measured according to the method of Habig *et al.* (1974) using 1-chloro-2, 4-dinitbenzene (CDNB) and GSH as substrates. A unit of GST was defined as the amount of glutathione conjugate formed using 1 mM GSH and CDNB min^{-1} mg protein. All data was expressed as the mean ± SD and analysed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

Glutathione content and protein carbonyl content assays

Tissues were homogenized in 5 vol of cold 1 M perchloric acid containing 2 mM EDTA and centrifuged at 30 000 g for 20 min. An aliquot of the pellet was used for PC content measurement. The supernatant was neutralized with 2 M KOH/0.3 M MOPS and centrifuged at 10 000 g for 10 min at 4°C. The total glutathione concentration was evaluated in the neutralized supernatant by the glutathione reductase enzymatic assay (Akerboom & Sies 1981). The reaction mixture contained 0.1 M potassium-phosphate buffer, 1 mM EDTA (pH 7), 0.2 mM NADPH, 0.06 mM DTNB, and 0.12 units glutathione reductase. The linear increase in absorbance at 412 nm and 25°C was recorded with a spectrophotometer (HP8453). A blank assay, without GSH, was run separately. For calibration, the procedure was performed using 100 uM GSSG instead of sample.

PC derivatives were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH). This compound reacts with PC groups that have been formed by ROS attack on side chains of amino acids. Resulting 2,4-dinitrophenylhydrazones were quantified spectrophotometrically at 370 nm using a molar extinction coefficient of 22×10^3 M^{-1} cm^{-1} (Lenz *et al.* 1989). The pellet from the TCA extract (above) was mixed with 1 ml of 10 mM DNPH in 2 M HCl. Control samples contained 1 ml of 2 M HCl instead the DNPH solution. Samples were incubated for 1 h at room temperature, and then centrifuged for 10 min at 5000 g. Supernatants were discarded and pellets were washed three times with 1 ml of ethanol–butylacetate (1:1 v/v) mixture, and pellets were dissolved in 1.5 ml of 6 M guanidine-HCl. The values were expressed as nanomoles of PC per gram wet weight of tissue. All data was expressed as the means ± SD and analysed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Figures 1a & 2a show the effects of thermal stress on SOD activity in digestive gland and gill tissues of *L. elliptica.*

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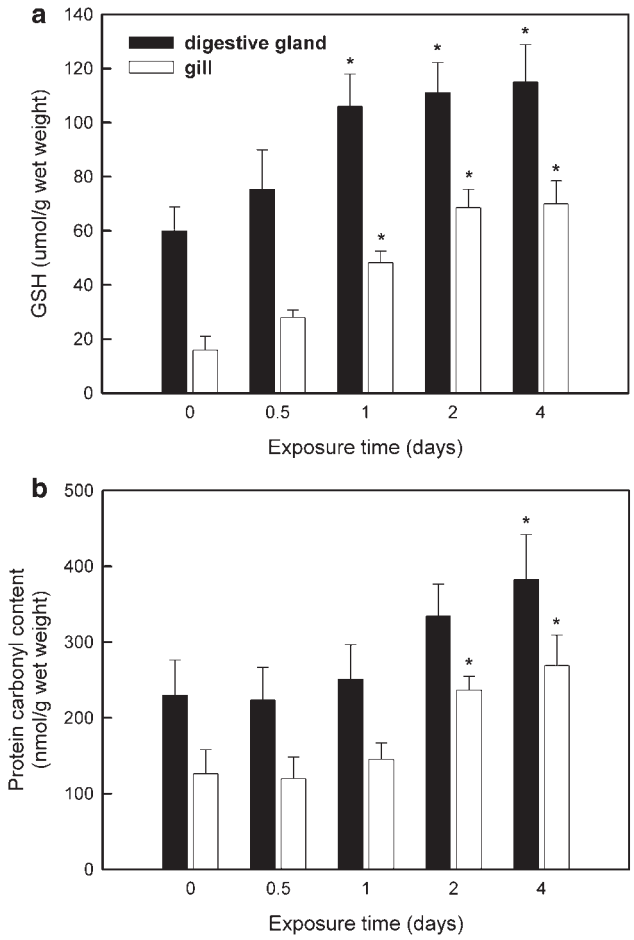

Fig. 3. Effect of heat exposure on **a.** GSH level, and **b.** PC content in digestive gland (filled bars) and gill (empty bars) tissues of *L. elliptica*. Data are mean ± SD, $n = 3-5$. Asterisks indicate significant differences ($p \leq 0.05$) from the control treatment.

After 12 h of exposure to heat stress, SOD activity increased slightly; it peaked after one day in both digestive gland and gill tissues. After one day, SOD activity decreased gradually and returned to the control level in both organs. Another primary antioxidant enzyme, CAT, was not significantly affected by experimental condition (Figs 1b & 2b).

The activities of GSH-related antioxidant enzymes were strongly affected by exposure to thermal stress. GPx activity increased in digestive gland (about -1.7 fold) and gill (about -1.8 fold) after two days of heat exposure, although it remained constant for the first 12 h of treatment (Figs 1c & 2c). The activity of GRd was significantly altered by 1.9-fold in digestive gland and 2.1-fold in gill at the two day exposures (Figs 1d & 2d). Compare to the control, GST activity was the most sensitive to heat stress, increasing more than 3-fold and 4-fold in digestive gland and gill, respectively (Figs 1e & 2e).

The concentration of total GSH in *L. elliptica* tissues continuously increased under heat stress. After two days of heat treatment, GSH levels had increased by 1.9- and 4.4-fold in digestive gland and gill tissues, respectively (Fig. 3a). Heat stress had little effect on PC content on the first day of heat exposure, but after two days, PC levels increased slightly (Fig. 3b).

Discussion

An increase in environmental temperature can lead to increased oxygen consumption, which in turn promotes ROS generation (Abele *et al.* 1998, 2001). Heise *et al.* (2003) reported the thermal stress induced significantly elevated ROS formation and significantly higher lipid radical generation in *L. elliptica* than in a temperate mud clam.

SOD activity, as it catalyses the removal of oxygen radical metabolically derived oxidative stress, has been shown to respond to thermal stress in digestive gland and gill tissues of *L. elliptica*, although the level of CAT activity increased slightly but not significantly in response to thermal stress. CAT directly reduces H_2O_2, which is produced by SOD, to produce water and oxygen. These two antioxidant enzymes are distributed differently in digestive gland and gill. SOD activity was much higher in gill than in digestive gland, whereas CAT and other antioxidant enzymes were more active in digestive gland than in gill. Whereas Viarengo *et al.* (1995) found higher SOD activity in digestive gland than in gill of the Antarctic mollusc *Adamussium colbecki*, Regoli *et al.* (1997) found that SOD activity is significantly higher in gill than in digestive gland. These contrasts with different enzyme activities between gill and digestive gland make it difficult to distinguish whether the antioxidant defences can be simultaneously or differentially modulated by stress in polar molluscs.

This is the first report on the relationship between GSH-related antioxidant enzymes and thermal stress in Antarctic stenothermal bivalves. Antioxidant enzymes connected to GSH metabolism showed significant responses to thermal stress in clam tissues. GPx activity increased over 1.7-fold in both tissues after heat exposure. Whereas CAT directly metabolizes H_2O_2 to ground-state O_2 and water, GPx, the major degradative enzyme for H_2O_2 and lipid hydroperoxides, couples the reduction of H_2O_2 to water with oxidation of reduced GSH. GSH, a ubiquitous cellular nonprotein sulphydryl compound, is involved in several cellular functions (Meister & Anderson 1983, Reed 1990). As a hydrogen donor and radical scavenger, GSH plays a major role in processes inducing or generating free radicals (Carmel-Harel & Storz 2000). It is not clear whether a certain level of GSH is necessary to develop thermal tolerance; intracellular concentrations of GSH increase after exposure to heat shock in several cells (Mitchell *et al.* 1983, Konings & Penninga 1985, Skibba *et al.* 1989, Harris *et al.* 1991), and it has been

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suggested that GSH is needed to mediate the initial response necessary for acquiring heat stress tolerance (Russo *et al.* 1984). Although few studies have reported on the influence of heat stress on GSH metabolism, these data suggest that the GSH redox system via GPx is robustly maintained in the face of heat-induced oxidative stress. GSH disulphide is reduced by GRd to form GSH; GRd activity is essential in the maintenance of GSH in its reduced form (Pena-Llopis *et al.* 2003). The activity of GRd was also significantly altered by heat stress. Heat stress appears to both produce oxidants and impair the enzyme system necessary for detoxification of H_2O_2 and organic hydroperoxides. Another GSH-related enzyme, GST, plays an important role in the detoxification of foreign compounds (Ceballos-Picot *et al.* 1992). In this study, GST activity was the most sensitive to heat stress. These increases in activity might have resulted from the increased lipophilic substances released during oxidative damage to polyunsaturated lipids, such as short-chain aldehydes and alkenals, which are substrates for GST (Galli *et al.* 1999). Peck *et al.* (2002) reported that 50% mortality was observed within seven days exposure at 9°C because *L. elliptica* has low upper temperature limits and it transfers to anaerobic metabolism in this temperature condition, whereas *L. elliptica* mortality was not observed during the experiments in this study. Pörtner *et al.* (2006) suggested that survival, at this extreme temperature, is supported by anaerobic metabolism and the protection of protein and membrane function by heat shock proteins and antioxidative defence. Previous studies have already shown that transcription level of heat shock protein to protein protection was instantly increased for thermal stress (Park *et al.* 2007). Our findings in this study indicate that phase II detoxification enzyme system was an important protection function at oxygen-limited thermal stress, and it might be involved in metabolism of accumulated anaerobic end products (Peck *et al.* 2002).

The levels of total GSH concentration were affected by heat stress. GSH may contribute to protection from ROS, but, at the same time, it is involved in the regulation of many redox-sensitive processes (Droge 2002). Under oxidative stress, GSH content often rises due to *de novo* synthesis (Ali *et al.* 2004, Lushchak *et al.* 2005). In our results, GSH increased continuously during thermal stress and changed in a similar manner to GSH-related antioxidant enzymes. We suggest that cellular GSH is involved in the major defence against thermal-induced oxidative stress. By contrast, PC contents in both organs slightly increased after two days of heat exposure. Compared to other oxidative modifications, PC are relatively difficult to induce and are irreversible modifications (Dalle-Donne *et al.* 2003). The level of carbonylated proteins is closely linked to ROS generation, so it is probable that increases in PC level might be shown as a results of irreversible physiological damage after exhausting the protective potential such as the action of chaperones.

Conclusions

This study represents the first comprehensive report of antioxidant defence enzyme activities and antioxidant components in the cold stenothermal *L. elliptica* from the Southern Ocean. Our results indicate that the GSH-related enzymes GRd, GPx, and GST are likely to be important in the antioxidant defence system at heat shock exposure, whereas SOD and CAT play a minor role in thermal stress. GSH metabolism is also greatly involved in the antioxidant defence system in *L. elliptica* under oxidative stress induced by thermal stress but protein oxidation was not as PC levels were not affected instantly by heat shock. Our present analysis is limited by extreme thermal stress, so further investigation will be needed to study antioxidative defences dependence on temperature variation under such critical temperatures, and with aerobic metabolism. Such integrative approaches to antioxidant defence system ins *L. elliptica* subjected to thermal stress contributes to our knowledge of adaptation in extremely cold environments.

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