Does starvation influence the antioxidant status of the digestive gland of *Nacella concinna* in experimental conditions?☆

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Abstract

In a previous study we analysed the effect of diesel seawater contamination in the digestive gland of the Antarctic limpet *Nacella concinna*. We observed that antioxidant enzyme activities decreased after one-week starvation prior to the experiment, and this was considered in the analysis of the obtained results. To know whether the digestive gland oxidant–antioxidant status may be altered by starvation and experimental conditions, we evaluated the food deprivation effect in limpets from the nearshore shallow waters of Potter Cove, Antarctica. Organisms were acclimated to laboratory conditions and were divided in fed and starved groups, and maintained in these conditions during one month. Every week 20 limpets were sampled from each group. Digestive glands were dissected and kept frozen until they were processed. Superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities, as well as lipid peroxidation (LPO) measured as thiobarbituric reactive substances (TBARS), protein oxidation (PO) and reduced glutathione (GSH) were measured. For both groups of limpets, SOD increased its activity in the first week of the exposure period, with a maximum in the second week. CAT activity increased significantly in the second week, only for the starved group. Similarly, GST activity also increased for starved group in the second week; but maintained this tendency for both groups until the fourth week. In fed and starved limpets, TBARS values increased significantly, during the first week and then returned to normal values. The PO levels in the starved group increased only during the first week. The GSH content, for the fed group, increased significantly after the third week. The obtained results indicate that biochemical or physiological studies conducted with *N. concinna* should consider the effects of food deprivation and time spent under experimental conditions.

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1. Introduction

The Antarctic marine environment is characterised by low but very stable temperatures and a highly seasonal food supply. Food availability and temperature are effectively uncoupled compared with tropical and temperate marine environments (Clarke, 1988; Clarke and Leakey, 1996). In general, the slow growth of marine Antarctic organisms is influenced by both food availability and temperature (Clarke, 1983). Many benthic species cease feeding in winter for periods ranging from a few weeks to many months (Brockington and Clarke, 2001). In contrast, the limpet *Nacella concinna* (Mollusca, Patelliidae) is unusual for Antarctic marine ectotherms because it feeds throughout the year, although feeding rates decrease significantly in winter (Fraser et al., 2002; Clarke et al., 2004). *N. concinna* is a common invertebrate of the Antarctic and Sub-Antarctic nearshore fauna. It has a circumpolar distribution and lives between the intertidal zones and depths around 100 m (Cadée, 1999). The littoral features of *N. concinna* make it a...
suitable model for studies involving: histology, physiology, biochemistry and pollution (Kennicutt et al., 1992; Cripps and Shears, 1997; Abele et al., 1998; Najle et al., 2000; Peck and Veal, 2001; Fraser et al., 2002; Ahn et al., 2002; Clarke et al., 2004; Ansaldo et al., 2005).

Starvation exerts a wide range of effects in molluscs: e.g., decline in heartbeat frequency of Patella caerulea and Patella rustica (Santini et al., 2002), alterations in lysosomal membrane integrity (Moore, 2004), depletion of glycogen in the nervous ganglia of Helix aspersa (Borges et al., 2004), decrease in the RNA/DNA ratio from muscle, digestive gland and gills in green mussels (Bracho et al., 2000), increased aminotransferases activity in the hemolymph of Bradybaena similaris (Pinheiro et al., 2001), and increased d-lactate dehydrogenase together with decreased d-lactate in the mantle of Octopus ocellatus (Fujisawa et al., 2005). Moreover, starvation has also been reported to have pro-oxidant effects in mammals, and has been considered responsible for most of the harmful effects derived from food deprivation, e.g. increased generation of reactive oxygen species (ROS) that is not effectively neutralized by antioxidant systems (Robinson et al., 1997; Domenicali et al., 2001).

Oxidative stress can be understood as a situation derived either from an enhanced rate of ROS generation and a reduced level of antioxidant defences (Sies, 1985). It may be estimated by the physicochemical condition in which an increase in the steady-state levels of oxidative species (i.e., O₂⁻, H₂O₂, HO, R and ROO⁻) occurs. This increased steady-state level of oxidants may consequently lead to reversible or irreversible cell damage, and eventually to cell death. ROS are produced in a series of biochemical reactions that will normally occur within the cellular compartments (i.e., mitochondrion and the endoplasmic reticulum are the most important ROS sources; Halliwell and Gutteridge, 1999). It is well known that a number of different enzymes and non-enzymatic compounds participate in the antioxidant chain in biological systems. Among the enzymes, superoxide dismutase (SOD) converts superoxide anion (O₂⁻) to hydrogen peroxide (H₂O₂), catalase (CAT) reduces H₂O₂ to water, and glutathione S-transferases (GSTs), which constitute a large family of multifunctional enzymes, conjugate the reduced glutathione (GSH) to xenobiotics and aldehydic products of lipid peroxidation such as 4-hydroxialkenals. As a non-enzymatic compound, GSH is the principal nonprotein thiol involved in the antioxidant cellular defence; it provides reducing capacity for several reactions, and plays an important role in detoxification of hydrogen peroxide, other peroxides and free radicals (Halliwell and Gutteridge, 1999; Hermes-Lima, 2004).

The effect of oxidative stress elicited by starvation was studied in several mammal models (see references in Pascual et al., 2003), but only a few studies were performed in non-mammal vertebrates like fish (Pascual et al., 2003; Morales et al., 2004), and aquatic invertebrates, like molluscs and crabs (Abele et al., 1998; Pinho et al., 2003; Ansaldo et al., 2005). Abele et al. (1998) have recorded reduced levels of SOD in the digestive gland and CAT in the gills of N. concinna after one month starvation. They also observed that gill CAT activity of starved animals decreased under pro-oxidant treatments as H₂O₂ exposure. Pinho et al. (2003) observed a decrease in CAT activity from Chasmagnathus granulatus hepatopancreas after one week of starvation, without any measurable modification in SOD activity. Ansaldo et al. (2005) reported that, during the acclimation week previous to the start of the experiments, SOD and CAT activities decreased significantly in the digestive gland of N. concinna. After this period, SOD activity of control limpets remained unchanged, while CAT activity continued decreasing. Hence, starvation must be considered as a pro-oxidant condition that could synergize external factors such as pollution and/or seasonality.

The aim of the present work was to determine whether food deprivation under experimental conditions could change the pro-oxidant/antioxidant balance, altering the normal oxidative status of the Antarctic limpet N. concinna.

2. Materials and methods

Specimens of Nacella concinna with a mean shell length of 28.3±3.0 mm (wet mass 3.04±0.60 g), were collected by SCUBA diving at a depths ranging from 2 to 4 m near Jubany Station (Argentina), 25 de Mayo (King George) Island, South Shetland Islands, during the summer of 2003. Two groups of 80 limpets were randomly assigned to two treatments: “fed” and “starved”. Twenty animals were kept in 20L Plexiglas aquaria filled with filtered seawater at 1±0.5 °C under natural photoperiodic conditions. Seawater was continuously aerated, and completely changed every 48 h.

Organisms in the fed group were fed fresh macroalgae (mainly: crustose red algae and the brown alga Ascoseira mirabilis; secondly: the red algae Iridea cordata, Gigartina skottsbergii and the brown alga Geminocarpus germinatus) which constitute their natural diet, and grow on the intertidal rocks present in the shallow waters ecosystem of Potter Cove (Iken et al., 1998). Every 48 h, the rocks covered with algae were replaced in each fed group in order to maintain the natural source of food. The replaced rocks were those without limpets on their surface avoiding any possible disturbance caused by handling.

Twenty animals from each group were sacrificed (cooking on ice, 6–8 min) at days 7, 14, 21 and 30 of the experiment (weeks 1–4 hereafter). The shell was carefully removed and the whole body washed in saline solution, placed on filter paper to drain extra fluids, and weighed. Then, the digestive gland was dissected, immediately immersed in liquid nitrogen and kept frozen until they were analysed in the laboratory. The "wild or natural" condition of antioxidant variables was studied on an additional group of 20 animals (week 0), randomly labelled fed or starved, by measuring the same parameters (see below) immediately after collection at the Jubany Station laboratory.

All procedures were performed at 0–2 °C. Digestive glands were homogenised (1:9 w/v) in a cold (4 °C) buffer solution containing Tris-base (125 mM), 2-mercaptoethanol (1 mM), and PMSF (0.1 mM) (Vijayavel et al., 2004) with pH adjusted to 6.8. Homogenates were centrifuged at 10,000 x g for 10 min at 4 °C, and the supernatant used for enzyme activity, LPO and PO studies.

SOD, CAT and GST activities were determined using the spectrophotometric methods described by Misra and Fridovich (1972), Aebi (1984) and Habig et al. (1974) respectively.
Specific enzyme activity was calculated considering the total protein content of the supernatant; results were expressed as enzyme units/min.mg of protein. One SOD unit is the amount of enzyme necessary to inhibit 50\% the rate of autocatalytic adrenochrome formation measured at 480 nm. One CAT unit is the amount of enzyme necessary to degrade 1 \( \mu \text{mol} \) of H\(_2\)O\(_2\), measured at 240 nm. One GST unit represents the amount of enzyme required to conjugate 1 \( \mu \text{mol} \) of 1-chloro-2,4-dinitrobenzene, determined at 340 nm.

The LPO level was measured according to Buege and Aust (1978), by the formation of thiobarbituric acid reactive substances (TBARS). Fresh homogenates were added to the reaction mixture (trichloroacetic acid 15\% (w/v), 2-thiobarbituric acid 0.375\% (w/v), and butylhydroxytoluene 0.147 mM) in a ratio of 1:5 (v/v). The mixture was vigorously shaken, maintained in boiling water for 60 min, and immediately cooled at 5 °C for 5 min (Ohkawa et al., 1979). Then it was centrifuged at 5000×\( g \) for 10 min, and the supernatant was measured spectrophotometrically at 535 nm.

The PO level was evaluated according to Reznick and Packer (1994), by detecting the formation of protein hydrazones as a result of the reaction of dinitrophenyl hydrazine (DNPH) with protein carbonyls. Some minor modifications were performed to the original protocol; briefly, after the protein hydrazone formation, they were precipitated using TCA 30\% (Fagan et al., 1999), and then washed 3 times with ethanol: ethyl acetate (1:1). After the final wash, the protein was solubilized in 1 mL of urea (6 M in 20 mM potassium phosphate, pH 2.5) instead of guanidine hydrochloride. To speed up the solubilization process, the samples were incubated at 37 °C in a water bath for 60 min. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 375 nm, using an absorption coefficient \( e=22,000 \ \text{M}^{-1} \ \text{cm}^{-1} \). It was verified that urea blanks showed the same absorbance peak at 375 nm as guanidine hydrochloride blanks.

The reduced glutathione (GSH) level of the starved group showed no significantly changes during the time of the assay (\( p>0.05 \)). In the fed group, the level of GSH began to increase in the first week, and was significantly different in the third week (\( p<0.01 \), remaining high during the fourth week (Fig. 1c).

The total protein quantity of the homogenates was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Data were analysed through an ANOVA followed by a posteriori mean comparisons (Tukey test) among wild (week 0) and experimental groups as well as between treatments (fed vs. starved). Previously, ANOVA assumptions were verified and transformations applied if lack of normality and/or variance heterogeneity were detected (Sokal and Rohlf, 1997).

3. Results

The TBARS levels for both groups augmented in the first week and this increment were significantly different (\( p<0.001 \)) compared to wild groups (week 0) (Fig. 1a). From the second week up to the end of the assay, the TBARS levels decreased to the control values, remaining unchanged. No differences in TBARS levels were observed between fed and starved limpets throughout the experiment (\( p>0.05 \)).

Protein oxidation, measured as carbonyl content level, only increased significantly (\( p<0.05 \)) in the first week for the starved animals (Fig. 1b). From the second week and until the end of the assay, values decreased back to those measured in the control group. The fed group remained unchanged throughout all the experiment (\( p>0.05 \)).

The GSH level of the starved group showed no significantly changes during the time of the assay (\( p>0.05 \)). In the fed group, the level of GSH began to increase in the first week, and was significantly different in the third week (\( p<0.01 \), remaining high during the fourth week (Fig. 1c).
The SOD activity increased significantly \((p < 0.05)\) for fed and starved groups in the end of the first week (Fig. 2a). Both groups of limpets registered the highest values during the second week \((p < 0.001)\). The SOD activity of the starved group remained significantly different \((p < 0.05)\) with similar levels to those observed in the first week of the assay \((p > 0.05)\). During the third week, the SOD activity of the fed group returned to the week 0 values \((p > 0.05)\). Fed and starved groups showed increased values in the end of the experimental period, although there was no significant difference between them \((p > 0.05)\), but they were significantly different \((p < 0.01)\) from the week 0 groups.

The CAT activity showed significantly \((p < 0.05)\) increased values for the starved group in the second and fourth weeks of the assay (Fig. 2b). During the third week, a decrease in CAT activity was observed, and this value was not significantly different \((p > 0.05)\) from the week 0 groups. The fed group only registered a significant increment \((p < 0.05)\) in the fourth week.

Starved limpets showed an increment of GST activity during the second week, which was not statistically different from week 0 group \((p > 0.05; \text{Fig. 2c})\), whereas the fed limpets did not show any change in the enzyme activity. From the third week and until the end of the assay, both groups showed increased values which were statistically different \((p < 0.05)\) from the week 0 groups.

4. Discussion

It has been reported that most of the negative effects of starvation could be mainly attributed to the participation of ROS generated under food deprivation (Pascual et al., 2003, Morales et al., 2004). However, taking into account the present results, we should consider that the antioxidant status of the Antarctic limpets may be influenced by starvation as well as by experimental conditions.

Our results showed a marked increment in the TBARS levels in the digestive gland of *N. concinna* during the first week of aquaria acclimation, probably due to the stress produced by experimental conditions, because this increment was observed both in fed and starved groups. Simultaneously, an increase was also observed for protein oxidation levels of the starved group. The SOD, CAT and GST activities increased in a synchronized way in the second week trying to counterbalance the oxidative damage. This fact was mainly evident in the starved group, and was reflected in the decrease of lipid and protein oxidation levels, which diminished to control values (week 0) after the second week. Throughout the experimental period, the CAT activity seems to be slightly higher in the starved group than in the fed one. Guderley et al. (2003) also reported increased CAT and GST activities in the liver of the starved fish *Gadus morhua*. Starvation decreases tissue metabolic capacities but, on the other hand, the food deprivation causes degradation of endogenous sources of energy (lipids, glycogen, and proteins) in order to maintain the fish physiological homeostasis. They found that the activity of certain lysosomal enzymes and antioxidant defences were maintained or enhanced even after the starvation period. These findings on fish antioxidant status addressed the interpretation that when facing energetic limitations such as food deprivation and/or severe hypoxia, aerobic organisms maintain relatively high levels of their antioxidant defences to cope with oxidative stress as metabolic priorities compared to other important functions such as weight gain and reproduction (Wilhelm Filho et al., 2005). Changes observed in the antioxidant status during the reproductive cycle of molluscs may be a determinant for the expression of the antioxidant enzymes, as well as for the synthesis of endogenous antioxidants such as GSH (Wilhelm Filho et al., 2001). In the present study, limpets were collected in the summer, and therefore no effect on the antioxidant status could be attributed to emission of gametes by the organism because this event only happens in spring, in a very narrow time-frame (Stanwell-Smith and Clarke, 1998).

![Fig. 2. The SOD, CAT and GST activities in the digestive gland of *N. concinna* in fed and starved groups at different time in aquaculture conditions. Data are expressed as mean±standard error. Different letters indicate significant differences \((P<0.05)\) among wild (week 0), fed and starved groups.](image-url)
The effect of starvation enhancing oxidative stress could also be attributed to the deficiency of the diet with low molecular weight components such as ascorbic acid, α-tocopherol and carotenoids, which are well known antioxidants in marine algae (Dummermuth et al., 2003). A deficit of these compounds could become a critical factor with regard to the antioxidant status of starved animals (Abele et al., 1998). However, in the present study, the GSH level for the starved group remained unchanged, probably due to the peptidases activities on peptides from the intracellular pool (Hermes-Lima, 2004) that kept the GSH level similar to the control group (0 week). By contrast, the fed group showed increasing values, probably due to the food intake.

The NADPH supply is critical because it is involved in the reduction of GSSG to GSH. The glucose-6-phosphate dehydrogenase (G6PDH), a key enzyme from the pentose phosphate pathway is involved in the production of NADPH. The inhibition or reduction of the G6PDH activity was reported during food deprivation (Barroso et al., 1998; Morales et al., 2004), or hypometabolism due to estivation (Ramnanan and Story, 2006). Thus, the generation of oxidative stress might be related to the inhibition of G6PDH activity in the digestive gland metabolism in the food-deprived limpets.

In a previous study, values of SOD and CAT activities 10-folded those registered in the present study for the 0 week group (Ansaldo et al., 2005). Similarly, comparing their studies, Fraser et al. (2002) and Peck and Veal (2001) observed differences in the oxygen consumption rates of N. concinna. Fraser et al. (2002) speculated that higher respiration rates reported by Peck and Veal (2001) had been the result of the starvation and feeding protocol used. Hence, the lower respiration rates reported by Fraser et al. (2002) were probably related to experimental protocols designed to minimise handling and experimental stress. In the present work, to avoid excessive handling stress, limpets were collected by scuba diving and immediately placed in the aquarium. By contrast, Ansaldo et al. (2005) collected the limpets by hand from a rocky shore during the low tide adding an extra stress for the limpets, and augmenting the stress parameters measured.

Furthermore, important climatic differences were recorded between Ansaldo et al. (2005) and the present study. Antarctic nearshore marine environments are characterised by a marked interannual variability in ice cover, temperature and the intensity and duration of the phytoplankton bloom (Clarke and Leakey, 1996). Limpets of Ansaldo’s study (2005) came from the summer of 2002 (mean temperature 1.9 °C), whereas limpets of the present study were sampled in the summer of 2003 (mean temperature 1.8 °C). Notwithstanding temperatures in both summers were similar. However, mean spring temperatures of each year were completely different (spring 2001: 0.1 °C vs. spring 2002: −1.5 °C). These marked differences in the spring temperatures may have conditioned the environmental physical conditions of the following summer. In the “warm” period, the snow and ice cover was scarce, with no debris of ice on the coast and in the intertidal–subtidal zone. This situation allowed limpets to easily access the intertidal zone, which is rich in food sources (Iken et al., 1998). The “cold” period corresponding to the summer of 2003, showed the opposite condition: permanent snow and ice covering the coast, with high amounts of ice debris in the intertidal zone turning it inaccessible for benthic fauna. These environmental characteristics determined by the extreme difference in temperature, might be influencing the antioxidant enzyme activities. Fraser et al. (2002) reported the seasonal decrease in the Antarctic limpet metabolism due to lowering temperatures. Hence, we conclude that differences observed in the antioxidant enzyme activities registered between the Ansaldo et al. (2005) and the present study, were also due to the temperature fluctuation registered in both sampling years.

In the present study, we observed that the starved and fed limpets maintained in laboratory conditions had relatively high levels of their antioxidant defences to cope with oxidative stress but the additional effect of experimental conditions probably masked the food deprivation effect per se.

In conclusion, and according to the present findings, we suggest that to avoid misinterpretation of experimental results, any assay involving N. concinna in aquarium conditions must take into account the acclimation period to this “artificial new environment”. Climatic and environmental conditions during animal sampling should also be considered due to the fact that they might affect the limpet’s physiological status.

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