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Effect of aerial exposure on the antioxidant status in the subantarctic stone crab *Paralomis granulosa* (Decapoda: Anomura)☆

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Abstract

In Tierra del Fuego (Southern South America), the stone or false king crab, *Paralomis granulosa* represents one of the most important crab fisheries. After capture, animals are kept in baskets and exposed to dryness for several hours, when the water flow through the gills is interrupted. As a consequence a concomitant increase of reactive oxygen species begins, triggering oxidative stress. The aim of this study was to determine oxidative stress and antioxidant enzyme activities due to air exposure in different tissues of *P. granulosa*. Fifty crabs (carapace length >82 mm) were captured in Beagle Channel (54° 50′S, 68° 20′W) during winter 2004. Five groups of 10 crabs each were exposed to dryness at 6°C for 0, 3, 6, 12 or 24 h, respectively. Activity of superoxide dismutase (SOD), catalase (CAT), glutathione S transferase (GST) protein and lipid oxidation were measured in gills, muscle, hepatopancreas and haemolymph samples. Almost all analyzed tissues showed antioxidant enzymes activity, which varied with time of air exposure. The maximum enzyme activity was measured after 6 h of air exposure. Protein oxidation levels varied significantly in gills. Lipid peroxidation levels increased significantly in muscle and hepatopancreas. The critical time of air exposure probably occurs at 6 h. Thereafter animals were unable to induce the synthesis of antioxidant enzymes or proteins. This should be taken into account to minimize the stress generated by the commercial capture process.

Keywords: Oxidative stress; Antioxidant enzymes; Air exposure; Protein oxidation; Lipid peroxidation; *Lithodidae*; *Paralomis granulosa*; King crab; Fishing processing; Beagle Channel

1. Introduction

The stone or false king crab *Paralomis granulosa* constitutes the main crab fishery in the southern tip of South America. Annual landings in Punta Arenas, Chile and Ushuaia, Argentina have totaled to around 3000 t during the last decade. Crabs are fished with baited traps that are submerged during 2–6 days and then recovered. Legal crabs i.e., males >80 mm carapace length, CL are retained onboard, and traps re-baited and re-submerged. Legal crabs are stocked in baskets on deck or in the hold. They spend several hours, in many cases up to 24 h, exposed to air, until their arrival to the factory. Crabs must arrive alive at the factory where they are slaughtered, boiled for few minutes, the muscle from the legs extracted and frozen. During trap handling and sorting, females and small males are also exposed to aerial conditions, yet for a shorter time, since they must be returned to water. This aerial exposure is unnatural since lithodid crabs are rarely out of seawater (but see Lovrich et al., 2002). In contrast to some brachyuran crabs, in which the degree of enclosure of the branchial chamber allows water to drain, lithodids are not well adapted to the aerial realm. Therefore, the flux of water running through the gills is interrupted, with the concomitant increase of reactive oxygen species, probably triggering

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oxidative stress. From the commercial perspective, one undesirable consequence of prolonged air exposure may be the oxidation of tissues that can eventually influence in the final product taste.

In *Paralithodes camtschaticus*, *Chionoecetes opilio*, and *C. bairdii* from the Bering Sea, aerial exposure was studied in relation to cold air temperatures affecting their survival. The windchill has been identified as the most severe stressor on snow crabs, and windchill values commonly occurring during the fishery result in mortality, limb loss and decreased activity (Carls and O'Clair, 1990, 1995; Warrenchuk and Shirley, 2002). For example in *C. opilio*, a 5-min exposure to windchill of −10 °C can produce 50% mortality (Warrenchuk and Shirley, 2002). Nevertheless, so far no studies have dealt with the physiological consequences of aerial exposure of lithodid crabs.

The normal oxygen consumption by aerobic organisms produces potentially deleterious reactive oxygen species (ROS) which may react in an aggressive manner, destroying cellular compartments, tissues and finally, killing the organism. These ROS include the superoxide anion radical ($O\cdot_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO\cdot$), the last being the most reactive and destructive species. Aerobic organisms are protected from ROS by several defenses including antioxidant enzymes such as superoxide dismutase (SOD) which converts $O\cdot_2^-$ to H_2O_2, catalase (CAT) converting H_2O_2 to water, and glutathione-*S*-transferase (GST) which is involved in the biotransformation of numerous xenobiotic compounds. Low molecular weight antioxidants, such as reduced glutathione and vitamins C and E, act in conjunction with these enzymatic defenses (Halliwell and Gutteridge, 1989; Hermes-Lima, 2004). The effectiveness of these antioxidants may vary with the stage of development and other physiological aspects of the organism (Livingstone, 2001). The balance between oxidants and antioxidants is vital in all aerobic organisms. Hence, when the rates of ROS formation overwhelm the antioxidant capacity of an organism, oxidative stress appears, which influences the individual fitness (Sies, 1986).

In the last decade, several studies have demonstrated potential ROS generation, antioxidant enzymes and free radical scavenger responses, and oxidative damage in aquatic invertebrates (e.g., Arun et al., 1999; Di Giulio et al., 1995; Correia et al., 2003). However, few studies have been undertaken on decapod crustaceans (e.g., Vinagre et al., 2003; Gamble et al., 1995), and so far, there are no data concerning the antioxidant status of any lithodid crab. The aim of this study was to determine the oxidative stress and the antioxidant enzyme activities in different tissues of *P. granulosa* due to air exposure.

2. Materials and methods

2.1. Animal collection and assay conditions

Fifty male *P. granulosa* were captured in the Beagle Channel (54° 50′S, 68° 20′W) with commercial traps in July–August 2004. All crabs were in intermoult stage and legal sized, i.e., >82 mm of carapace length. Animals were transported to the laboratory and kept in individual tanks with running seawater at 6±0.5 °C for 1 week to acclimate to aquaria conditions. They were fed twice with fresh squid mantle. Light cycle was 12:12-h light:dark. After the acclimation period, five groups of ten crabs each were exposed to air for different time periods: 0 (control), 3, 6, 12 and 24 h. Crabs were placed in individual plastic boxes without water and with cloths fully embedded in seawater to maintain the air humidity, and placed at 6±0.5 °C. Air exposure of all crab groups started at 8 am, in order to avoid overlapping of antioxidant enzyme activities with a possible circadian rhythm, as occur in other species (e.g., Maciel et al., 2004; Fanjul-Moles et al., 2003).

After the exposure period, haemolymph samples were withdrawn from the ventral sinus via the arthrodial membrane at the base of the 4th or 5th pair of pereiopods using 1 ml disposable plastic syringes. Samples of 500 µl were transferred to pre-chilled 1.5 ml centrifuge tubes that contained 1 ml of ice-cold Tris–HCl buffer (pH 6.8, 125 mm Tris, 1 mM 2-mercaptoethanol, and 0.1 mm PMSF) as was described by Vijayavel et al. (2004), to avoid haemolymph clotting. Samples were centrifuged for 10 min at 9000×*g* at 4 °C. The supernatant was collected and immediately analyzed. After haemolymph sampling, the 7th gill, hepatopancreas and the muscular mass from the 5th pair of pereiopods were dissected, frozen at −20 °C and analyzed within the following 30 days.

2.2. Sample preparation

The homogenates were prepared using 0.3 g of gill or 0.1 g of muscle or hepatopancreas tissue in 1.2 or 1.4 ml of cold (4 °C) buffer solution containing 125 mm Tris, 1 mM 2-mercaptoethanol, and 0.1 mM PMSF (Vijayavel et al., 2004). The pH was adjusted to 6.8 with HCl. Samples were processed using a Potter-Teflon homogenizer, and immediately centrifuged for 15 min at 11,000×*g* at 4 °C. The supernatants were collected and employed as antioxidant enzyme sources.

2.3. Biochemical analyses

Superoxide dismutase (SOD) activity was measured by the inhibition of the auto-oxidation of epinephrine (2 mM) in 50 mM glycine buffer (pH 10.2) according to Misra and Fridovich (1972). The reaction was analyzed spectrophotometrically at 480 nm with different volumes of enzyme sample. One unit of SOD was defined as the amount of enzyme inhibiting the oxidation of epinephrine by 50%.

Catalase (CAT) activity was measured by the method of Chance (1954). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 3 mM H_2O_2, and it was analyzed spectrophotometrically at 240 nm. One unit of CAT was defined as 1 nmol of H_2O_2 degraded per minute per milligram of protein.

Glutathione-*S*-transferase (GST) was determined by the method of Habig et al. (1974). GST activity was measured as increase in absorbance at 340 nm, using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates. The reaction mixture contained 0.1 M phosphate buffer (pH 6.5), GSH 1 mM and CDNB 1 mM. One unit was defined as 1 µmol of GSH conjugated per minute per mg of protein.

Lipid peroxidation was measured according to Hermes-Lima et al. (1995). Frozen tissue samples were homogenized in 100% cold HPLC grade methanol (1:5 w/v for gill and muscle, and 1:9 for hepatopancreas). Homogenates were centrifuged 10 min at 5000×*g* and the supernatant was collected. Sample absorbance (580 nm) was registered after 3 h of incubation at room temperature. Levels of lipid peroxides are expressed as cumene hydroperoxide (CHP) equivalents per gram of wet weight tissue.

The determination of carbonyl content in proteins can be used as a measure of oxidative protein damage (Fagan et al., 1999). Protein oxidation was measured by the method of Reznick and Packer (1994). The number of carbonyl groups on proteins was quantified spectrophotometrically (375 nm) using 2,4-dinitrophenylhydrazine (DNPH).

The total protein content was determined following the method of Lowry et al. (1951), using bovine serum albumin as standard. The range 30–120 μg ml^{-1} was used to prepare calibration curves.

2.4. Statistical analyses

Data are presented as mean±standard error. Analyses of variance (one-way ANOVA) were performed to determine aerial exposure effects on each antioxidant enzyme. Data were checked for normality and homogeneity of variance by Kolmogorov–Smirnov and Levene tests, respectively (Sokal and Rohlf, 1995). Significant differences ($p<0.05$) were compared using Tukey post hoc test (Sokal and Rohlf, 1995).

3. Results

The activity of the enzymes involved in the antioxidant defense of *P. granulosa* varied with the time of air exposure.

All analyzed tissues showed SOD activity (Fig. 1A; ANOVAs for each tissue $p<0.05$). The highest SOD activity was observed in the muscle at 6 h of air exposure, and twofold higher than that registered in the hepatopancreas at the same time. After 3 h of air exposure, haemolymph showed high significant differences (Tukey, $p<0.001$). Gills were the only tissue where SOD activity decreased with time of air exposure (ANOVA, $F=5.18$, $p=0.003$). After 3 h of air exposure SOD activity was significantly reduced to 50% (Tukey, $p=0.03$), and this value remained constant until the end of the experimental time (Fig. 1A).

Similar to SOD the CAT activity of gills, muscle and haemolymph varied with time of exposure (ANOVAs, $p<0.05$). CAT and SOD showed coordinated activity in muscle, hepatopancreas and haemolymph (Fig. 1A and B). Air exposure caused a significant increase in CAT activity among time 0 values and 3 and/or 6 h in gills, muscle and haemolymph (Tukey, $p<0.05$; Fig. 1B). Maximum values of CAT activity were ~80% higher for gills and muscle, and 122% higher for haemolymph than time 0 values. After these maxima, CAT activity decreased to values similar to those at time 0 (Tukey, $p>0.05$; Fig. 1B). Furthermore, gills showed the highest CAT activity which duplicated CAT activity of muscle and hepatopancreas at 6 h of air exposure. Haemolymph showed the lowest CAT activity. CAT activity patterns observed in the hepatopancreas was similar to other tissues (Fig. 1B), and the 46% increment of CAT activity after 6 h of exposure was not significantly different from the observed at time 0.

The GST activity showed different patterns among tissues (Fig. 1C), although all of them showed differences along the experimental time (ANOVAs, $p<0.05$). Muscle tissue showed the highest GST activity, with a decrease (40%) in the first hours and increase values at 12 h (Fig. 1C). During the assay, hepatopancreas and haemolymph GST activities were reduced in ~50% compared with values registered at time 0 (Tukey, $p<0.05$). In the muscle, GST activity first decreased in a 40% and at 12 h increased to values similar to those at time 0 (Fig. 1C). The gill GST activity significantly increased at 6 h of air exposure (Tukey, $p<0.05$, Fig. 1C).

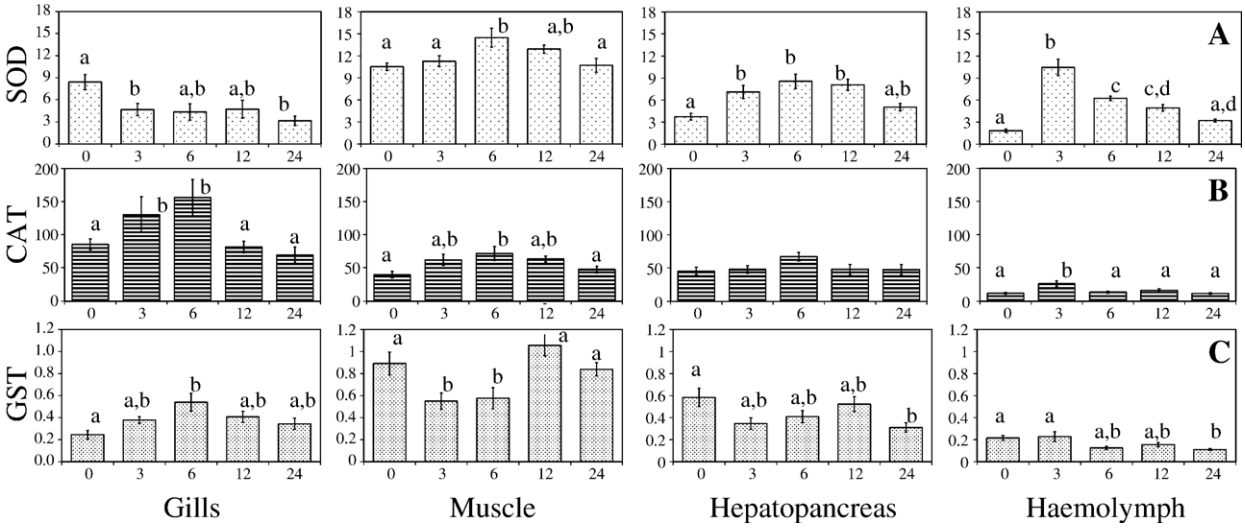

Fig. 1. Antioxidant enzyme activities of SOD (U SOD/mg protein), CAT (nmol/mg protein) and GST (mU GST/mg protein) during different times of air exposure in different tissues of *Paralomis granulosa*. Data are expressed as mean±standard error of a sample of 10 animals. Values accompanied by the same letter are not statistically different (Tukey post hoc test, $p<0.05$).

Table 1
Protein oxidation and lipid peroxidation levels in haemolymph, gills, muscles and hepatopancreas of the crab *Paralomis granulosa* during air exposure

Time (h)	0	3	6	12	24
Protein oxidation ([carbonyl] nmol/mg protein)					
Haemolymph	0.15±0.01	0.14±0.01	0.20±0.02	0.17±0.02	0.20±0.03
Gills	0.19±0.03[a]	0.34±0.04[b]	0.56±0.04[c]	0.32±0.04[b]	0.44±0.04[b,c]
Muscle	0.58±0.05	0.41±0.04	0.44±0.04	0.45±0.07	0.40±0.02
Hepatopancreas	0.29±0.03	0.34±0.03	0.34±0.03	0.37±0.02	0.27±0.02
Lipid peroxidation (CHP eq/mg wet weight)					
Gills	1.68±0.20	1.27±0.06	1.17±0.10	1.26±0.10	1.25±0.20
Muscle	2.0±0.15[a,b]	1.7±0.25[a]	2.9±0.32[b,c]	3.1±0.32[b,c]	3.3±0.26[c]
Hepatopancreas	32.0±2.9[a]	39.7±3.7[a,b]	51.5±5.9[b]	48.9±2.5[b]	48.8±2.3[b]

Data are expressed as mean±standard error of a sample of 10 animals. Values accompanied by the same letter are not statistically different (Tukey post hoc test, $p<0.05$).

Gills were the only tissue in which protein oxidation varied significantly (ANOVA, $F=12.47$, $p<0.001$, Table 1). After 6 h of air exposure, protein oxidation reached its maximum and 3-folded the time 0 values (Tukey, $p<0.001$). In the other assayed tissues, protein oxidation levels remained unchanged throughout the experiment (ANOVAs, $p>0.05$, Table 1).

In gills, lipid peroxidation level was statistically similar throughout the time of air exposure (ANOVA, $F=1.22$, $p=0.32$), whereas muscle and hepatopancreas showed significant differences (ANOVAs, $p<0.001$ and $p=0.002$, respectively; Table 1). In these latter tissues, lipid peroxidation levels increased after the air exposure. In both cases the maximum peak appeared at 6 h of exposure, and remained constant thereafter and until the end of the experiment (Table 1). In the hepatopancreas lipid peroxidation level 17-folded those registered in the other studied tissues.

4. Discussion

4.1. Enzymatic antioxidants

This study showed that air exposure affects the oxidative status of *P. granulosa* exerting oxidative stress. Compared to brachyurans *P. granulosa* has no means to keep water in the gill chamber (cf., Luquet and Ansaldo, 1997). Hence, during the air exposure of *P. granulosa* the oxygen interchange at the gill level is reduced to almost zero. Gills are the only tissue that did not show an increase of SOD activity throughout the experimental time. This means that hydrogen peroxide, which is the substrate for CAT enzyme activity, is probably produced by any other intracellular pathway. In fact, as a general pattern, CAT activity increased until 6 h of air exposure and decreased afterwards. An alternative explanation for this situation would be related with the possibility that the CAT activity increased as a physiological strategy. This increase may be possible as an anticipatory response for protection to the oncoming oxidative stress during reoxygenation (Hermes-Lima and Zenteno-Savín, 2002; De Oliveira et al., 2005). This process was named "preparation for oxidative stress" (Hermes-Lima et al., 1998), and involves a build-up of antioxidant capacity before the actual occurrence of oxidative stress happens. The increase in the activity of antioxidant enzymes would reflect an increase in the rate of their synthesis and/or decrease in their degradation (Hermes-Lima and Zenteno-Savín, 2002).

After 3 h of air exposure, the SOD and CAT activities showed a coordinated response in haemolymph, muscle and hepatopancreas throughout the experiment. After 6 h of air exposure, however, the animals could not maintain the high rhythm of antioxidant production, and their values decreased down to time 0 values. In crab tissues the physiological response might be the same: a clear preparatory strategy to deal with oxidative stress during the post-anoxia recovery. Under oxygen-limited state, several vertebrates and invertebrates species showed this pattern of increment of some antioxidant enzyme activities (Hermes-Lima and Zenteno-Savín, 2002; Almeida et al., 2005). The high activities of antioxidant enzymes during the anoxic condition, seems to be the key to minimize tissue damage during the reoxygenation, since tissues are prepared to deal with oxyradicals (Hermes-Lima et al., 1998; Storey, 1996).

The crustacean hepatopancreas is a site of maximal free radical generation due to the multiple oxidative reactions that take place herein (Arun and Subramanian, 1998). Since we did not observe high values of antioxidant enzyme activities in this tissue after air exposure, we suggest that mainly non-enzymatic antioxidants could act in this organ. In fact, carotenoids and their derivatives, as β-carotene, were found in high concentration in the hepatopancreas of crustaceans (Sagi et al., 1995), and are known to deactive chemical species such as singlet oxygen, triplet photochemical sensitizers, and free radicals (Miki, 1991; Matsuno, 2001).

Protein oxidation levels varied significantly in the gill as a consequence of air exposure. This fact might be related with the fluctuation of CAT and GST activities, which also varied to counterbalance the oxidative damage. Moreover, lipid peroxidation levels were affected significantly in muscle and hepatopancreas, which might be associated with SOD and CAT fluctuations observed in the same tissues.

4.2. Ecological and evolutionary approach

In *P. granulosa*, responses in the gill antioxidant enzyme observed under air exposure were similar to that found in the gills of the estuarine, intertidal crab *Chasmagnathus granulatus*

(De Oliveira et al., 2005). In *C. granulatus*, gill SOD activity decreased, yet CAT and GST activities significantly increased after 8 h of anoxia. In an intertidal crab, this situation is considered adaptative since this species normally undergoes cyclic periods of oxygen availability/deprivation due to tidal changes.

Lithodid depth distribution is very wide, from mid-intertidal to abyssal depths (>4000 m) (Thatje and Arntz, 2004; Thatje et al., 2005). Lithodid radiation likely began in the intertidal zone of the northeastern Pacific (see Zaklan, 2002 for a review). The family is of recent origin, arising between 13 and 25 million years a suggested by molecular (Cunningham et al., 1992) and fossil (Feldmann, 1998) evidence. At the present times, southern lithodid crabs perform vertical migrations in summer to moult and mate in shallow waters. In the Southern Hemisphere, the only representative of the family found in the intertidal during the spring ebb tides is *Lithodes confundens* (Lovrich et al., 2002). The intertidal species of the family (e.g., *Hapalogaster* spp., *Cryptolithodes* spp.) are those mainly occurring in the North Pacific/Bering Sea and are considered ancestral representatives (Zaklan, 2002). Our results in *P. granulosa* suggest that this "preparation for oxidative stress" – with a maximum of enzymatic activity at 6 h of air response –, could be a feature of the intertidal lineage of this group that has been retained by current species. However, the adaptative value of this "preparation for oxidative stress" in lithodids is still intriguing.

In summary, the air exposure of *P. granulosa* alters its oxidant–antioxidant status and triggers antioxidant responses of its enzymes. Crabs are severely affected at 6 h of air exposure although they seem in a good health condition. This should be taken into account in order to minimize the stress generated by the commercial capture process. Furthermore, future studies on the reoxygenation phase should be done, for a better understanding of antioxidant enzymes in this species. Particularly, these data could be useful to know the environmental conditions (time, temperature) that they can be exposed to the air and returned to the water without suffering any irreversible damage.

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