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Aquaculture 319 (2011) 205–210

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Oxidative stress in the subantarctic false king crab *Paralomis granulosa* during air exposure and subsequent re-submersion

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A R T I C L E I N F O

Article history:
Received 4 November 2010
Received in revised form 30 May 2011
Accepted 26 June 2011
Available online 3 July 2011

Keywords:
Antioxidant enzymes
Lithodidae
Palatability assay
Stone crab
Live transport

A B S T R A C T

In the Southern South America, the stone or false king crab *Paralomis granulosa* constitutes the main crab fishery. One of the extremely stressful situations to fished crustaceans is the air-exposure handling once they are on deck. The aims of this study were to determine effects of air-exposure and re-submersion on: (1) the antioxidant enzyme activities and lipid peroxidation in different tissues; and (2) changes in the meat palatability in *Paralomis granulosa*. One hundred and twenty male crabs were captured in Beagle Channel (54°S, 68°W) and randomly assigned to the two planned experiments. For the air-exposure, four groups of 8 animals each were exposed to dryness at 7 °C for 6 h, and then re-submerged for 0.5, 1, 2 and 24 h respectively, whereas a fifth group was used as control. Activities of superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), as well as lipid peroxidation were measured in gills, muscle, hepatopancreas and haemolymph. Almost all tissues showed a significant increase of antioxidant enzyme activities until 2 h of recovery ($\alpha = 0.05$). The highest antioxidant enzyme activities were found in gills, in which SOD, CAT and GST activities were similar to the control values after 24 h of recovery. Moreover, gills and hepatopancreas showed significantly increased ($\alpha = 0.05$) lipid peroxidation at 0.5 h of re-submersion. For the palatability assay, three groups of twenty crabs each were air-exposed at 7 °C for: 6, 12 and 18 h and then re-submersed for 24 h, whereas a fourth group was used as control. Four dishes of ~10 g meat each were offered to 30 judges who performed a taste ranking with the meat samples. Even if differences in the meat flavor of *P. granulosa* were detected, participants could not rank samples according to the increasing air exposure of animals. Live transportation of *P. granulosa* is promising and crabs can be re-submerged and offered alive.

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1. Introduction

In the Southern South America the stone or false king crab *Paralomis granulosa* constitutes the main crab fishery. Landings in Punta Arenas – Chile – and in Ushuaia – Argentina – have totaled around 3,000 t per year in the last decade (Guzmán et al., 2004). The king crab fishery of the Beagle Channel develops at an artisan scale. Fishers own their boats, which are small (<10 m length) and limited to manage a small number of traps. Fishing revenues are normally low and fishers have limited means to increase the fishing effort since the number of traps is fixed by law in 1000 traps for the all channel. During the fishing operations, legal male crabs (>80 mm carapace length) are fished with baited traps, retained onboard and stored in baskets on deck. Thus, crabs spend several hours exposed to air before arriving at the factory, where they have to be delivered alive, as stated by Argentine law. The whole capture is sold to retailers who own the factories where crabs are sectioned, boiled for few minutes, the crab meat is extracted from pereiopods and is frozen in packs to 0.5 kg or 2.4 kg. This product is called "fancy" and is mainly exported.

In recent years, the fishing industry has started to focus on the transport of live crustaceans as lobsters *Nephrops norvegicus* (Lund et al., 2009; Ridgway, 2007), *Panulirus interruptus* (Márquez-Ríos et al., 2007), *Jasus edwardsii* (Morris and Oliver, 1999), and crabs *Cancer pagurus*, *Carcinus maenas*, *Maja* spp., *Necora puber* (Barrento et al., 2008). Currently, live transport is normally done without water. The marketing of live animals could constitute an opportunity for local fishers to diversify their markets and increase their incomes without augmenting the fishing effort. The crab capture could be sorted into small animals sold to retailers to produce "fancy packaged meat" and large animals sold for live transportation out of Tierra del Fuego.

Lithodids are not well adapted to the aerial realm since air exposure of lithodid is unnatural and these crabs are rarely out of seawater (but see Lovrich et al., 2002). One of the extremely stressful situations to crustaceans is the air-exposure handling, causing both severe metabolic and respiratory disturbance (Danford et al., 2001a; Lund et al., 2009). From the commercial point of view, one of the

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doi:10.1016/j.aquaculture.2011.06.041

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undesirable consequences of a prolonged air-exposure may be the oxidation of tissues that could eventually influence the taste of the final product.

Inside cells, reactive oxygen species (ROS) as by-products of cell respiration are continuously produced. The most common ROS include the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO.) (Halliwell and Gutteridge, 2007). All organisms present both enzymatic and non-enzymatic antioxidant defensive systems against ROS generation. The enzymatic antioxidant defenses include superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST). These enzymes remove or transform ROS into less toxic metabolites (Halliwell and Gutteridge, 2007). Furthermore, antioxidants of low molecular weight such as reduced glutathione (GSH) and vitamins C and E, act in conjunction with these enzymatic defenses (Halliwell and Gutteridge, 2007; Hermes-Lima, 2004). Hence, when the rates of ROS formation overwhelm the antioxidant capacity of an organism, oxidative stress is produced (Sies, 1986), leading to an increase in oxidative damage to different cellular targets (Almeida et al., 2005), such as oxidation of DNA, lipids and proteins that modify the normal cellular functions (Halliwell and Gutteridge, 2007). This may occur under various conditions, one of the most important being the exposure of the organism to hypoxia or anoxia followed by re-oxygenation (Hermes-Lima and Zenteno-Savín, 2002).

In the last two decades, several studies have demonstrated different strategies used by animals to cope with oxidative stress (*e.g.* Arun et al., 1999; Correia et al., 2003; Di Giulio et al., 1995). However, only a few studies were performed on decapod crustaceans (*e.g.* de Oliveira et al., 2005; Gamble et al., 1995; Vinagre et al., 2003). Particularly, antioxidant enzymatic activities have been studied in *P. granulosa* as indicators of the damage caused by different times of air exposure (Romero et al., 2007). In lithodids no further information has been published about the response of antioxidant enzyme activities to air exposure followed by re-submersion, neither about the flavor changes associated with prolonged air exposure. Thus, the aims of this study were to determine the effects of air exposure and re-submersion on: (1) the antioxidant enzyme activities in different tissues of *Paralomis granulosa* and (2) flavor changes in the meat palatability of *P. granulosa*. We hypothesize that the time of air exposure for *P. granulosa* is directly related to the flavor of the meat. Since lipid oxidation may increase with air exposure (Romero et al., 2007), meat from animals that suffer a higher air exposure will degrade their taste as exposure time increases.

2. Materials and methods

2.1. Acclimation and air exposure

One hundred and twenty male crab *Paralomis granulosa* were captured in the Beagle Channel (54°S, 68°W) with commercial baited traps in May 2007. All crabs were in intermoult stage and of legal size. Animals were transported to the laboratory and kept in individual tanks with running seawater at 7 ± 0.5 °C for one week in order to acclimate them to aquaria conditions. During this week they were fed twice with fresh squid mantle. Light cycle was 12:12 h light:dark.

2.2. Experiment 1: Antioxidant enzyme activities and lipid peroxidation level after 6 h of air exposure and different times of recovery

Since the maximum enzyme activity was measured after 6 h of air exposure (Romero et al., 2007) this period was selected as air exposure time to analyze antioxidant enzyme activities at different times of recovery. After the acclimation period, four of five groups of eight crabs each were exposed to air for 6 h, whereas the fifth group was used as control without air exposure. Air exposure was performed by placing the animals in individual plastic boxes without water and with cloths fully soaked in seawater to maintain the air-humidity, and placed at 7 ± 0.5 °C air temperature. After 6 h of air exposure each group of animals was re-submersed in sea water at 7 ± 0.5 °C water temperature for: 0.5, 1, 2, and 24 h. These times were selected because we expected high antioxidant enzyme activities at least in the first 3 groups (0.5, 1 and 2 h) of recovery (de Oliveira et al., 2005; Malanga et al., 2007). Therefore, from the physiological point of view these first hours are the most important. The last group (24 h of recovery) was selected to know whether activity of antioxidant enzymes returned to the basal levels after 24 h of recovery.

After re-submersion haemolymph samples from each animal were withdrawn from the ventral sinus via the arthropodial membrane at the base of the 4th pair of pereiopods using 1 mL disposable plastic syringes. Samples of 500 μL were transferred to pre-chilled 1.5 mL centrifuge tubes which contained 1 mL ice-cold Tris–HCl buffer (0.125 M, pH 6.8) to avoid haemolymph clotting. Samples were centrifuged for 10 min at 9000 ×*g* at 4 °C. The supernatant was collected and stored at −80 °C. After haemolymph sampling, crabs were dissected by removing the carapace, and the 7th gill, hepatopancreas and the muscular mass from the 4th pair of pereiopods were dissected and frozen at −80 °C until analysis.

2.2.1. Sample preparation

The homogenates were prepared using 0.3 g of gills or 0.1 g of muscles or hepatopancreas tissue in 1.2 or 1.4 mL of cold (4 °C) Tris–HCl buffer (0.125 M, pH 6.8), respectively. Samples were processed using a homogenizer, and immediately they were centrifuged for 15 min at 11,000 ×*g* at 4 °C. The supernatants were collected and employed as antioxidant enzyme source.

2.2.2. Biochemical analyses

Superoxide dismutase (SOD) was evaluated using the method of Misra and Fridovich (1972). SOD activity was measured by the inhibition on the auto-oxidation of epinephrine (2 mM, pH 2) in 50 mM glycine buffer (pH 10.2). The reaction was recorded 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 Aebi (1984). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 3 mM H_2O_2, and it was registered 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 by increasing 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), 1 mM GSH and 1 mM CDNB. 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 for 10 min at 5000 ×*g* and the supernatant was collected. Samples 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 total protein content was determined following the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.2.3. Statistical analyses

Data are presented as mean ± standard error. Analyses of variance (one-way ANOVA) were performed to determine air exposure and re-submersion effects on antioxidant enzymes. Data were checked for normality and homogeneity of variance by Kolmogorov-Smirnov and

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Levene tests, respectively (Sokal and Rohlf, 1995). Some data were ln-transformed to meet model assumptions. Significant differences ($p<0.05$) were compared using Tukey post hoc test (Sokal and Rohlf, 1995).

2.3. Experiment 2: Palatability assay

After the acclimation period, four groups of twenty crabs each were exposed to air at different times: 0 (control), 6, 12 and 18 h. We used different air exposure times because the idea was to simulate several hours of live crab transport followed by a re-immersion on the water for 24 h, which is a routinely used period after extended time transportation in other species (Chen et al., 2007). Crabs were placed in individual plastic boxes without water and with cloths fully soaked in seawater to maintain the air-humidity, and placed at 7 ± 0.5 °C air temperature. After the air exposure, animals were returned to the tanks for a re-submersion period of 24 h.

To obtain the meat for the palatability assay, animals were processed in a local factory (Pesquera del Beagle S.A.) following commercial standards. From each of the four treatments, portions of 10 g were offered to an experienced panel of 30 members. The judges involved in this assay were qualified chefs, sommeliers and local people (including scientific researchers) who frequently eat king crab, and were not professional tasters. Samples were placed on white ceramic dishes and coded with numbers 1 to 4, not related with the time of air exposure, so that judges could not associate the dish number with the treatment. Water and unsalted bread were allowed to be taken between sample tasting. Judges were asked to perform a ranking with the sample flavors giving the number one to the best taste sample.

2.3.1. Statistical analyses

A non-parametric Friedman ANOVA by ranks (Siegel, 1956) was used to test whether judges differentially selected the four meat flavors. A Kendall concordance coefficient (Kendall, 1948) was used to test the hypothesis that the meat flavor ranking was in agreement among participants more than that expected by chance. The range of Kendall concordance is from 0 to 1, where values close to 1 represent perfect agreement in the rankings of the meat flavor among judges.

3. Results

3.1. Antioxidant enzyme activities and lipid peroxidation level after air exposure and re-submersion times

Almost all analyzed tissues showed an increase of enzyme activities after air exposure and re-submersion. In all periods, the highest antioxidant enzyme activities were found in gills.

All analyzed tissues showed a similar pattern of SOD activity (Fig. 1; ANOVAs for each tissue $p<0.05$). A significant activity increase of this enzyme was observed during the first 2 h of re-submersion (Tukey, $p<0.05$ in all cases). This increase of SOD activity varied between 90% in gills and 40% in muscle after 30 min of re-submersion. After 24 h of re-submersion SOD activity was similar to control values in all analyzed tissues (Fig. 1; Tukey, $p>0.05$ in all cases).

The CAT activity varied differentially with air exposure and re-submersion times (ANOVAs, $p<0.05$ in all cases). All tissues showed a similar pattern of CAT activity (Fig. 1). Compared to muscle and gills, the activity of CAT was much lower in haemolymph and hepatopancreas. In all analyzed tissues, the response of CAT and SOD activities showed a similar pattern (Fig. 1). Animals that were air exposed followed by a re-submersion period presented a significant increase in CAT activity between control values and 30 min, 1 h and 2 h values in all tissues (Tukey, $p<0.05$; Fig. 1). Maximum values of CAT activity in gills and hepatopancreas peaked after 2 h of re-submersion and were ~50% higher than control values. In muscle and haemolymph the maximum CAT activity values were registered after 30 min of re-submersion and were close to 90 and 150% higher than control values, respectively. Similar to SOD activity, in all analyzed tissues CAT activity decreased to control values after 24 h of re-submersion (Tukey, $p>0.05$; Fig. 1).

We observed GST activity in all analyzed tissues (Fig. 1). The activity pattern of this enzyme was similar to those for SOD and CAT (Fig. 1; ANOVAs, $p<0.05$). Gill was the tissue with the highest GST activity (Fig. 1). Haemolymph presented the highest increases of GST activity and was ~200% after 30 min of re-submersion. Gills and muscles presented an increment of GST activity of about ~130 and ~92% after 1 h or 30 min of re-submersion, respectively (Tukey, $p<0.05$, Fig. 1). Similarly to SOD and CAT activities, GST in all tissues decreased to control values after 24 h of re-submersion (Fig. 1).

Lipid peroxidation remained constant in muscles throughout the time of the assay (ANOVA, $F=2.26$, $p=0.083$). However, gills and hepatopancreas showed a significantly higher lipid peroxidation related to air exposure and re-submersion (ANOVAs, $F=4.90$, $p=0.003$ and $F=4.44$, $p=0.006$, respectively; Table 1). For gills and hepatopancreas the maximum peaks appeared after 30 min of re-submersion, and were ~38 and ~50% higher than the control values, respectively (Table 1). The hepatopancreas presented the highest lipid peroxidation level, and was an order of magnitude higher than those registered in the other studied tissues.

3.2. Palatability assay

In each ranking category, *P. granulosa* meat quality as measured by its taste corresponded with the time of air exposure, e.g., >40% of the panel selected the meat from air-unexposed animals as the best one, or *ca.* 35% of the panel selected the meat from animals air-exposed for 18 h as the worst one (Fig. 2). There was a significant difference in the meat selection between judges (Friedman Test, $n=30$, $df=3$, $p<0.02$). About 27% of the tasters agreed to rank the four samples according to the increase of air exposure of crabs. However, the ranking of the meat flavor done by participants was not confident ($\text{Kendall}_{\text{coefficient of concordance}}=0.11$). Hence, a change in the meat flavor of *Paralomis granulosa* was not clearly and statistically detected after different times of air exposure and 24 h of recovery.

4. Discussion

The results presented here show the physiological strategies used by the subantarctic false king crab *P. granulosa* to deal with stress associated with extended air exposure. Results of this study demonstrate that male crabs exposed to air until 18 h followed by 24 h of recovery could be transported to markets where they can be offered alive. Moreover, flavor changes of crab meat due to the air-exposure and re-submersion seems not to be affected as judged by an experienced panel.

Aerobic organisms possess a baseline status of antioxidant systems to assure the maintenance of a balance between production and removal of endogenous ROS and other pro-oxidants (Correia et al., 2003). Particularly, the oxidative status of *P. granulosa* is significantly affected by emersion and re-submersion periods. The highest antioxidant enzyme activities found in gills of *P. granulosa* allow us to assume that this organ has an important role in antioxidant defenses. In many crustaceans, gills are responsible for exchanging gases, highly functional in maintaining osmotic balance and have an important role in the immune response (Burnett et al., 2006 and references therein). Usually, gills are exposed to higher oxygen concentrations compared to other tissues. This feature anticipates an increase in the rate of production of ROS (Zielinski and Pörtner, 2000), and a higher protection level in this organ would be necessary to protect animals from oxidative stress. Thus, enzyme antioxidant defenses of all these organisms are a complex adaptive system to preserve cellular functions and to prevent oxidative stress.

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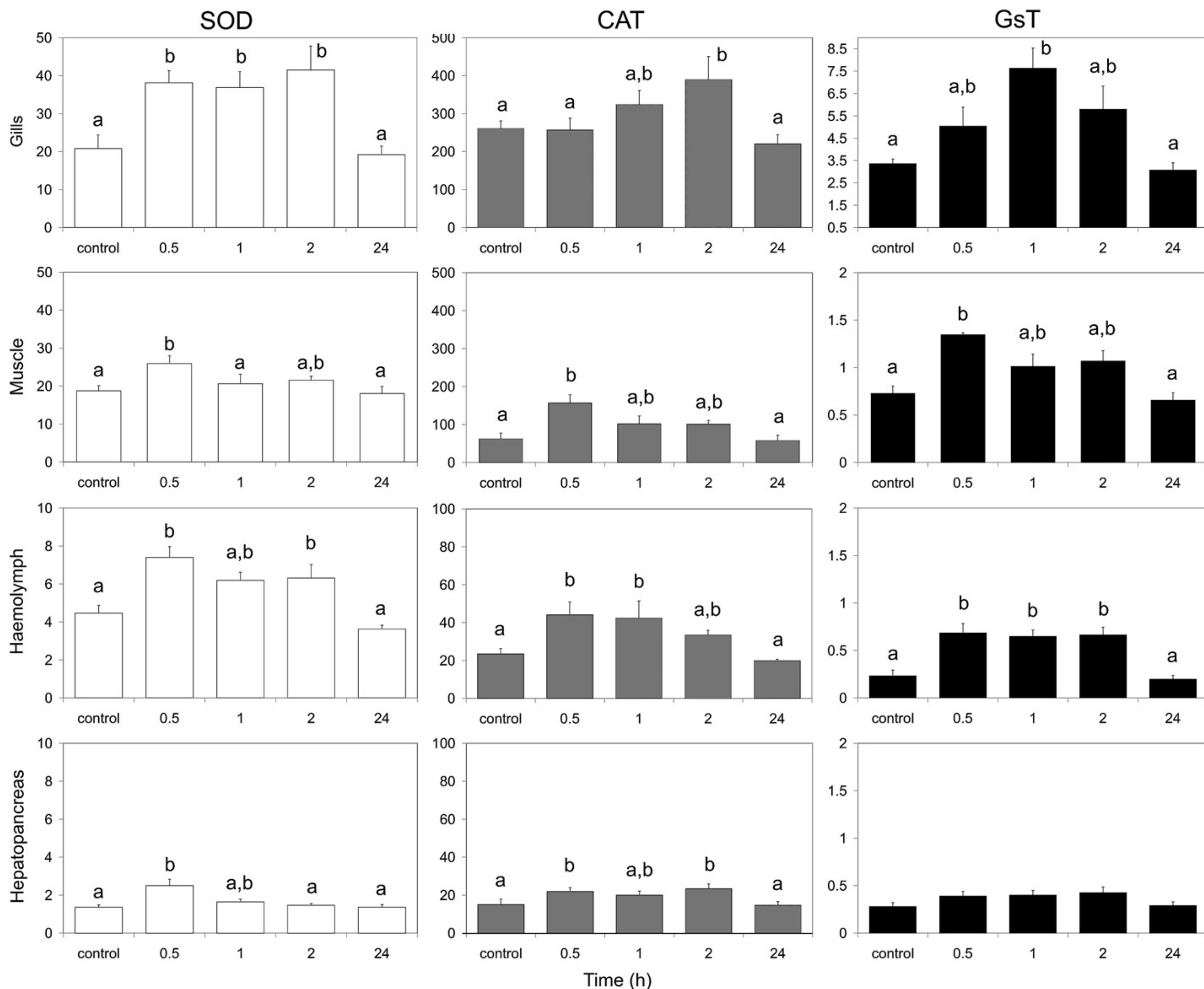

Fig. 1. *Paralomis granulosa*. Antioxidant enzyme activities of SOD (U SOD / mg protein), CAT (nmol / mg protein) and GST (mU GST / mg protein) during 6 h of air exposure and re-submersion in different tissues of False King Crab. Data are expressed as mean ± standard error of a sample of 8 animals. Different letters indicate significant differences (Tukey post hoc test, $p<0.05$). Note that ordinate scale of haemolymph, hepatopancreas and muscle is different than that of gills.

Almost all analyzed tissues in *P. granulosa* showed an increase of SOD, CAT and GST enzyme activities during the first 2 h of re-submersion. This fact could be probably related to the presence of augmented concentration of oxidant substrates like O_2^-, H_2O_2 and ROOH respectively, generated by excretion of final anaerobic products which are accumulated during the air exposure period. Furthermore, recent evidence showed that GST isoforms have also peroxidase activity (Almeida et al., 2005 and references therein). A similar pattern of increase in CAT and GST activities was found in gills of *Neohelice granulata* crab after 20 and 40 min of aerobic recovery (de Oliveira et al., 2005). The gastropod *Littorina littorea* showed a SOD and CAT activity increase in the recovery period from anoxia (Hermes-Lima and Zenteno-Savín, 2002). The subantartic limpet *Nacella (P.) deaurata* also experience increased SOD, CAT and GPx antioxidant enzyme activities between 30 min and 3 h of recovery after 5 h of anoxia (Malanga et al., 2007). The processes that lead to the production of ROS vary significantly related to changes in the environmental factors. Marine organisms have to adjust their antioxidant defenses in order to maintain the concentration of ROS formation at low levels. Thus, organisms prevent both cellular damage and oxidative stress (Lesser, 2006). Previous studies on *P. granulosa* showed an increase in the antioxidant activities during anoxia

Table 1
Paralomis granulosa. Lipid peroxidation levels in different tissues of False King Crab during air exposure and re-submersion. Data are expressed as mean ± standard error of a sample of 8 animals. Different letters indicate significant differences (Tukey post hoc test, $p<0.05$).

Lipid peroxidation (CHP eq/mg wet weight)					
Time (h)	0	0.5	1	2	24
Muscle	2.42 ± 0.23	2.10 ± 0.20	1.78 ± 0.10	2.00 ± 0.14	1.73 ± 0.21
Gills	1.60 ± 2.21^{a}	2.21 ± 0.16^{b}	1.93 ± 0.10^{a,b}	1.64 ± 0.08^{a}	1.68 ± 0.10^{a}
Hepatopancreas	23.81 ± 2.52^{a}	35.97 ± 2.70^{b}	22.87 ± 1.66^{a}	24.13 ± 3.84^{a}	24.28 ± 2.66^{a}

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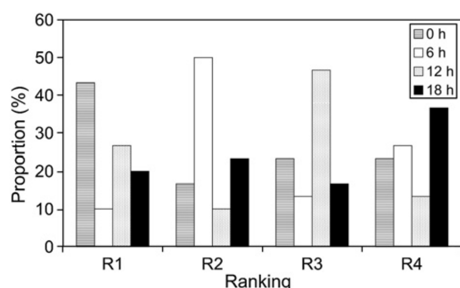

Fig. 2. *Paralomis granulosa*. Ranking of tasting the meat samples of False King Crab performed by experienced judges. Meat samples were from crabs air-exposed to three different periods. Data are expressed as percentage of judges that selected samples from R1 (sample with the best palatability) to R4 (sample with the worst palatability).

periods, as a strategy to protect the tissues against oxidative damage during the re-oxygenation (Romero et al., 2007). This process was named "preparation for oxidative stress" (Hermes-Lima et al., 1998) and it is used by several invertebrates and vertebrates species (Almeida et al., 2005; Hermes-Lima and Zenteno-Savín, 2002) helping tissues to be prepared to deal with oxyradicals (Storey, 1996).

Polyunsaturated fatty acids are vulnerable to oxidation due to their conjugated double bond structures (Storey, 1996). In meat products lipid oxidation is considered as the main factor responsible for the loss of quality (Nassu et al., 2003). Particularly in *P. granulosa*, lipid peroxidation after anoxia and re-submersion periods showed damage in gills and hepatopancreas, although this damage is quickly reverted once the animals return to water. Even if both the preparation for oxidative stress (Romero et al., 2007) and the increase of antioxidant enzymes during the recovery period are considered, they are not enough defenses to prevent the lipid peroxidation in tissues. It is known that *P. granulosa* experiences significantly increased lipid peroxidation in hepatopancreas between 6 and 24 h of air exposure (Romero et al., 2007). *Paralomis granulosa* has no means to keep water in the gill chamber, as occurs in brachyurans (c.f. Luquet and Ansaldo, 1997). Hence, during the air exposure of this lithodid crab the oxygen interchange at the gill level probably is minimum or nil. This pattern points to the need of oxygen to support metabolic processes related to oxidative damage repair (Almeida et al., 2005).

The initial freshness state of meat is gradually modified by a degradation processes, reducing its quality for human consumption. The general quality of frozen meat undergoes difference changes during post mortem time, even if it is stored on ice. This process was studied specially on fish meat, where loss of cell integrity (such as hyper acidophilic cytoplasm, indistinct margins and loss of cytoplasmic details) was found on the myofibres of the muscle after 10–14 days post mortem. Furthermore, other parameters as gumminess and chewiness, fracturing and hardness also declined significantly in the refrigerated post mortem muscle (Caballero et al., 2009). On the other hand, keeping alive animals with sea water in holding facilities until they are needed for cooking is a traditional guarantee of freshness (Barrento et al., 2008).

After an air exposure, animals could show an increment of ATP, pH (*Panulirus interruptus*, Márquez-Ríos et al., 2007), metabolic end products as ammonia, lactate and/or urate (*Homarus americanus*, Danford et al., 2001a), (*Callinectes sapidus*, *C. rathbunae*, *C. danae*, Danford et al., 2001b), haemolymph glucose concentration (*Jasus edwardsii*, Morris and Oliver, 1999; *Cancer Pagurus*, Barrento et al., 2010) and hyperglycaemic hormone titres (*Nephros norvegicus*, Lund et al., 2009), among others. All these physiological changes could be reverted once animals are returned to the aquarium. However, in many cases the stress suffered by animals is irreversible and conduce them to death. The tolerance to air exposure and the time needed to return to basal metabolism is known to be different among species. Parameters as temperature, salinity and/or seawater quality during air exposure and re-immersion, have a key role in the animal survival. For example, a winter harvest is suggested for several species (Barrento et al., 2008 and references therein; Giomi et al., 2008; Lund et al., 2009) in order to avoid losses due to deaths associated to high (summer) temperatures.

Nowadays, transporting of live shellfish as crab, lobster and prawn are a common practice in international trading (Danford et al., 2001b). Thus, in Southern South America, the possibility to offer live southern king crabs in both national and international markets would be an excellent trade alternative. However, more research on physiological requirements of *P. granulosa* is needed before starting with living transport systems on this species. Additionally, if this practice becomes common the effects of the selection of crabs by size on the population should be assessed to know whether the exploitation pressure on the resource affects its sustainability.

Acknowledgments

We are grateful to O. Florentin and G. Malanga for field and laboratory assistance. We also thank two anonymous reviewers for their helpful comments on the manuscript. Animals were supplied by the Pesquera del Beagle S.A. and Secretaría de Desarrollo Sustentable y Ambiente, Province of Tierra del Fuego. The palatability assay was performed during the gastronomic festival "Ushuaia a Fuego Lento III – 2008". Funds were provided by the Agencia de Promoción Científica y Tecnológica (MINCyT, PICTs 06-1230, 1308 and AR 07-004) and the CONICET (PIPs 0200, 0268 and 0335).

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