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Daily variations of the antioxidant defense system of the lithodid crab *Lithodes santolla*

Natasha Schvezov *, Gustavo A. Lovrich, Federico Tapella, M. Carolina Romero

Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET. Centro Austral de Investigaciones Científicas, CADIC, Houssay 200, V9410CAB Ushuaia, Tierra del Fuego, Argentina

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ABSTRACT

Several physiological processes can induce daily variations in aerobic metabolism. *Lithodes santolla* is a decapod crustacean of special concern since it is a sub-Antarctic species of commercial interest. The aim of this work was to study in *L. santolla* the daily variations in levels of enzymatic and non-enzymatic antioxidants, lipid peroxidation and protein oxidation, and haemolymphatic pH. Males of *L. santolla* of commercial size were randomly dissected every 4 h during a period of 24 h. Enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase and glutathione peroxidase were determined in samples of gills, muscle, hepatopancreas and haemolymph. Ascorbic acid, total glutathione, lipid peroxidation and protein oxidation were also determined in all tissues. Gills showed the highest enzymatic activity and hepatopancreas the highest concentration of non-enzymatic antioxidants. Maximum antioxidant activity was during the dark phase in gills and during the photophase in the haemolymph. Muscle showed significant daily variations, with peaks during the photophase and scotophase. Overall, an antioxidant protective mechanism is present in all tissues, where SOD and CAT represent the first line of defense. The defense mechanism in *L. santolla* seems to be more active during the dark phase, with slight differences among the analyzed tissues, indicating a higher metabolic rate.

1. Introduction

There are several physiological processes that induce daily variations in aerobic metabolism. Physiological parameters like oxygen consumption and locomotor activity vary rhythmically along the day/night cycle, and imply alterations in aerobic metabolism (Maciel et al., 2010). Of special importance in crustacean species are photoperiodically controlled phenomena in locomotor activity, moulting, and reproduction including the release of eggs or dispersal of larvae, not at last interesting for fisheries in terms of predictions of catches (Strauss and Dircksen, 2010). In the crab *Neohelice granulata* oxygen consumption was augmented at night in laboratory conditions, which coincided with the circadian rhythm of the locomotor activity reported for this species (Pereyra et al., 1996; Maciel et al., 2004).

Alteration in aerobic metabolism produces changes in radical oxygen species (ROS) production (Maciel et al., 2004) which affects macromolecules such as proteins, carbohydrates, nucleic acids and lipids (Kong et al., 2008). Organisms have developed mechanisms to defend against these ROS constituted by enzymatic and non-enzymatic molecules. Among the antioxidant enzymes are superoxide dismutase (SOD) that converts the superoxide anion (O^{2-}) to hydrogen peroxide (H_2O_2), catalase (CAT) that converts H_2O_2 to water, glutathione peroxidase (GPx) that reduces lipid hydroperoxides to their corresponding alcohols and free H_2O_2 to water, and glutathione-S-transferase (GST) that is involved in the biotransformation of numerous xenobiotic compounds. Furthermore, low molecular weight antioxidants such as reduced glutathione and vitamins C and E, act together with these enzymatic defenses (Halliwell and Gutteridge, 1999; Hermes-Lima, 2004).

Studies on daily changes in the antioxidant system have been done in vertebrate and invertebrate species. CAT activity in mouse has been studied, and showed the existence of time-related changes along a 24-h period (Sani et al., 2006). In rats daily variations in lipid peroxidation levels were correlated with daily variations in aerobic metabolism (Baydas et al., 2002). In contrast, Fanjul-Moles et al. (2003, 2009) who studied the glutathione system of the crayfish *Procambarus clarki*, demonstrated daily variations in the oxidative status of the animal. The involvement of rhythmicity in free radical formation, detoxification and, perhaps, also radical generation avoidance, in fact, suggests a particular adaptive value for programmed, anticipatory temporal organization (Hardeland et al., 2003).

Lithodes santolla, commonly known as Southern king crab, is a decapod crustacean of special concern since it is a sub-Antarctic species with a commercial interest. The fishery of *L. santolla* has developed south to 40°S since the 1930s, especially in the Beagle Channel and Straits of Magellan. As for the last few years, total annual landings for the Argentinean and Chilean fisheries have totaled ca. 64,000 t.

Abbreviations: SOD, Superoxide dismutase; CAT, Catalase; GPx, Glutathione peroxidase; GST, Glutathione-S-transferase; AA, Ascorbic acid; TG, Total glutathione; LPO, Lipid peroxidation; PO, Protein oxidation; ROS, Reactive Oxygen Species; HL, Haemolymph; Mc, Muscle; HP, Hepatopancreas.

* Corresponding author at: Bernardo Houssay 200, V9410CAB Ushuaia – Tierra del Fuego, Argentina. Tel.: +54 2901 422310, +54 2901 422314, +54 2901 422278x130; fax: +54 2901 430644.
E-mail address: nschvezov@cadic-conicet.gob.ar (N. Schvezov).

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Regulations impose that only males above 110 mm of carapace length can be retained and commercialized. To our knowledge, studies on daily variations of the antioxidant defense system have been done previously in crustaceans, only in species of Cambariidae (Fanjul-Moles and Prieto-Sagredo, 2003) and in the estuarine crab *N. granulata* (Maciel et al., 2004). Even though several studies have been done in different aspects of *L. santolla* (*e.g.* Vinuesa et al., 1990; Lovrich and Vinuesa, 1999; Lovrich et al., 2003; Thatje et al., 2003; Anger et al., 2004; Calcagno et al., 2005), only a few of them were focused on its physiological aspect (Comoglio and Amin, 2008; Paschke et al., 2009). Rhythmicity as an ecophysiological adaptation involves almost every facet of the crustacean biology and comprises developmental, physiological, sensory, and behavioural aspects (Strauss and Dircksen, 2010) that could eventually affect the biology. Commercial animals suffer different physiological changes due to air exposure or handling that could change the flesh quality (c.f. Romero et al., 2011). This article is part of a borader study on dry transportation of commercial crabs.

The aim of the present work was to analyze in *L. santolla* the daily variations in *i*) activities of the enzymes: superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx); and glutathione-S-transferase (GST); *ii*) concentration of non-enzymatic antioxidant molecules (ascorbic acid and glutathione); *iii*) concentration of oxidative damage parameters (lipid peroxidation and protein oxidation); and *iv*) haemolymphatic pH. Results of this study will help in understanding the physiological and biochemical basis of *L. santolla*, as well as providing additional contribution to the field of oxidative stress in decapod crustaceans.

2. Material and methods

2.1. Acclimation and assay condition

Male *L. santolla* were captured in Beagle Channel (ca. 55ºS; 68ºW) in August 2010 by baited commercial traps and transported to the laboratory for 3 weeks of acclimation. Animals were sorted into 6 groups of 8 animals each and kept in tanks. Animals were fed with fresh squid mantle and they were maintained in aquaria with filtered sea water at 6 ± 0.5 °C, pH 8 and under 12:12 h light cycles, with the onset of light at 8:00 h. Selected photoperiodic conditions resembled the natural period of L/D hours in the Beagle Channel during late August, and used as a standard throughout the literature. All crabs were in intermoult stage and of legal size (119 ± 5 mm of carapace length). One group of animals was dissected randomly every 4 h during a 24 h cycle.

Before dissection, samples of haemolymph were withdrawn from the ventral sinus via the arthrodial membrane at the base of the 3rd or 4th pair of pereiopods using 10 mL disposable plastic syringes. Two subsamples of 1 mL were transferred to pre-chilled 1.5 mL centrifuge tubes that contained 500 μL 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 Vijavayel et al. (2004), to avoid haemolymph clotting. The rest of haemolymph sample was used to analyze haemolymph pH in situ (THERMO pHMETER Model ALFA5, Fields Instrumental). After haemolymph sampling, the 7th gill, hepatopancreas and the muscular mass from the 4th pair of pereiopods were dissected and frozen at −80 °C. The chocie of the 7th gill and the 4th pair of pereiopods were selected to standardize the protocol and to make results more comparable.

2.2. Sample preparation

The homogenates were prepared using 0.3 g of gills or muscle or 0.15 g of hepatopancreas tissue in 1.5 mL of cold (4 °C) Tris–HCl buffer (0.125 M, pH 6.8). Samples were homogenized between 0 and 4 °C, and then they were centrifuged at 11,000 *g* at 4 °C for 15 min. The supernatants were collected and employed as antioxidant and oxidative damage source.

The enzymatic activity of superoxide dismutase (EC 1.15.1.1; SOD), catalase (EC 1.11.1.6; CAT), glutathione peroxidase (EC 1.11.1.9; GPx) and glutathione-S-transferase (EC 2.5.1.18; GST) and the antioxidants ascorbate (AA) and total glutathione (TG) were determined in each sample. Additionally, lipid peroxidation (LPO) and protein oxidation (PO) were determined as indicators of oxidative stress. All enzymatic analyses were expressed in relation to total proteins in the sample, and non-enzymatic analyses in relation to wet tissue weight or mL haemolymph.

2.3. Apparatus and reagents

All analyses were performed in a GBC Cintra 10e Spectrophotometer. Enzymes and compounds used to determine enzymatic activities were from Sigma, solvents and acids used were from Cicarelli and reagents used for buffers were from Anedra. All reagents were pro-analysis quality.

2.4. Antioxidant enzymes assays

The activity of CAT was determined according to the decrease in the concentration of hydrogen peroxide at 240 nm (Aebi, 1984). The reaction mixture contained 30 mM H_2O_2 and 50 mM phosphate buffer (pH 7.0). The activity of SOD was quantified measuring the inhibition of the reaction of the superoxide anion (O_2^-) with cytochrome c, which forms reduced cytochrome c and has a maximum of absorbance at 550 nm (McCord and Fridovich, 1969). The superoxide anion is generated by 50 μM xanthine and 6 nM xanthine oxidase, and reacts with 20 μM cytochrome c in a phosphate buffer (50 mM, pH 7.8, 0.1 mM EDTA). GST activity was measured by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2.4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). The reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 1 mM GSH and 1 mM CNDB. GPx activity was determined measuring the velocity by which 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) diminishes in a system containing 1 mM reduced glutathione (GSH), in the presence of glutathione reductase. The determination was done in a buffer containing 2 mM EDTA, 1 mM azide and 50 mM Tris–HCl, pH 7.6. The lectures were performed at 340 nm ($\varepsilon_{340} = 6{,}23\ mM^{-1}\ cm^{-1}$, Flohé and Gunzler, 1984). GPx activity in haemolymph and hepatopancreas could not be measured due to loss of sample. Enzymatic activities of GST, GPx and CAT were expressed as U mg protein^{-1}, where 1 U is equivalent to 1 μmol of substrate consumed per minute at 25 °C. One unit of SOD (USOD) was defined as the amount of enzyme inhibiting the oxidation of product by 50% at 30 °C.

2.5. Non-enzymatic antioxidant assays

The determination of total glutathione (TG) was based on the reaction generated by 5,5′-dithiobis-2 nitrobenzoic acid (DTNB), which resulted on oxidized glutathione (GSSG) and 2-nitrobenzoate (TNB), this last one having an absorbance at 412 nm. Previously, samples were deproteinized with perchloric acid 2 M. Results are expressed in nmol GSH · mg wet tissue^{-1} (Beutler et al., 1963). In hepatopancreas and haemolymph TG could not be analyzed because measurements were non detectable with the used technique.

Ascorbic acid (AA) was determined according to the method of Mitsui and Ohta (1961). Samples were deproteinized with TCA 5%, and mixed with sodium molybdate 0.66% p/V, sulfuric acid 25 mM and sodium phosphate 0.025 mM. This mixture was heated for 40 min at 60 °C, then centrifuged at 8000 *g* for 5 min, and measured at 660 nm. Ascorbic acid was used for the calibration curve, and data is presented as ng AA · g wet tissue^{-1}.

2.6. Lipid peroxidation and protein oxidation

Lipid peroxidation was determined by the method proposed by Ohkawa et al. (1979), based on the formation of a Shiff base after the reaction of thiobarbituric acid (TBA) with the secondary products of the lipid peroxidation measured at 535 nm. Data are expressed as μmol TBARS · g wet tissue^{-1}. LPO was determined in all tissues, except in haemolymph, due to non-detectable measurements with the used technique.

Protein oxidation (PO) was analyzed based on the reaction of 2,4-dinitrophenyl hydrazine (DNPH) with protein carbonyl groups to form hydrazones derivates. The content of carbonyl was quantified by its maximum absorbance at 375 nm using the extinction coefficient of 22,000 M^{-1} cm^{-1} (Reznick and Packer, 1994; Ansaldo et al., 2007), and expressed as nmol carbonyl · mg protein^{-1}.

Total protein concentration was estimated following Lowry method (Lowry et al., 1951), using bovine albumin as standard.

2.7. Data analysis

Analysis of variance (ANOVA) was performed to determine the effects of the 24 h cycle on each parameter considered. Normality and homogeneity were checked by Shapiro–Wilk and Levene and Brown–Forsythe tests (Sokal and Rohlf, 1995). Some data were transformed using the logarithm or square-root to accomplish with normality and homoscedasticity criteria. Significant differences ($p<0.05$) were compared by Tukey post hoc test. If after transformations data did not attain the ANOVA assumptions, they were analyzed with a Kruskal–Wallis test as a nonparametric one-way analysis of variance method. Data are presented as mean ± standard error.

3. Results

3.1. Gills

Gills presented the highest antioxidant enzyme activities and along the 24 h cycle SOD, CAT and GST activities varied significantly (Fig. 1; $F=2.89$, $p<0.05$, F 7.54, $p<0.001$ and $F=3.34$, $p<0.001$, respectively). Maximum SOD activity in gills was found during the dark cycle at 20 and 4 h, with 14 ± 3 and 12 ± 1 USOD mg protein^{-1} respectively. The lowest value was at the onset of the light phase, at 8 h (7 ± 1 USOD mg protein^{-1}; Fig. 1A). Peaks of CAT activity were also found at 20 h and 4 h (11.4 ± 0.8 and 9 ± 1 mUCAT mg protein^{-1}, respectively), whereas the minimum activity was observed at 0 h (4.1 ± 0.4 mUCAT mg protein^{-1}; Fig. 1B). GST activity peaked at 4 h (13 ± 2 UGST mg protein^{-1}) and had minimum activity at 8 h (4 ± 1 UGST mg protein^{-1}; Fig. 1C). GPx in gills was constant ($H=5.7$ and $p>0.05$, Fig. 1D) and presented mean value of $3.2 \pm 0.1 \times 10^{-3}$ UGPx mg protein^{-1}.

TG values were also similar along the 24 h cycle (Table 1; $H=4.92$, $p=0.43$), with mean concentration of 0.8 ± 0.1 nmol GSH mg wt^{-1}. AA values did not present significant differences during 24 h (Table 1; $F=2.09$, $p=0.09$).

Lipid peroxidation showed a significant daily variation (Table 2; $F=8.3$, $p<<0.001$), with a peak at 0 h. On the other hand, PO did not present significant differences in the analyzed period (Table 2; $F=1.51$, $p=0.21$).

3.2. Muscle

Muscle showed antioxidant enzyme activity, with significant variations in SOD ($F=11.9$, $p<0.001$) and CAT ($F=4.29$, $p<0.01$) during

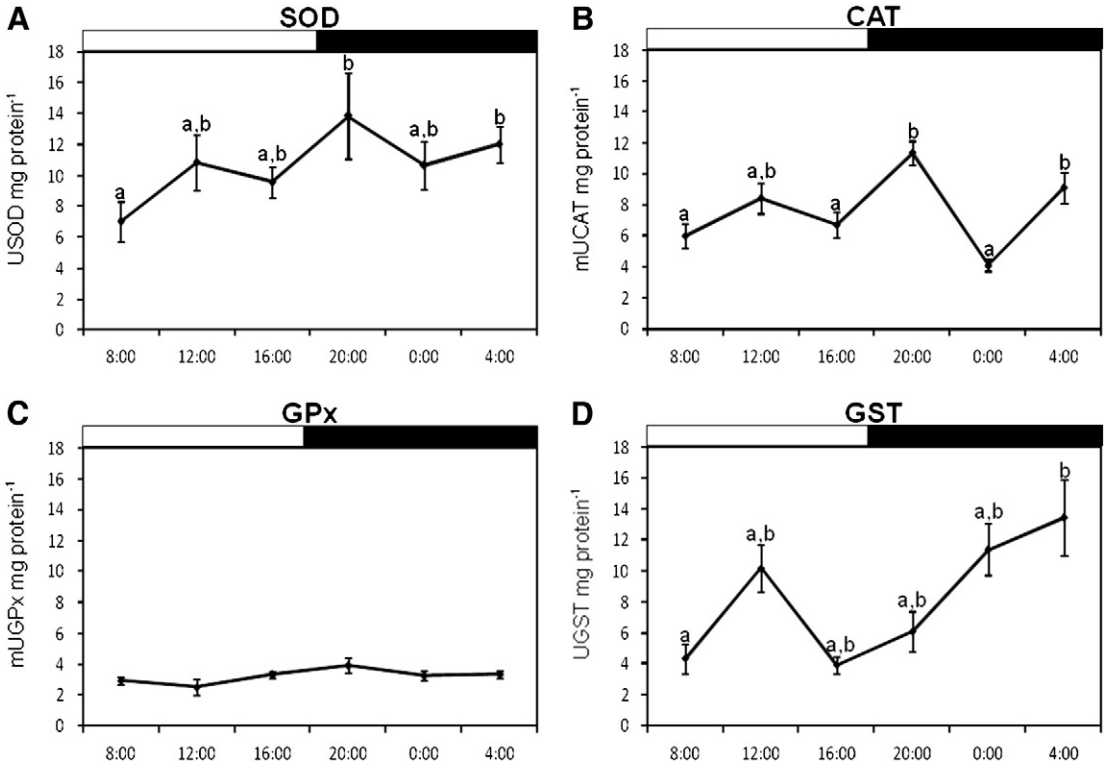

Fig. 1. Daily variations of CAT, SOD, GPx and GST in gills of *Lithodes santolla*. Data are expressed as mean ± standard error. Different letters indicate significant differences ($p<0.05$). Activity levels of: (A) SOD in SOD units USOD mg proteins^{-1}, (B) CAT in mUCAT mg protein^{-1}, (C) GPx in mUGPx mg protein^{-1}; and (D) GST in UGST mg proteins^{-1}. Open and solid bars indicate light and dark phases, respectively.

Table 1
Daily variations of ascorbic acid (AA) and total glutathione (TG) in *Lithodes santolla*. Data are expressed as mean ± standard error; AA values are presented as ng AA gwt^{-1} for gills, muscle and hepatopancreas, and as ng AA $mL\ HL^{-1}$ for haemolymph. TG is presented as nmol GSH $mgwt^{-1}$.

	Time (h)	Gills	Muscle	Haemolymph	Hepatopancreas
AA					
	8:00	85 ± 12	114 ± 7	3.3 ± 0.9	237.7 ± 0.6
	12:00	50 ± 6	102 ± 9	2.1 ± 0.4	209.3 ± 1.0
	16:00	60 ± 9	116 ± 3	2.9 ± 0.7	231.8 ± 0.4
	20:00	67 ± 9	98 ± 7	1.9 ± 0.2	279.8 ± 0.6
	0:00	88 ± 14	115 ± 7	2.8 ± 0.5	250.8 ± 0.7
	4:00	81 ± 10	121 ± 14	2.8 ± 0.6	328.6 ± 0.9
TG					
	8:00	0.8 ± 0.3	13 ± 2	–	–
	12:00	1.6 ± 0.5	14 ± 2	–	–
	16:00	0.4 ± 0.1	16 ± 2	–	–
	20:00	0.5 ± 0.2	14 ± 1	–	–
	0:00	0.4 ± 0.3	16 ± 2	–	–
	4:00	1.3 ± 0.5	14 ± 2	–	–

the 24 h cycle (Fig. 2). Peaks of SOD activity were observed at the onset of light and in the dark phase, and a minimum activity at noon (12 h, 1.3 ± 0.2 U SOD mg $protein^{-1}$. Fig. 2A). CAT showed a peak of enzymatic activity at 12 h (3.2 ± 0.3 mU CAT mg $protein^{-1}$), and a decrease at 0 and at the onset of light at 8 h (1.9 ± 0.2 and 2.0 ± 0.2 mU CAT mg $protein^{-1}$, respectively. Fig. 2B). GPx and GST were rather constant in the 24 h cycle ($F = 1.17$, $p > 0.05$ and $H = 4.85$, $p > 0.05$, respectively), with mean values of $2.8 \pm 0.9 \times 10^{-3}$ U GPx mg $protein^{-1}$ and 2.8 ± 0.1 U GST mg $protein^{-1}$ (Fig. 2C and D).

Ascorbic acid was also present in muscles, with 110 ± 4 ng AA gwt^{-1} of mean concentration, with no significant differences in its concentration along the 24 h cycle ($F = 1.01$, $p = 0.43$; Table 1). As in gills, TG did not present a daily variation ($F = 0.36$, $p = 0.87$), and a mean concentration of 14.6 ± 0.7 nmol GSH $mgwt^{-1}$ was observed (Table 1).

Oxidative damage as lipid peroxidation and protein oxidation was recorded in muscle. Only LPO presented significant variation during 24 h ($F = 10.76$, $p < 0.001$), with a peak of concentration at 8 h, whereas PO values were similar ($F = 1.11$, p 0.37; Table 2).

3.3. Haemolymph

The pH of haemolymph presented significant variations along the day, ($F = 5.60$, $p < 0.001$), lower pH was observed during the first hours of the photophase and then increased throughout the studied period (Table 3).

Table 2
Daily variations in lipid peroxidation (LPO — μmol TBARS gwt^{-1}) and protein oxidation (PO — nmol carbonyl mg $protein^{-1}$) analyzed in tissues of *Lithodes santolla*. Data are expressed as mean ± standard error. Different letters indicate significant differences ($p < 0.05$).

	Time (h)	Gills	Muscle	Haemolymph	Hepatopancreas
LPO					
	8:00	15 ± 2^{a}	7.4 ± 0.5^{a}	–	70 ± 6
	12:00	14 ± 1^{a}	$5.1 \pm 0.4^{b,c}$	–	77 ± 7
	16:00	12 ± 1^{a}	$5.4 \pm 0.2^{a,c}$	–	74 ± 5
	20:00	10 ± 1^{a}	4.1 ± 0.4^{b}	–	70 ± 5
	0:00	28 ± 3^{b}	5.5 ± 0.4^{a}	–	75 ± 3
	4:00	14 ± 2^{a}	7.2 ± 0.5^{a}	–	76 ± 4
PO					
	8:00	0.37 ± 0.08	0.17 ± 0.01	0.31 ± 0.03^{a}	0.48 ± 0.07
	12:00	0.40 ± 0.09	0.23 ± 0.03	$0.53 \pm 0.03^{a,b}$	0.41 ± 0.05
	16:00	0.31 ± 0.07	0.16 ± 0.01	$0.64 \pm 0.07^{a,b}$	0.26 ± 0.02
	20:00	0.28 ± 0.06	0.16 ± 0.02	0.70 ± 0.08^{b}	0.41 ± 0.06
	0:00	0.22 ± 0.05	0.19 ± 0.03	0.44 ± 0.03^{a}	0.39 ± 0.03
	4:00	0.20 ± 0.03	0.19 ± 0.02	0.48 ± 0.02^{a}	0.41 ± 0.05

Enzymatic activities were observed also in this tissue (Fig. 3). Although SOD activity in the haemolymph was similar along the 24 h cycle (Fig. 3A: $F = 2.24$, $p = 0.07$), a peak of activity was observed at 12 h. Furthermore, this enzyme presented 4.5 ± 0.4 USOD mg $protein^{-1}$ of mean activity. CAT and GST activities presented significant differences ($F = 7.97$, $p < 0.001$ and $H = 22.2$, $p < 0.001$, respectively) along the 24 h cycle. Peaks of CAT activity were observed during the photophase, and in the scotophase at 0 h (Fig. 3B). GST activity was the highest at 12 and 20 h (Fig. 3C, 0.52 ± 0.05 and 0.59 ± 0.08 U GST mg $protein^{-1}$, respectively), whereas the minimum activity was registered at 8 h (0.26 ± 0.01 U GST mg $protein^{-1}$. Fig. 3C).

Haemolymph showed an average AA concentration of 2.7 ± 0.2 ng AA mL^{-1}, and this value was similar during the 24 h cycle (Table 1; $F = 0.41$, $p = 0.84$). PO values were significantly different (Table 3; $F = 7.2$, $p < 0.001$), with maximum concentrations of carbonyl at 16 and 20 h.

3.4. Hepatopancreas

Even though this organ presented enzymatic activity of SOD, CAT and GST, no significant differences during the 24 h cycle were found (Fig. 4; $F = 1.53$ $p = 0.20$; $F = 1.1$, $p = 0.37$ and $F = 1.62$, $p > 0.05$, for each enzyme respectively). The mean enzymatic activities during the period studied was 8.3 ± 0.3 U SOD mg $protein^{-1}$, 2.2 ± 0.1 mU CAT mg $protein^{-1}$ and 0.77 ± 0.06 U GST mg $protein^{-1}$, for SOD, CAT and GST, respectively.

This organ presented an AA mean concentration of 258 ± 12 ng AA gwt^{-1}, with no significant differences during the 24 h cycle (Table 2; $H = 6.44$, $p = 0.27$). Furthermore, LPO and PO levels were similar throughout the 24 h ($F = 0.35$, $p = 0.88$ and $H = 7.69$, $p = 0.17$, respectively), with a mean concentrations of 74 ± 2 TBARS gwt^{-1} and 0.39 ± 0.02 nmol carbonyl mg $protein^{-1}$ for LPO and PO, respectively.

4. Discussion

L. santolla showed variations in its antioxidant defense system during a 24 h cycle with differences among tissues studied. This is the first study showing daily variations in the oxidative metabolism of a sub-Antarctic species that inhabits the subtidal realm.

Particularly, gills of *L. santolla* presented the highest enzymatic antioxidant activities, specifically of CAT, SOD and GST. In crabs, gills are primarily responsible for respiration, acid–base balance and osmotic and ionic regulation (Paital and Chainy, 2010). The generation of ROS in this organ is presumed to be high since oxygen diffuses through the gill cells (Maciel et al., 2004). Therefore, gills exhibit a low threshold response to oxidative stress, and provide a first line of antioxidant defense since this organ is the first one to come into contact with any changes occurring in the water (e.g. oxygen concentration, temperature, contaminants, etc., Borković et al., 2008; Romero et al., 2011).

In aerobic organisms, the antioxidant defense system works in a concerted way. Superoxide dismutase is one of the first enzymes to respond in the presence of ROS (Hermes-Lima, 2004), but high levels of SOD do not necessarily improve the efficiency of the antioxidant defense system. Thus, rises in SOD activity will only be effective if H_2O_2 is eliminated at a sufficiently effective rate (Hardeland et al., 2000). This action can be accomplished by a simultaneous activity of CAT or GPx, which is the case observed in gills of *L. santolla* where SOD and CAT activities show a similar profile. Specifically, all antioxidant enzymes increase their activity in gills at 20 h, which coincides with the minimum in oxidative damage found. At the initial dark phase, LPO increases, whereas later in the dark phase LPO levels diminished following the increase of CAT and GST activities. In general, the antioxidant defense system in gills was more active during the dark phase, showing peaks of enzymatic activity and non-enzymatic antioxidants, which indicate a higher metabolic activity during the night probably due to an increase in oxygen uptake. Likewise, in gills of the

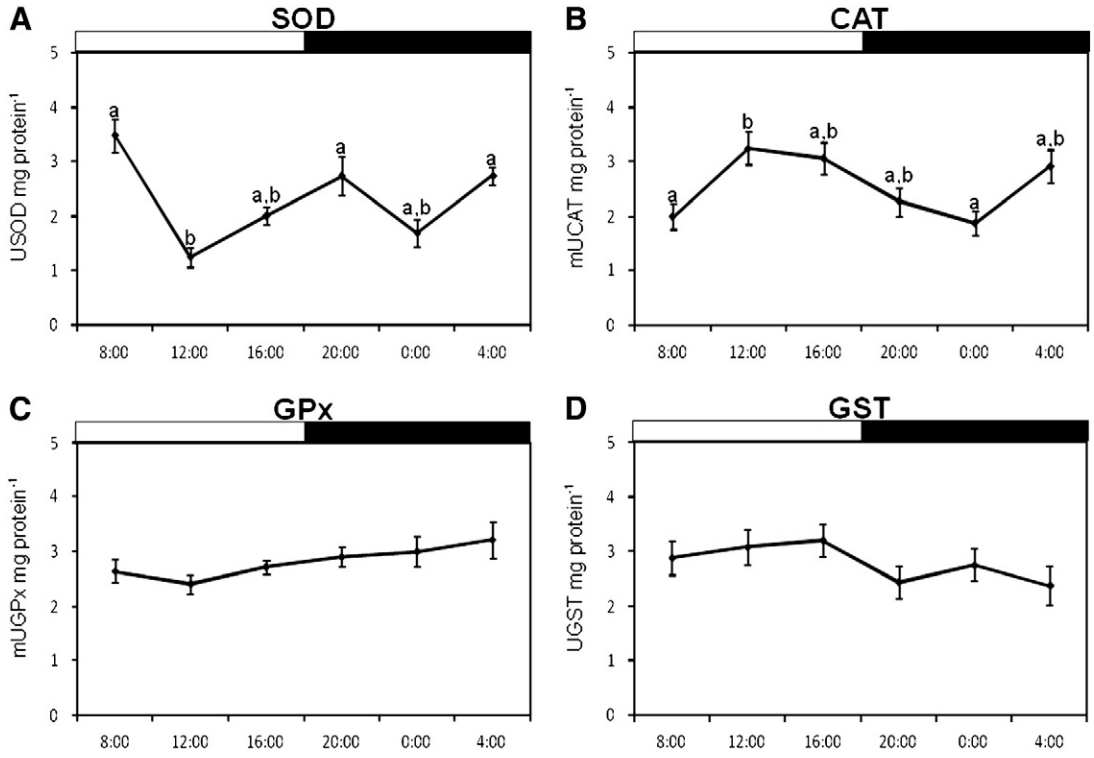

Fig. 2. Daily variations of CAT, SOD, GPx and GST in muscle of *Lithodes santolla*. Data are expressed as mean ± standard error. Different letters indicate significant differences ($p<0.05$). Activity levels of: (A) SOD in SOD units USOD mg proteins^{-1}, (B) CAT in mUCAT mg protein^{-1}, (C) GPx in mUGPx mg protein^{-1}; and (D) GST in UGST mg proteins^{-1}. Open and solid bars indicate light and dark phases, respectively.

crab *N. granulata* higher GST activity and non-proteic sulfhydryl concentration was found during night, which was related to higher oxygen consumption (Maciel et al., 2004).

The enzymatic antioxidant defense system in the hepatopancreas of *L. santolla* was lower than in gills. Levels of lipid peroxidation in the hepatopancreas were high, compared to gills, muscle and haemolymph of *L. santolla*. It is known that the crustacean hepatopancreas is a major organ mainly associated with diverse metabolic activities, ranging from digestion and absorption to nurture the ovary (Paital and Chainy, 2010) and can be considered metabolically more active than gills and muscle tissue. Furthermore, in our study *L. santolla* showed a constant antioxidant defense against the production of ROS, since it is a site of maximal free radical generation due to the multiple oxidative reactions that take place herein (Arun and Subramanian, 1998; Romero et al., 2007). *Paralomis granulosa*, another lithodid crab that inhabits the same waters as *L. santolla*, shows a similar profile in the enzymatic antioxidant defense, since CAT and SOD activity in hepatopancreas was lower than in gills and muscle, and presented the highest lipid peroxidation values (Romero et al., 2007). Romero et al. (2007) proposed the presence of other non-enzymatic antioxidants present in this organ. Particularly, in *L. santolla* the highest concentration of ascorbic acid was found in the hepatopancreas. Ascorbic acid, apart from the metabolic functions, it is a potent non-enzymatic antioxidant defense, able to recycle vitamin E, scavenge ROS and inhibit lipid peroxidation and oxyradical mediated damage to proteins and DNA (Hermes-Lima, 2004). This fact suggests that in this organ the non-enzymatic defense system participates actively against the production of ROS. Total glutathione is also considered a non-enzymatic antioxidant usually found in crustacean species (Dandapat et al., 2003; Elia et al., 2006; Hoguet and Key, 2007), and although it could not be detected in hepatopnacreas of *L. santolla* probably due to concentrations lower than the detection limit, we do not discard the presence of it.

The antioxidant defense system in muscles of *L. santolla* varies along the 24 h period as well as in other tissues. The oxidative damage could have been originated by the action of high concentrations of H_2O_2, since during the dark phase there is an increase of SOD activity and a reduction of CAT activity, coinciding with high lipid peroxidation. The time at which the increment of CAT activity occurs, corresponds to a minimum lipid peroxidation. Furthermore, periodic rhythms found in all enzymatic antioxidants in muscle of *L. santolla* show a concerted mechanism of defense as well, where peaks of activities are found during both the photophase and scotophase. Our results suggest that the high enzymatic activity in muscles principally before the photophase begins, and the production of lipid peroxidation during the night may be due to locomotor activity. Muscular exercise results in an increase of oxygen consumptions and eventually in a raise of ROS production (Lawler and Powers, 1998; Geihs et al., 2010).

From cellular studies and analyses performed at the level of whole organism, arguments in favor of the role of pH in haemolymph in changing the activity of metabolic pathways have been developed (Forgue et al., 2001). Particularly, the increment in pH observed in the haemolymph of *L. santolla* during the night could indicate a higher metabolic rate due to an increment of oxygen uptake and diminishment of pCO_2 in haemolymph. In *Astacus leptodactylus*, blood pO_2 changes occurring during the circadian rhythm are associated with a statistically

Table 3
Lithodes santolla pH in haemolymph. Data are expressed as mean ± standard error. Different letters indicate significant differences for the null hypothesis of equality of mean pH ($p<0.05$).

Time (h)	pH
8:00	7.46 ± 0.04^{a}
12:00	$7.69 \pm 0.03^{a,b}$
16:00	$7.70 \pm 0.08^{a,b}$
20:00	7.93 ± 0.07^{b}
0:00	8.00 ± 0.11^{b}
4:00	$7.75 \pm 0.12^{a,b}$

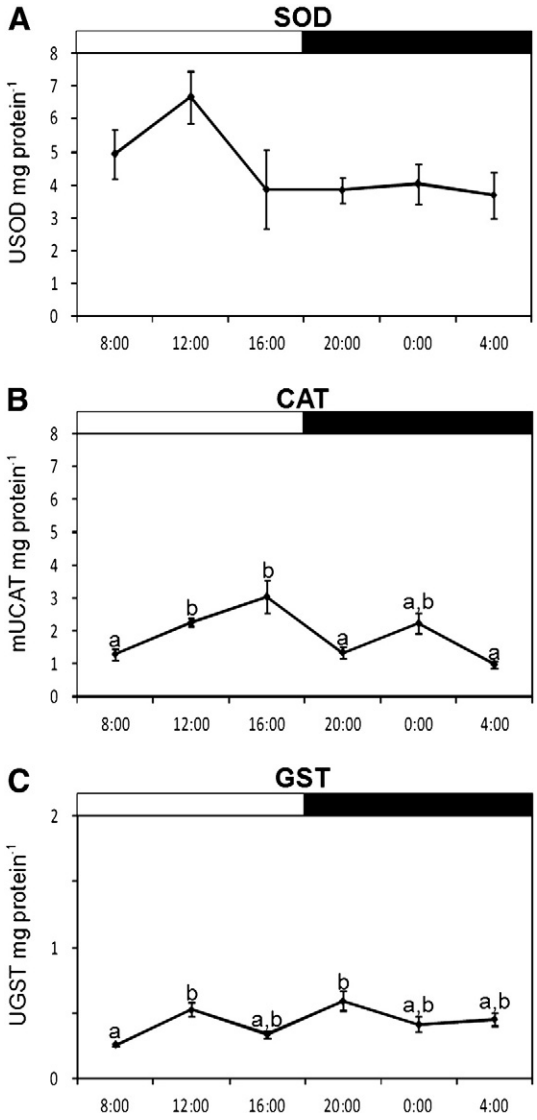

Fig. 3. Daily variations of CAT, SOD, and GST in haemolymph of *Lithodes santolla*. Data are expressed as mean ± standard error. Different letters indicate significant differences ($p<0.05$). Activity levels of: (A) SOD in SOD units USOD mg proteins^{-1}, (B) CAT in mUCAT mg protein^{-1}, and (C) GST in UGST mg proteins^{-1}. Open and solid bars indicate light and dark phases, respectively.

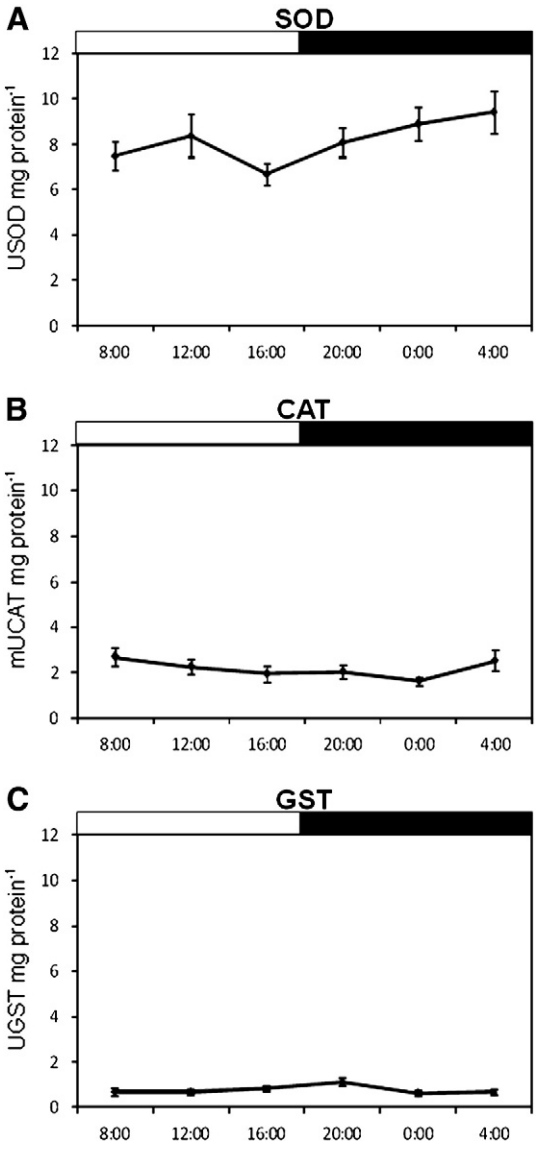

Fig. 4. Daily variations of CAT, SOD, and GST in hepatopancreas of *Lithodes santolla*. Data are expressed as mean ± standard error. Different letters indicate significant differences ($p<0.05$). Activity levels of: (A) SOD in SOD units USOD mg proteins^{-1}, (B) CAT in mUCAT mg protein^{-1}, and (C) GST in UGST mg proteins^{-1}. Open and solid bars indicate light and dark phases, respectively.

significant daytime hypercapnic acidosis and a night-time hypocapnic alkalosis (the blood pCO_2 change is approximately 0.1 kPa and the blood pH change is 0.07–0.1 irrespective of the water pO_2 between 10 and 30 kPa; Sakakibara et al., 1987). The increase in oxygen consumption and activity at night has been observed in other subtidal decapods (Crear and Forteath, 2000 and references therein).

Along the 24 h cycle, during the photophase, the antioxidant defense system in haemolymph of *L. santolla* is active, probably as a method to be prepared for the increase in metabolic rate that occurs later. Although, during the night oxidative damage occurs, observed by the peak of protein oxidation at 0 hs, when the antioxidant defense system is low.

Overall, an antioxidant defense mechanism in *L. santolla* occurs in all tissues, where SOD and CAT are working as the first line of defense. GPx activity, although with no significant differences found in this work, is of fundamental relevance for safeguarding cell homeostasis when there is an overproduction of H_2O_2 (Hermes-Lima, 2004). GST activity, on the other hand, was different along the 24 h cycle in all tissues except hepatopancreas, indicating an important role in the defense mechanism of *L. santolla*. Particularly, GST is a phase II enzyme catalyzing GSH conjugation with several molecules, including LPO, and it is considered to ameliorate the oxidative damage (Maciel et al., 2004). The enzymatic defense system seems to be efficient in *L. santolla*, with no need of a strong increase of non-enzymatic antioxidants, such as glutathione and ascorbic acid measured in this work. We take into account that other non-enzymatic antioxidants may be acting as well, such as melatonin, which can inhibit lipid peroxidation and neutralize several ROS, and is also involved in the regulation of the circadian cycle (Hardeland et al., 2000, 2003; Hermes-Lima, 2004).

In conclusion, *L. santolla* has an antioxidant defense system in gills, muscle, hepatopancreas and heamolymph operating coordinately, with variations during a 24 h cycle. To our knowledge, this is the first study of daily variations in the antioxidant defense system of a sub-Antarctic crustacean. The defense mechanism in *L. santolla* seems to be working actively during the dark phase due to a higher metabolic rate. The increase in metabolic rate could be due to an increment in oxygen uptake and locomotor activity, causing the production of ROS, which needs to be verified in future works.

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References

Aebi, H., 1984. Catalase *In vitro*. Methods Enzymol. 105, 121–126.
Anger, K., Lovrich, G.A., Thatje, S., Calcagno, J.A., 2004. Larval and early juvenile development of *Lithodes santolla* (Molina, 1782) (Decapoda: Anomura: Lithodidae) reared at different temperatures in the laboratory. J. Exp. Mar. Biol. Ecol. 306, 217–230.
Ansaldo, M., Sacristán, H., Wider, E., 2007. Does starvation influence the antioxidant status of the digestive gland of *Nacella concinna* in experimental conditions? Comp. Biochem. Physiol. C 146, 118–123.
Arun, S., Subramanian, P., 1998. Antioxidant enzymes in freshwater prawn *Macrobrachium malcolmsonii* during embryonic and larval development. Comp. Biochem. Physiol. B 121, 273–277.
Baydas, G., Gursu, M.F., Yilmaz, S., Canpolat, S., Yasar, A., Cikim, G., Canatan, H., 2002. Daily rhythm of glutathione peroxidase activity, lipid peroxidation and glutathione levels in tissues of pinealectomized rats. Neurosci. Lett. 323, 195–198.
Beutler, E., Durán, O., Kelly, B.M., 1963. Improved method for determination of blood glutathione. J. Lab. Clin. Med. 61, 882–890.
Borković, S.S., Pavlović, S.Z., Kovacevic, T.B., Stajn, A.S., Petrovic, V.M., Saicić, Z.S., 2008. Antioxidant defense enzyme activities in hepatopancreas, gills and muscle of Spiny cheek crayfish (*Orconectes limosus*) from the River Danube. Comp. Biochem. Physiol. C 147, 122–128.
Calcagno, J.A., Lovrich, G.A., Thatje, S., Nettelmann, U., Anger, K., 2005. Growth of first juvenile stages of *Lithodes santolla* and *Paralomis granulosa* at different temperatures. J. Sea Res. 54, 221–230.
Comoglio, L., Amin, O.A., 2008. Starvation effects on physiological parameters and biochemical composition of the hepatopancreas of the southern king crab *Lithodes santolla* (Molina, 1782). Rev. Biol. Mar. Oceanogr. 43, 345–353.
Crear, B., Forteath, G.N.R., 2000. The effect of extrinsic and intrinsic factors on oxygen consumption by the southern rock lobster, *Jasus edwardsii*. J. Exp. Mar. Biol. Ecol. 252, 129–147.
Dandapat, J., Chainy, G.B.N., Rao, K.J., 2003. Lipid peroxidation and antioxidant defence status during larval development and metamorphosis of giant prawn, *Macrobrachium rosenbergii*. Comp. Biochem. Physiol. C 135, 221–233.
Elia, A.C., Dörr, A.J., Mastrangelo, C., Prearo, M., Abete, M.C., 2006. Glutahtione and antioxidant enzymes in the hepatopancreas of crayfish *Procambarus clarkii* (Girard, 1852) of lake Trasimeno (Italy). Bull. Fr. Pêche. Piscicult. 380 (1351-1351).
Fanjul-Moles, M.L., Prieto-Sagredo, J., 2003. The circadian system of crayfish: a developmental approach. Microsc. Res. Tech. 60, 291–301.
Fanjul-Moles, M.L., Durán-Lizarraga, M.E., Gonsebatt, M.E., Prieto-Sagredo, J., 2003. The effect of photoperiod and light irradiance on the antioxidant circadian system of two species of crayfish from different latitudes: *Procambarus clarkii* and *P. digueti*. Photochem. Photobiol. 77, 210–218.
Fanjul-Moles, M.L., Prieto-Sagredo, J., López, D.S., Bartolo-Orozco, R., Cruz-Rosas, H., 2009. Crayfish *Procambarus clarkii* retina and nervous system exhibit antioxidant circadian rhythms coupled with metabolic and luminous daily cycles. Photochem. Photobiol. 85, 78–87.
Flohé, L., Gunzler, W.A., 1984. Assays of glutathione peroxidase. Methods Enzymol. 5, 114–121.
Forgue, J., Legeay, A., Massabuau, J.C., 2001. Is the resting rate of oxygen consumption of locomotos muscles in *Crustacea limites* by the low blood oxygenation strategy? J. Exp. Biol. 204, 933–940.
Geihs, M.A., Alves Vargas, M., Maciel, F.E., Caldas, S.S., Cruz, B.P., Primel, E.G., Monserrat, J.M., Nery, L.E.M., 2010. Effect of melatonin in the antioxidant defense system in the locomotor muscles of estuarin crab *Neohelice granulata* (Decapoda, Brachyura). Gen. Comp. Endocrinol. 166, 72–82.
Habig, W., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-S-transferase. The first step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Halliwell, B., Gutteridge, J.M.C., 1999. Free radicals, other reactive species and disease, Free Radicals in Biology and Medicine, Third ed. Oxford University Press, pp. 617–783.
Hardeland, R., Coto-Montes, A., Burkhardt, S., Zsizsik, B.K., 2000. Circadian rhythms and oxidative stress in non-vertebrate organisms. In: Driessche, T.V. (Ed.), The Redox State and Circadian Rhythms. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121–140.
Hardeland, R., Coto-Montes, A., Poeggeler, B., 2003. Circadian rhythms, oxidative stress, and antioxidant defense mechanisms. Chronobiol. Int. 20, 921–962.
Hermes-Lima, M., 2004. Oxygen in biology and biochemistry: role of free radicals. In: Storey, K.B. (Ed.), Functional Metabolism: Regulation and Adaptation. John Wiley and Sons, Inc., New York, pp. 319–368.
Hoguet, J., Key, P., 2007. Activities of biomarkers in multiple life stages of the model crustacean, *Palaemonetes pugio*. J. Exp. Mar. Biol. Ecol. 353, 235–244.
Kong, X., Wang, G., Li, S., 2008. Seasonal variations of ATPase activity and antioxidant defenses in gills of the mud crab *Scylla serrata* (Crustacea, Decapoda). Mar. Biol. 154, 269–276.
Lawler, J.M., Powers, S.K., 1998. Oxidative stress, antioxidant status and the contracting diaphragm. Can. J. Appl. Physiol. 23, 23–55.
Lovrich, G.A., Vinuesa, J.H., 1999. Reproductive potential of the lithodids *Lithodes santolla* and *Paralomis granulosa* in the Beagle Channel. Argentina. Sci. Mar. 63, 355–360.
Lovrich, G.A., Thatje, S., Calcagno, J.A., Anger, K., Kaffenberger, A., 2003. Changes in biomass and chemical composition during lecithotrophic larval development of the Southern king crab *Lithodes santolla* (Molina). J. Exp. Mar. Biol. Ecol. 288, 65–79.
Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Maciel, F.E., Rosa, C.E., Santos, E.A., Monserrat, J.M., Nery, L.E.M., 2004. Daily variations in oxygen consumption, antioxidant defenses, and lipid peroxidation in the gills and hepatopancreas of an estuarine crab. Can. J. Zool. 82, 1871–1877.
Maciel, F.E., Geihs, M.A., Monserrat, J.M., Nery, L.E.M., 2010. Antioxidant defense system rhythms in crustaceans and possible role for melatonin. Front. Biosci. E2, 1448–1459.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.
Mitsui, A., Ohta, T., 1961. Photooxidative consumption and photoreductive formation of ascorbic acid in green leaves. Plant Cell Physiol. 2, 31–44.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Asssay of lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Paital, B., Chainy, G.B.N., 2010. Antioxidant defenses and oxidative stress parameters in tissues of mud crab (*Scylla serrata*) with reference to changing salinity. Comp. Biochem. Physiol. C 151, 142–151.
Paschke, K., Cumillaf, J.P., Loyola, S., Gebauer, P., Urbina, M., Chimal, M.E., Pascual, C., Rosas, C., 2009. Effect of dissolved oxygen level on respiratory metabolism, nutritional physiology, and immune condition of southern king crab *Lithodes santolla* (Molina, 1782) (Decapoda, Lithodidae). Mar. Biol. 157, 7–18.
Pereyra, P., De la Iglesia, H.O., Maldonado, H., 1996. Training-to-testing intervals different from 24 h impair habituation in the crab Chasmagnathus. Physiol. Behav. 59, 19–25.
Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 233, 357–363.
Romero, M.C., Ansaldo, M., Lovrich, G.A., 2007. Effect of aerial exposure on the antioxidant status in the subantarctic ston crab *Parolomis granulosa* (Decapoda: Anomura). Comp. Biochem. Physiol. C 146, 54–59.
Romero, M.C., Tapella, F., Sotelano, M.P., Ansaldo, M., Lovrich, G.A., 2011. Oxidative stress in the subantarctic false king crab *Paralomis granulosa* during air exposure and subsequent re-submersion. Aquaculture 319, 205–210.
Sakakibara, Y., Burtin, B., Massabuau, J.C., 1987. Circadian rhythm of extracellular pH in crayfish at different levels of oxygenation. Respir. Physiol. 69, 359–367.
Sani, M., Sebaï, H., Gadacha, W., Boughattas, N.A., Reinberg, A., Mossadok, B.A., 2006. Catalase activity and rhythmic patterns in mouse brain, kidney and liver. Comp. Biochem. Physiol. B 145, 331–337.
Sokal, R.R., Rohlf, F.J., 1995. Biometry. The Principles and Practice of Statistics in Biological Research, 3rd ed. W.H. Freeman and Co., NY.
Strauss, J., Dircksen, H., 2010. Circadian clocks in crustaceans: identified neuronal and cellular systems. Front. Biosci. 15, 1040–1074.
Thatje, S., Calcagno, J.A., Lovrich, G.A., Sartoris, F.J., Anger, K., 2003. Extended hatching rhythms in the Subantarctic lithodid crabs *Lithodes santolla* and *Paralomis granulosa* (Crustacea: Decapoda: Lithodidae). Helgol. Mar. Res. 57, 110–113.
Vijavayel, K., Gomathi, R.D., Durgabhavani, K., Balasubramanian, M.P., 2004. Sublethal effect of naphtalene on lipid peroxidation and antioxidant status in the edible crab *Scylla serrata*. Mar. Pollut. Bull. 48, 429–433.
Vinuesa, J.H., Comoglio, L., Lovrich, G.A., 1990. Growth of immature southern king crab *Lithodes santolla* in the Beagle Channel. Proc. Int. Symp. King Tanner Crabs 90–0, 259–271.