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Baseline defense system of commercial male king crab *Lithodes santolla* from the Beagle Channel

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ABSTRACT

Environmental and physiological variations influence the steady-state concentration of free oxygen radicals in cells. Because of the seasonal life cycle of *Lithodes santolla* in the Beagle Channel, a baseline study of the antioxidant physiological variations along the seasons is necessary for a better understanding of its ecophysiology. The aim of this study was to evaluate the seasonal variations in gills, hemolymph, muscle and hepatopancreas of the: i) enzymatic activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione transferase; ii) ascorbic acid and total glutathione; iii) lipid peroxidation and protein oxidation; iv) glucose, proteins and pH. Seasonality found in the antioxidant defense system of *L. santolla* from the Beagle Channel acts in a collaborative way during the most relevant life cycle phases (reproduction and molting), avoiding a long term oxidative stress. The antioxidant system also shows changes in the enzymatic activities likely caused by the environmental factors, such as low temperatures during winter and spring seasons.

1. Introduction

At high latitudes, as ectothermal animals, crustaceans are prone to annual fluctuations in metabolic rates, locomotor activity, and growth rates induced by environmental parameters such as photoperiod, temperature, and food availability (Pörtner, 2002; Fanjul-Moles and Prieto-Sagredo, 2003; Malanga et al., 2009; Palma-Anzures et al., 2012). In addition to variations mediated by external factors, physiological events such as reproduction and molting can change feeding and growth cycles (Geracitano et al., 2004).

Marine organisms adjust to a wide variety of environmental factors on different temporal and spatial scales, such as oscillations in the environmental temperature and photoperiod (Filho et al., 2001; Borković et al., 2008). Just like any other metabolic pathway, the processes that lead to the production of radical oxygen species (ROS) vary significantly over large gradients in many environmental and metabolic factors, and adjustments in antioxidant defenses are required in order to maintain the steady-state concentration of ROS at low levels and thus prevent oxidative stress and cellular damage (Lesser, 2006). The antioxidant defense system is constituted of antioxidant enzymes such as superoxide dismutase (SOD) converting the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), catalase (CAT) that converts H_2O_2 to water, glutathione peroxidase (GPx) which reduces lipid hydroperoxides to their corresponding alcohols and free H_2O_2 to water, and the phase II detoxification enzyme, glutathione transferase (GST) which is involved in the biotransformation of numerous xenobiotic compounds. Together with these enzymatic defenses, reduced glutathione (GSH) and vitamins C and E occurring inside the cell are low molecular weight antioxidants (Halliwell and Gutteridget, 1984; Hermes-Lima, 2004). Generation of ROS, oxidation rates and antioxidant status related to the environmental temperature and the metabolic activity (Filho et al., 2001) have been previously shown for mussels (Borković et al., 2008), snails (Despotovic et al., 2012), fishes (Da Rocha et al., 2009), crabs (Elia et al., 2006), limpets (Malanga et al., 2005) and sea urchins (Malanga et al., 2009). Particularly in crabs, environmental and physiological variations cause changes in free radical production (Paschke et al., 2010), that could cause oxidative damage and eventually produce changes in the taste of meat or even death (Romero et al., 2011).

Lithodid crabs are particularly numerous at high latitudes, where seasonality is the chief source of biological variations. Abundant populations of several lithodid species have sustained profitable fisheries in both hemispheres and most of the scientific knowledge was motivated by their fishery management demands (Zaklan, 2002). Particularly *Lithodes santolla* distributes in the coastal waters of the southern tip of South America, between 45°S and 55°S. This is a typical sub-Antarctic species with an annual life cycle: mating occurs during November–December, females bear eggs for approximately 10 months, larvae hatch during September and males molt during April–May (see review by Lovrich and Tapella, 2014). During the last decade fisheries for *L. santolla* in both Chile and Argentina have yielded about 6000–

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10,000 tons per year. Management is based on the extraction of only the males above legal size, which in the Beagle Channel is 110 mm of carapace length (CL). Even though the available board of information for this species is substantial (Lovrich and Tapella, 2014), physiological studies were focused on juveniles (e.g. Paschke et al., 2010, 2013), but those dealing on commercial males are still scarce (e.g. Comoglio et al., 2008; Romero et al., 2013). The Beagle Channel (ca. 55°S 67°W) is a typical sub-Antarctic coastal environment with a marked seasonality. Surface temperature oscillates between approximately 4.3 °C and 9.8 °C in August and January, respectively (Balestrini et al., 1998). Surface salinity is highly variable, ranging from 15 to 20 psu in November–March to 31.5 psu in June, depending on the precipitation regime and distance to the river discharges (Balestrini et al., 1998; Curelovich et al., 2009; Aguirre et al., 2012).

Because of the seasonal life cycle, a baseline study of the physiological variations of king crabs along the seasons is necessary for further studies, as for example eco-toxicological studies or decision taking in the fishery management. This work evaluates the physiology in male adults of *L. santolla*, specifically its oxidative and antioxidant status. The aim of this study was to evaluate the seasonal variations of the: i) enzymatic activities of the antioxidant defense system (such as SOD, CAT, GPx and GST); ii) water-soluble non-enzymatic antioxidant molecules (ascorbic acid — AA and GSH); iii) oxidative damage parameters (lipid peroxidation — LPO and protein oxidation — PO); and iv) other parameters as glucose, proteins and pH. This investigation will lead to have a better understanding of the ecophysiology of this species.

2. Material and methods

2.1. Sampling

Captures of 10 to 15 males of *L. santolla* were done in September 2010, December 2010 (mating season), March 2011 (before molting season) and July 2011 each, with baited commercial traps and immediately transported to laboratory. To avoid any handling effect, animals were acclimated for at least 24 h in aquaria with filtered seawater at temperature and photoperiod according to the season. All crabs were of legal size, and averaged 119 ± 5 mm CL. Since crabs were captured by a fisherman we did not record the in situ temperatures and considered a temporal series used as standard for the Beagle Channel (Fig. 1). Moreover, sampling was done only for one year because the fishery is collapsed, commercial males are scarce and difficult to obtain.

Before dissection, samples of hemolymph were withdrawn from the ventral sinus via the arthrodial membrane at the base of the 4th or 5th 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 hemolymph clotting. The rest of the sample was used to analyze hemolymph pH in situ. After the hemolymph sampling, the 7th gill, the hepatopancreas and the muscular mass from the 4th pair of pereiopods were dissected, immediately frozen at −20 °C, and lyophilized within a week and kept dry until analysis.

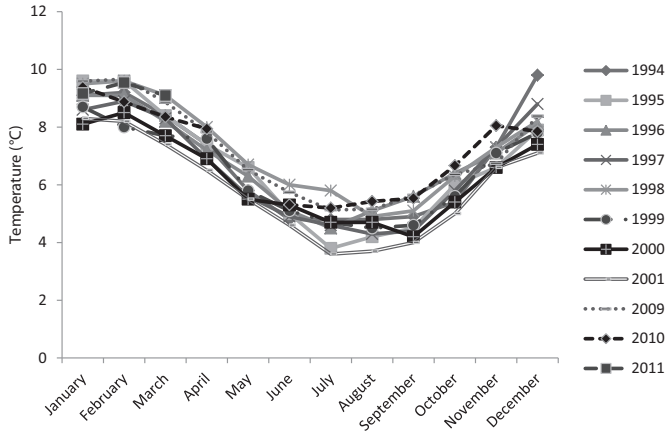

Fig. 1. Water temperature in the Beagle Channel from 1994 to 2001, and 2009–2011. Data sources: Servicio Centralizado de Documentación — CADIC (1994–2001) and Curelovich, 2013 (2009–2011).

2.2. Sample preparation

The homogenates were prepared using a relation wet tissue:buffer of 1:6 for gills, muscle and hemolymph, and 1:11 for hepatopancreas, using cold Tris–HCl (pH 6.8, 125 mM Tris, 1 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride — PMSF) as buffer. 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 an antioxidant source. The enzymatic activity of SOD, CAT, GPx and GST and the antioxidants AA and GSH were determined in each sample. Additionally, LPO and PO were determined as indicators of oxidative damage. 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 of hemolymph.

2.3. Antioxidant enzymes assays

The activity of SOD was quantified by measuring the inhibition of the reaction of superoxide anion 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 ethylenediaminetetraacetic acid — EDTA). 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). GPx activity was determined by measuring the velocity by which 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) diminishes in a system that contains 1 mM GSH, in the presence of glutathione reductase and using H_2O_2 as substrate. The determination is done in a buffer containing 2 mM EDTA, 1 mM azide and 50 mM Tris–HCl, pH 7.6. The lectures were done at 340 nm ($\varepsilon_{340} = 6.23\ mM^{-1}\ cm^{-1}$ — Flohé and Gunzler, 1984). GPx activity in hemolymph and hepatopancreas could not be measured due to loss of samples. GST activity was measured by monitoring the rate of conjugation of 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. Enzymatic activities of CAT, GST, and GPx were expressed as U mg protein^{-1}, where 1 U is equivalent to 1 μmol of substrate consumed or formed product per minute. One unit of SOD (USOD) was defined as the quantity of enzyme able to inhibit by 50% the superoxide-related reduction of cytochrome c.

2.4. Non-enzymatic antioxidants assays

Low molecular weight thiols were determined by the reaction generated by 5,5′-dithiobis-2 nitrobenzoic acid (DTNB). This reaction results in oxidized glutathione (GSSG) and 2-nitrobenzoate (TNB); this last one has an absorbance at 412 nm. Previously, samples were deproteinized with perchloric acid 2 M. Since glutathione is the major low molecular weight thiol in eukaryotes, we use this technique as a proxy of GSH levels (Lushchak et al., 2005). Results are expressed in nmol GSH mg wet tissue^{-1} (Beutler et al., 1963). GSH could not be analyzed in hepatopancreas and hemolymph because measurements were non detectable with this technique.

Concentration of AA was determined according to the method of Mitsui and Ohta (1961). Samples were deproteinized with trichloroacetic acid 5%, and mixed with sodium molibdate 0.66% p/V, sulfuric acid

0.025 M 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. AA was used for the calibration curve, and data is presented as ng AA g wet tissue^{-1}.

2.5. Total protein and glucose

Total protein concentration was estimated following the Lowry method (Lowry et al., 1951), using bovine albumin as standard. Glucose was determined with a commercial kit (Weiner Lab), using glucose as standard.

2.6. Lipid peroxidation and protein oxidation

The LPO was determined by the method proposed by Ohkawa et al. (1979), based on the formation of a Shiff base from the union of thiobarbituric acid (TBA) with the secondary products of the LPO measured at 535 nm. Data is expressed as μmol TBARS g wet tissue^{-1}. LPO was determined in all tissues, except hemolymph, due to non detectable measurements with this technique.

Oxidative damage in protein was analyzed based on the reaction of 2,4-dinitrophenyl hydrazine (DNPH) with protein carbonyls to form protein hydrazone derivatives. The content of carbonyl is quantified by its maximum absorbance at 375 nm using the extinction coefficient of 22,000 M^{-1} cm^{-1} (Reznick and Packer, 1994), and expressed as nmol carbonyl mg protein^{-1}. PO was determined in all tissues.

2.7. Data analysis

All analyses were done in R Core Team (2012). Normality and homogeneity of data were checked by Shapiro–Wilk and Levene tests (Sokal and Rohlf, 1995). Since most data were non-normal and heteroscedastic, Kruskal–Wallis was used as a nonparametric analysis of variance method. When ANOVA assumptions were met this analysis was used to determine the effects of the season on each studied parameter. 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 after ANOVA, or by multiple comparison test after Kruskal–Wallis. Data are presented as mean ± standard deviation of 10–15 samples, depending on the tissue and physiological parameter.

3. Results

3.1. Antioxidant enzymes

Enzymatic activity was found in all analyzed tissues of *L. santolla*. SOD activity varied along the year in gills ($H = 14.85$, $p < 0.01$), hemolymph ($H = 23.57$, $p < 0.001$) and muscle ($H = 43.03$, $p < 0.001$; Fig. 1). A similar activity pattern was observed in gills and hemolymph, in which a low activity of SOD occurred during December (Fig. 2). SOD activity in muscle, on the other hand, presented its highest activity during December and July (Fig. 2). In hepatopancreas, SOD activity was invariant ($H = 6.13$, $p > 0.05$), with a mean value of 9.3 ± 3.4 USOD mg protein^{-1}.

All tissues presented CAT activity with seasonal differences (Fig. 3). Particularly, during September a higher CAT activity was observed in gills ($H = 15.89$, $p < 0.01$) and muscle ($H = 21.22$, $p < 0.001$), opposite to hepatopancreas ($H = 18.32$, $p < 0.001$) which presented the lowest CAT activity at this time of the year (Fig. 3). In December, only in gills a significant decrease of CAT activity was observed, coincident with an increase of its activity in hepatopancreas. This latter organ maintained a high CAT activity in March as well, contrary to hemolymph ($H = 11.54$, $p < 0.01$) and muscle, which showed a decreased activity of CAT. During July the muscle still presented a low CAT activity, and only in hemolymph CAT activity increased (Fig. 3).

The enzymatic activity of GPx presented significant differences only in muscle ($H = 9.06$, $p < 0.05$ — Fig. 4). In this tissue, GPx activity was the lowest in September, yet it did not show significant differences with the values observed in December and March. Throughout the year GPx in all other tissues showed mean values of 0.06 ± 0.02 ($H = 7.46$, $p > 0.05$), 6.6 ± 2.3 ($F = 0.76$, $p > 0.05$) and 7.3 ± 2.8 ($F = 2.74$, $p > 0.05$) mUGPx mg protein^{-1} in gills, hemolymph and hepatopancreas, respectively (Fig. 4).

GST activity in *L. santolla* was present in all analyzed tissues, with seasonal differences in gills ($H = 9.74$, $p < 0.05$), hemolymph ($H = 21.72$, $p < 0.001$) and muscle ($H = 9.33$, $p < 0.01$ — Fig. 3 5). The pattern of GST activity differed among tissues, with low activities in general in September. Hepatopancreas showed a constant activity of 0.90 ± 0.56 UGST mg protein^{-1} along the year ($H = 3.99$, $p > 0.05$ — Fig. 5).

3.2. Non-enzymatic antioxidants

During September and December gills had concentrations of GSH two orders of magnitude lower compared to the rest of the year

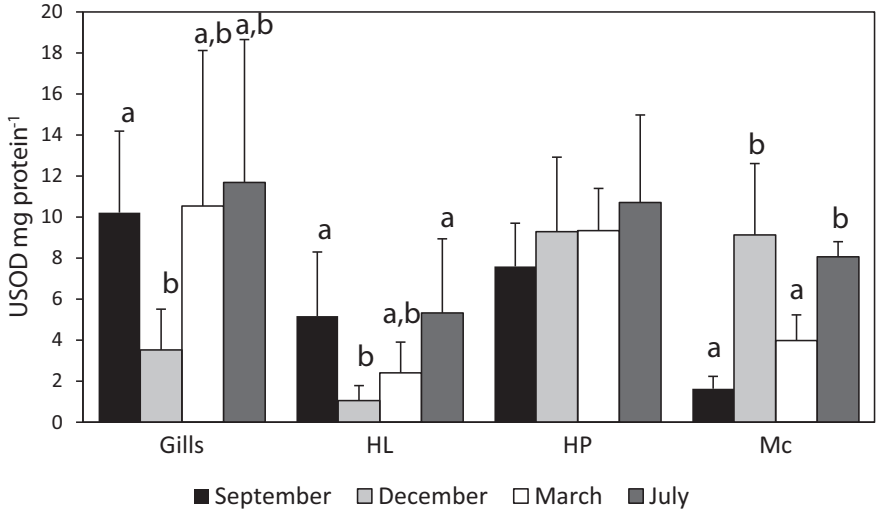

Fig. 2. Superoxide Dismutase (SOD) activities in gills, hemolymph (HL), hepatopancreas (HP) and muscle (Mc) of male *Lithodes santolla*. Activities are expressed as USOD mg protein^{-1}, mean ± SD. Different letters indicate significant differences between seasons.

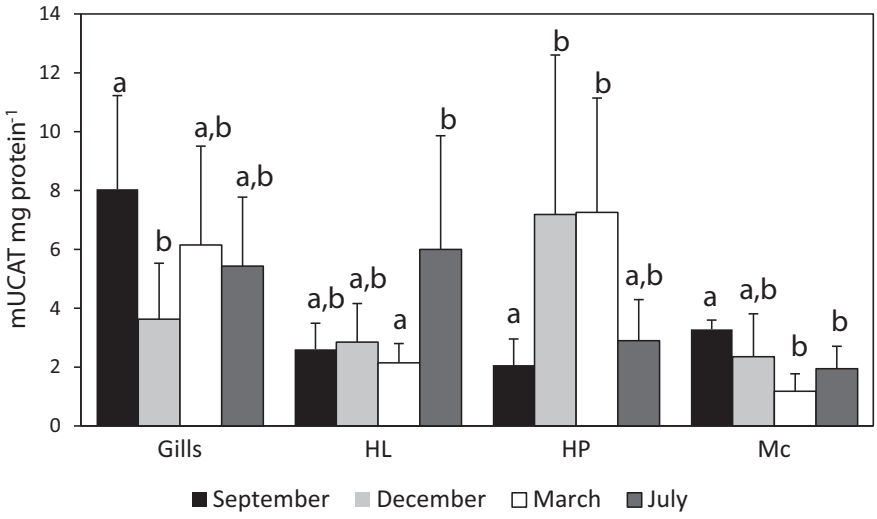

Fig. 3. Catalase (CAT) activities in gills, hemolymph (HL), hepatopancreas (HP) and muscle (Mc) of male *Lithodes santolla*. Activities are expressed as mUCAT mg protein^{-1}, mean ± SD. Different letters indicate significant differences between seasons.

(H = 42.1, p < 0.001 — Table 1). In muscle, GSH concentration was the lowest in September (H = 42.09, p < 0.001; Table 1).

The AA was the only non-enzymatic antioxidant that could be determined in hemolymph and hepatopancreas, since GSH could not be detected with the used technique. Muscle was the tissue with the highest content of AA along the studied year (Table 2). Seasonal variations of AA among tissues differed, yet in September a low concentration of AA was registered in all tissues (Table 2).

3.3. Protein, glucose and pH

Along the year protein and glucose presented significant differences in all tissues (Table 3). Particularly in gills, the lowest concentrations of glucose and proteins were found in September; whereas in July protein concentration lowered, differing with glucose which presents a high concentration in that month (Table 3). In hemolymph low concentrations of glucose and protein were also found during July and September (F_{gluc} = 18.92, F_{prot} = 33.59, p < 0.001 in both cases; Table 3). Hepatopancreas showed the highest concentration of glucose, compared to the other studied tissues (Table 3). Glucose content increased in this organ during December and March (H = 40.27, p < 0.001 — Table 3), whereas protein content was highest during September (H = 17.94, p < 0.001), although it did not differ significantly from December. In muscle, glucose concentration was minimum in July (H = 26.5, p < 0.001). Protein concentration in muscle was also minimum in July (F = 8.2, p < 0.001 — Table 3), yet not differing significantly from December and March. The pH in hemolymph presented significant variations along the year (H = 41.89, p < 0.01), with the highest values recorded in September and December (Table 4).

3.4. Oxidative damage

Oxidative damage, determined as LPO and PO, showed differences throughout the year in all analyzed tissues (Table 5). LPO was highest in the hepatopancreas. On a seasonal basis, LPO was minimum during September in hepatopancreas (H = 35.7, p < 0.001) and muscle (H = 28.8, p < 0.001), and in gills the minimum occurred in July (H = 24.09, p < 0.001). In all tissues analyzed, protein damage was consistently the lowest in July (Table 5).

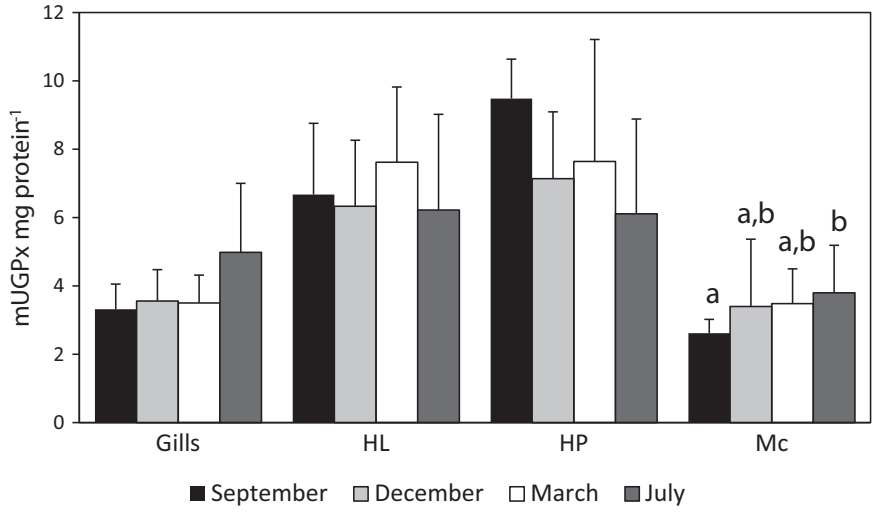

Fig. 4. Glutathione peroxidase (GPx) activities in gills, hemolymph (HL), hepatopancreas (HP) and muscle (Mc) of male *Lithodes santolla*. Activities are expressed as mUGPx mg protein^{-1}, mean ± SD. Different letters indicate significant differences between seasons.

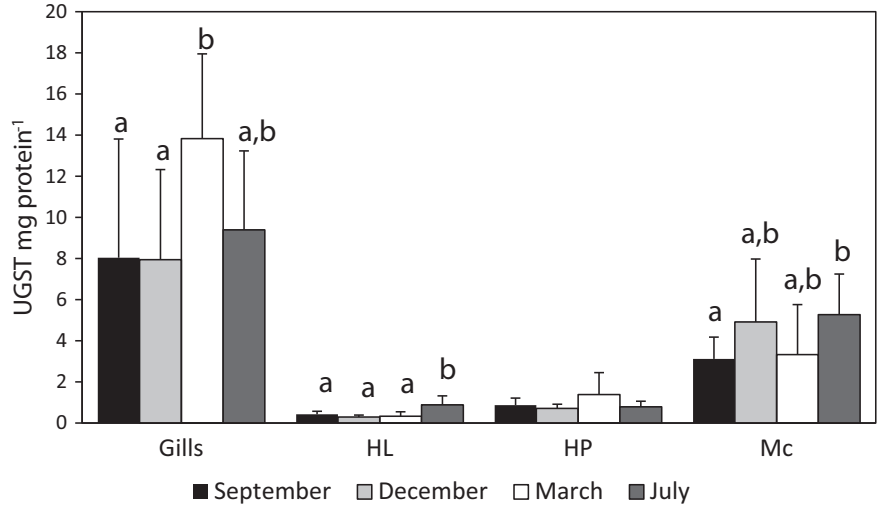

Fig. 5. Glutathione-S-transferase (GST) activities in gills, hemolymph (HL), hepatopancreas (HP) and muscle (Mc) of male *Lithodes santolla*. Activities are expressed as mUCAT mg protein^{-1}, mean ± SD. Different letters indicate significant differences between seasons.

4. Discussion

4.1. Enzymatic antioxidant defense system

The enzymatic activity of the different studied tissues of male *L. santolla* was seasonal. In our work, the enzymatic antioxidant defense system is acting in a concerted way against the overproduction of ROS during the most relevant life cycle phases of a crustacean, as reproduction and molting. The antioxidant system also shows higher activities likely caused by the environmental factors, such as low temperatures during winter and spring seasons.

The increased enzymatic activities of SOD and CAT in gills were observed at the same time of the year and the decrease of activities of both enzymes was during December. SOD is an enzyme that can have protective and regulatory functions in the cell. First, the combination of oxygen and SOD can effectively eliminate other radicals from the system, with superoxide acting as a radical sink (Winterbourn, 1993). Second, SOD is part of a metabolic pathway that could influence the redox state of the cell and regulate functions that are under the redox control (Koppenol, 1993; Winterbourn, 1993). The removal of H_2O_2 generated during the dismutation of superoxide anion is produced by CAT and GPx, which act as hydrogen peroxide scavengers. This action, in which SOD and CAT act in a concerted way, is observed in gills of *L. santolla*.

In crabs, the gill is a sensitive organ, since it is the first contact with any change in the water, besides being the main tissue in which oxygen diffuses and ROS production is presumed to be the maximum (Borković et al., 2008; Maciel et al., 2010). Although the activities observed in March and July were not different, the strong differences between September and December may be indicating the influence of temperature in the enzymatic activities of SOD and CAT in gills. In the Beagle Channel, temperature has values of approximately 5 °C from June to September, and then increases to average temperatures near 9 °C in December–March (Curelovich et al., 2009; Duarte et al., 2011; Giarratano et al., 2011). Since we attribute physiological changes to seasonal temperature changes of about 4 °C, we are confident that any inter-annual change in monthly temperatures did not influence our results. Moreover, surface salinity is particularly locally variable and therefore its direct effects on the defense system are difficult to assess from our study.

Oxidative stress induced by low temperatures occurs in shrimps *Macrobrachium nipponense* (Wang et al., 2006), *Litopeneus vannamei* (Qiu et al., 2011), in zebrafish *Danio rerio* (Malek et al., 2004) and the barnacle *Balanus balanoides* (Niyogi et al., 2001). There are two speculative reasons leading to this: i) a temperature decrease weakens the system of ROS elimination, since temperature can directly affect the activity of enzymes by changing their physical structure and thereby changing their catalytic efficiency or binding capacity (Cailleaud et al., 2007), and/or ii) enhancing of ROS production (Lushchak, 2011). The mechanism involved is still not clear, yet in gills of *L. santolla* the first line of enzymatic defense (SOD and CAT) is active during low temperature seasons, which may be a consequence of the possible overproduction of ROS. Alternatively, this intensification of the enzymatic defense can be part of an annual cycle as a "preparation" for the temperature increment occurring in spring, in late September/October, that can cause oxidative stress. Environmental parameters account for metabolic depression generating a hypometabolic state (Alves de Almeida and Di Mascio, 2011), in which the organism may respond as in a hypoxic state towards changes of salinity (Freire et al., 2011) or temperature (Frederich and Pörtner, 2000). During this hypometabolic state, oxygen consumption is diminished with the subsequent ROS production. In several vertebrates and invertebrates, during this state there is a production of ROS (Welker et al., 2013), and the antioxidant defense system is strongly activated (Hermes-Lima and Zenteno-Savín, 2002; Lushchak, 2011). Therefore, the animal's antioxidant defense system stays activated and is ready for the over-generation of ROS produced once the oxygen consumption is normalized (Hermes-Lima et al., 1998).

Table 1
Reduced glutathione (GSH) concentration in male *Lithodes santolla*. Data are expressed as mean ± SD; TG is presented as nmol GSH mgwt^{-1} in gills and muscle (Mc). Different letters indicate significant differences between seasons.

GSH	Gills	Mc
September	$(9 \pm 1) \times 10^{-4a}$	0.015 ± 0.004^{a}
December	$(8 \pm 2) \times 10^{-4a}$	0.47 ± 0.06^{b}
March	0.25 ± 0.14^{b}	0.67 ± 0.12^{c}
July	0.14 ± 0.09^{b}	$0.58 \pm 0.07^{b,c}$

Table 2
Ascorbic acid (AA) in male *Lithodes santolla*. Data are expressed as mean ± SD; AA values are presented as ng AA gwt^{-1} for gills, muscle (Mc) and hepatopancreas (HP), and as ng AA mL HL^{-1} for hemolymph (HL). Different letters indicate significant differences between seasons.

AA	Gills	HL	HP	Mc
September	55 ± 21^{a}	3 ± 1^{a}	227 ± 37^{a}	108 ± 28^{a}
December	$37 \pm 17^{a,c}$	7 ± 1^{b}	483 ± 81^{b}	1509 ± 1130^{b}
March	117 ± 84^{b}	8 ± 1^{b}	$405 \pm 35^{a,b}$	1165 ± 312^{b}
July	32 ± 10^{c}	4 ± 1^{a}	537 ± 99^{b}	867 ± 209^{b}

Table 3
Protein and glucose concentration in hepatopancreas (HP), gills, hemolymph (HL) and muscle (Mc) of male *Lithodes santolla*. Protein is expressed as mg mL^{-1}, and glucose as mg glucose g $tissue^{-1}$ in HP, Gills and Mc, and as mg glucose mL^{-1} in HL. Data expressed as mean ± SD. Different letters indicate significant differences between seasons.

Protein	Gills	HL	HP	Mc
September	25 ± 5^{a}	6 ± 2^{a}	5 ± 2^{a}	8.6 ± 0.8^{a}
December	35 ± 5^{b}	13 ± 2^{b}	$4.1 \pm 0.8^{a,b}$	7.4 ± 0.5^{b}
March	33 ± 6^{b}	12 ± 4^{b}	3.3 ± 0.6^{b}	$7.8 \pm 0.9^{a,b}$
July	20 ± 4^{a}	4 ± 3^{a}	3.5 ± 0.4^{b}	7 ± 1^{b}
Glucose				
September	0.05 ± 0.03^{a}	0.013 ± 0.005^{a}	2 ± 1^{b}	0.8 ± 0.2^{a}
December	0.19 ± 0.07^{b}	0.022 ± 0.008^{b}	5 ± 1^{a}	1.0 ± 0.5^{a}
March	0.16 ± 0.04^{b}	$0.014 \pm 0.004^{a,b}$	9 ± 3^{a}	1.1 ± 0.4^{a}
July	0.11 ± 0.04^{b}	0.011 ± 0.004^{a}	1.3 ± 0.6^{b}	0.3 ± 0.2^{b}

Table 5
Lipid peroxidation (LPO — μmo TBARS gwt^{-1}) and protein oxidation (PO — nmol -carbonyl mg $protein^{-1}$) analyzed in tissues of male *Lithodes santolla*. Data are expressed as mean ± standard deviation. Different letters indicate significant differences between seasons.

LPO	Gills	HL	HP	Mc
September	13 ± 5^{a}	–	76 ± 24^{a}	5 ± 1^{a}
December	15 ± 4^{a}	–	246 ± 84^{b}	15 ± 6^{b}
March	12 ± 2^{a}	–	186 ± 17^{b}	18 ± 5^{b}
July	8 ± 2^{b}	–	188 ± 44^{b}	13 ± 5^{b}
PO				
September	0.6 ± 0.2^{a}	$0.6 \pm 0.2^{a,b}$	0.4 ± 0.1^{a}	0.2 ± 0.1^{a}
December	1.0 ± 0.1^{b}	0.5 ± 0.2^{a}	1.6 ± 0.3^{b}	1.4 ± 0.5^{b}
March	1.0 ± 0.1^{b}	$0.9 \pm 0.3^{b,c}$	1.6 ± 0.3^{b}	1.2 ± 0.1^{b}
July	1.2 ± 0.2^{b}	1.8 ± 0.9^{c}	1.5 ± 0.3^{b}	1.2 ± 0.3^{b}

In the hemolymph, this combined action of SOD and CAT against LPO and PO was also recorded. Particularly, in September a high oxidative damage was prevented by the high activities of CAT and SOD. On the other hand, the combined increase of the three enzymatic activities determined in hemolymph – SOD, CAT and GST – in July were not enough to prevent the damage, inducing protein oxidation in this tissue. Furthermore, we do not disregard the presence of other enzymatic or non-enzymatic defenses not analyzed in this work that may be acting against the imbalance of ROS concentration in the hemolymph, especially in December and March, when PO is low, as well as the enzymatic activities.

The hepatopancreas of *L. santolla* presented no seasonality in neither of the enzymes, except CAT. In crustaceans the hepatopancreas is mainly associated with diverse metabolic activities, as for example digestion, absorption and energetic reservoir for nurturing all the organs (Paital and Chainy, 2010), and can be considered metabolically more active than other tissues as gills and muscle. Furthermore, in our study the hepatopancreas of *L. santolla* showed a stable antioxidant defense against the production of ROS, since this organ is a site of maximal free radical generation due to the multiple oxidative reactions occurring here (Arun and Subramanian, 1998; Romero et al., 2007). In December and March CAT activity in the hepatopancreas had values twice as high than in September. This increase of activity coincides with: (1) the warmer seasons in the Beagle Channel, and (2) the mating season of this species during November and December, in which males likely fast during the long (4 weeks) precopulatory embrace (Gowland & Lovrich unpubl. data). Both factors imply physiological changes in the metabolism that may be causing an increase in the production of H_2O_2, hence increasing CAT activity. Notwithstanding, there may be other antioxidants preventing oxidative damage in the hepatopancreas during the year. The reproductive activity and temperature changes associated to patterns of food intake, storage and utilization are likely causing changes in the hormonal and nutritional status which might also affect the levels of antioxidant defense enzymes (Borković et al., 2005).

During July the high activities of the enzymatic defense system in muscle, particularly of SOD, GPx and GST, are indicating that a collaborative antioxidant defense mechanism is preventing oxidative damage at this time of the year. Specifically, the studied enzymatic glutathione system in the muscle of *L. santolla*, composed of GPx and GST, decreases the activities in September, remaining unchanged along the year, and then significantly increases in July. We hypothesize that metabolism in the muscle is highly active in July due to post-molting condition. During this period, cell division is elevated resulting from the muscle growth and the replacement of the water uptake before molting by tissue-cells (Hartnoll, 1982), a process that lasts about 3–4 months in another sympatric lithodid (Lovrich et al., 2003).

On the other hand, during December, the enzymatic defense system is highly active, which may be attributed to energetic mobilization due to reproduction. Reproduction in male *L. santolla* is associated with strong competition for guarding females (Gowland unpublished results) with the consequent muscular activity in seizing the female and having agonistic interactions with other males. In the deep sea crustaceans *Nephrops norvegicus* and *Aristeus antennatus*, CAT, the glutathione system and cholinesterase activities in hepatopancreas and muscle are acting in accordance to the molting and reproductive seasons of the species, where the environmental factors such as temperature and salinity, are constant (Antó et al., 2009).

Furthermore, CAT activity differs from the other enzymes, since in muscle it peaks in September, when the other enzymatic activities are lowered, and this unique action seems enough to prevent the oxidative damage, reflected by the low LPO and PO at that time of the year. This action may be again a "preparation for oxidative stress" during a possible hypometabolic state during September, and the activity remains unchanged during the reproduction season in December.

Table 4
Hemolymphatic pH in male *Lithodes santolla*. Data are expressed as mean ± SD; different letters indicate significant difference.

	pH
September	7.74 ± 0.04^{a}
December	7.57 ± 0.02^{a}
March	7.20 ± 0.03^{b}
July	7.11 ± 0.03^{b}

4.2. Non-enzymatic antioxidants

Concentration of GSH was seasonal in gills and muscle. GSH is the most important water-soluble low-molecular weight antioxidant. Numerous physiological functions have been attributed to GSH: scavenging of H_2O_2 and other peroxides and free radicals, acting as a co-enzyme, translocation of amino acids across cell membranes, and catalysis of disulfide exchange reactions (Hermes-Lima, 2004; Kovacevic et al., 2008). GSH depletion leads to cell death and has been documented in many degenerative conditions.

In gills of *L. santolla* GSH showed a strong diminishment of concentration in September and December. The overwhelming difference of GSH in gills of *L. santolla* indicates an important role to prevent cellular damage, in September probably attributable to the seasonal environmental changes in the Beagle Channel, as aforementioned. In contrast, during December, we attribute low GSH concentration to the temperature increases in coincidence with the reproductive season of king crabs in the Beagle Channel. The low GSH concentration in muscle during September indicates that GSH is used to prevent oxidative damage caused mainly by the post-molting process along with environmental changes. For example, in the amphipod *Gammarus locusta*, reductions of GSH have been observed due to environmental changes which occasioned an increase of oxygen uptake (Gismondi et al., 2012), and

in the mussel *Perna perna* declines of GSH concentration have been attributed to the breeding season of the mussel (Filho et al., 2001).

The concentration of AA showed a strong seasonality in all tissues; and it was higher in muscle and hepatopancreas than in gills and hemolymph. For example, AA content in tissues of the crab *Scylla serrata* exhibited seasonal variation being higher in summer (Paital and Chainy, 2013). Seasonal variations in AA in this crab may be ascribed to their metabolic status which induces oxidative stress related to seasonal factors such as salinity, oxygen tension and temperature (Lushchak, 2011). Because of high metabolism, higher ratio of ascorbyl radical to ascorbate (hydrophilic oxidative stress index) was recorded in the summer season in the digestive gland of sub-polar limpet (Malanga et al., 2007). However, our results showed that in all tissues, except gills, high concentrations of AA are found during all the year, except in September. These differences can also be related to diet. Macroalgal consumption in *L. santolla* is higher in summer and autumn (60–80% frequency of occurrence; Comoglio, 1994). Seasonal variations in the antioxidant capacity of the scallop *Adamussium colbecki* and freshwater and estuarine fishes were attributed to a higher algal food supply that could alter the concentration of low molecular weight antioxidants, such as carotenoids and α–tocopherol (Regoli et al., 2002; Da Rocha et al., 2009). Moreover, in sea urchins *Loxechinus albus* annual variations of ascorbic acid content likely occur via dietary supplementation. In this species food intake could vary during the year increasing ascorbate content to preserve the tissue from oxidative damage in the hydrophilic milieu (Malanga et al., 2009). Therefore, we conclude that the non-enzymatic antioxidant concentrations in *L. santolla* are minimal in September because they are a main antioxidant source in this time of the year, along with SOD and CAT, and coincidently with the low oxidative damage (see Section 4.4).

4.3. Metabolic status of L. santolla

Glucose and protein showed seasonality in all tissues of *L. santolla*. Particularly, hepatopancreas and muscle increased their glucose concentration during the mating period (late spring — November–December) and pre-molting season (autumn, April–June). This rise of glucose concentration may be related to the need for an increased supply of substrate for glycolysis (da Silva-Castiglioni et al., 2010), as a source of energy. In gills and hemolymph of *L. santolla*, high glucose concentrations in December and March were found, and it may be acting as a buffer so animals can respond quickly to environmental variations (da Silva-Castiglioni et al., 2010). Hyperglycemia has been observed as a response to stress factors such as hypoxia in the crayfishes *Parastacus defossus* and *Parastacus brasiliensis* (da Silva-Castiglioni et al., 2010), environmental anaerobiosis in the crab *Chasmagnathus granulata* (Oliveira et al., 2001) and molting in the crayfish *Procambarus clarkii* (Fanjul-Moles and Gonsebatt, 2011). Experimental diets rich in carbohydrates produced high concentrations of glucose in different tissues, mainly hemolymph (Oliveira et al., 2004; Vinagre et al., 2007).

From cellular studies and analyses performed at the level of whole organism, arguments in favor of the role of pH in hemolymph in changing the activity of metabolic pathways have been developed (Forgue et al., 2001). In our study hemolymphatic pH was acidified in March and July, which could be attributed to the metabolic changes occurring before and after the molting season.

Protein content was seasonal in all tissues of *L. santolla*, among which gills and hemolymph had increased its concentration during December and March. In contrast, hepatopancreas and muscle had a higher protein concentration in September. In the Beagle Channel, at the time of the year when the water temperature is low and photoperiod short (June–September), food availability decreases (Diez et al., 2009). Hence, crab protein may be a source of energy during winter, evidenced by the low protein concentration in muscle and hepatopancreas in July. The decrease of proteins in March and only in the hepatopancreas could be related to the surplus of energy required for molting during April–June. In crustaceans, protein contents and its seasonal variation have been attributed to its role as an energy source, as well as with the availability and type of food (Oliveira et al., 2005; Vinagre et al., 2007). A decrease of protein content in the muscle of the crayfish *Parastocus varicosus* during the period of food scarcity may contribute to the maintenance and survival (da Silva-Castiglioni et al., 2007). Similarly during the period of starvation of the shrimp *Penaeus esculentus* (Barclay et al., 1983), the lobster *Nephrops norvegicus* (Dall, 1981) and the amphipod *Hyalella curvispina* (Dutra et al., 2008) the abdominal muscle mass makes the largest contribution of protein to energy metabolism, in which a small change in this tissue is sufficient to make a substantial contribution to the overall maintenance. During experimental starvation, *L. santolla* uses protein reserves from the hepatopancreas as energy source (Comoglio et al., 2008), and hence the protein decrease we found in hepatopancreas in March and July. Furthermore, since protein is a structural, functional and energetic constituent of tissues (Dutra et al., 2008), it may be used during the reproduction, when high energy demand is needed, and males are starving during the pre-copulatory embrace, as mentioned before.

4.4. Oxidative damage

Accordingly, in *L. santolla* oxidative stress parameters were seasonal in all tissues. Particularly, LPO values were the highest in the hepatopancreas. This pattern was also observed in *Lithodes confundens* of the Atlantic coast of Tierra del Fuego and *L. santolla* from the San Jorge Gulf, in which LPO values in the hepatopancreas were an order of magnitude higher than in other organs (Romero et al., 2013).

In general, in most of the tissues this damage presented the lowest values during September. In many works, a higher oxidative stress parameter determined as LPO, is observed in summer, usually related to the increase in temperature or due to the reproductive seasons of the studied species (Power and Sheehan, 1996; Sheehan and Power, 1999; Malanga et al., 2007). However, in our work on *L. santolla*, the higher oxidative stress parameters during almost all the year are showing a high metabolic rate. Particularly, this increased metabolic rate could be caused by the physiological processes involved during mating, as for example pheromone production or fasting (see Duffy and Thiel, 2007). It has been postulated that short term low level oxidative stress influences cellular physiology by disturbing ROS linked cell signaling mechanisms (Finkel and Holbrook, 2000; Gutteridge and Halliwell, 2000; Paital and Chainy, 2013). In the sea urchin *Loxechinus albus* from the Beagle Channel, LPO is a required physiological event for the spawning process, making lipids more available for the separation of reproductive cells from the gonadal tissue (Malanga et al., 2009) Therefore, we hypothesize that in *L. santolla* LPO and PO may be important in physiological processes, such as reproduction and molting.

5. Conclusion

We propose that the studied tissues in *L. santolla* reflect different antioxidant defense status. The antioxidant defense system is influenced principally by environmental changes in gills and hemolymph, and by reproduction and molting in hepatopancreas and muscle. The seasonality found in the antioxidant defense system of *L. santolla* from the Beagle Channel acts in a concerted way avoiding a long term oxidative stress that could cause damage in its reproduction and immunity.

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