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Responses of free radical metabolism to air exposure or salinity stress, in crabs (*Callinectes danae* and *C. ornatus*) with different estuarine distributions ☆

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ABSTRACT

The swimming crabs *Callinectes danae* and *C. ornatus* are found in bays and estuaries, but *C. danae* is more abundant in lower salinities, while *C. ornatus* remains restricted to areas of higher salinity. Experimental crabs of both species were submitted to: air exposure (Ae, 3 h), reimmersion in 33‰ (control) sea water (SW) (Ri, 1 h) following air exposure; hyposaline (Ho, 10‰ for 2 h) or hypersaline (He, 40‰ for 2 h) SW, then return to control 33‰ SW (RHo and RHe, for 1 h). Hemolymph was sampled for osmolality and chloride determinations. Activity of antioxidant enzymes [glutathione peroxidase (GPX), catalase, glutathione-S-transferase] and levels of carbonyl proteins and lipid peroxidation (TBARS) were evaluated in hepatopancreas, muscle, anterior and posterior gills. In Ho groups, hemolymph concentrations were lower in both species, compared to He groups. *C. danae* displayed higher control activities of GPX (hepatopancreas and muscle) and catalase (all four tissues) than *C. ornatus*. *C. ornatus* presented increased activities of catalase and GPX in Ae, Ri, and He groups. Increased TBARS was seen in *C. ornatus* tissues (He group). The more euryhaline species displayed higher constitutive activities of antioxidant enzymes, and the less euryhaline species exhibited activation of these enzymes when exposed to air or hyper-salinity.

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1. Introduction

Aerobic metabolism necessitated the development of an endogenous system of defense against toxic byproducts of oxygen metabolism, the reactive oxygen species (ROS). If the cell's antioxidant system is not able to manage ROS that are produced, a disruption of redox signaling and oxidative damage to cell components may take place (Jones, 2006). A special situation that can challenge the antioxidant system of animals is when they routinely endure cycles of hypoxia/anoxia/ischemia, followed by reoxigenation. Reperfusion or reoxigenation after a period of hypoxia or anoxia favors intense formation of ROS, potentially leading to oxidative stress. This problem may affect a vast array of living organisms, under a wide range of time frames (Hermes-Lima, 2004; Bickler and Buck, 2007; Freire et al., 2011).

Cyclic changes (within hours) in dissolved oxygen are typical of estuarine systems. Estuaries are open coastal water bodies constantly and cyclically under the influence of the tides, thus presenting a mixture of fresh water and sea water of variable proportions along the day (Nybakken, 1988). Estuarine species have been studied concerning their antioxidant responses (reviewed in Freire et al., 2011). For example, increased activities of catalase and glutathione-S-transferase (GST) during anoxia exposure have been detected in the gills of the estuarine crab, *Chasmagnathus* (*Neohelice*) *granulata* (Oliveira et al., 2005). Moreover, it has been increasingly evident that inhabitants of special environmentally challenging habitats, such as tidally-affected ecosystems (subject to the stressful variations in oxygenation, salinity and water temperature; see below), exhibit higher constitutive expression/activities of several stress indicators, when compared to their relatives that inhabit more stable sublittoral habitats. This has been shown for the heat-shock proteins in invertebrates (e.g., Dong et al., 2008; Gracey et al., 2008), blood cortisol in fishes (Yamashita et al., 2003), and the antioxidant system in mollusks (limpets, Malanga et al., 2004), and fish (Ross et al., 2001).

Dissolved oxygen is not the single fluctuating variable in estuaries; variability in temperature and salinity interact with O_2, and are also responsible for modulation of endogenous ROS production, which affect the endogenous antioxidant response in estuarine residents (Ross et al., 2001; Cailleaud et al., 2007; An and Choi, 2010). However, the effect of salinity variations on the antioxidant system in intertidal/estuarine animals has only recently been investigated (see Freire et al., 2011). Changes in salinity (either increases or decreases) affect expression/activities of enzymes of

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the antioxidant system, as demonstrated in mollusks, crustaceans and in fish. As examples in crustaceans: GST in the estuarine copepod *Eurytemora affinis* (Cailleaud et al., 2007), glutathione peroxidase (GPX) of the cultivated shrimp *Litopenaeus vannamei* (Liu et al., 2007), superoxide dismutase (SOD), catalase and GPX in different tissues of the mud crab *Scylla serrata* (Paital and Chainy, 2010), and peroxiredoxin in the flatback mud crab *Eurypanopeus depressus* (Van Horn et al., 2010). In mollusks, SOD in the gills of the Pacific oyster, *Crassostrea gigas* (Jo et al., 2008), SOD and catalase of the ark shell *Scapharca broughtonii* in digestive glands and gills (An and Choi, 2010).

Callinectes danae Smith, 1869 and *Callinectes ornatus* Ordway, 1863 are brachyuran crabs of the family Portunidae, and are very abundant in bays along the southern and southeastern coast of Brazil, frequently in brackish water areas, of salinity below that of full-strength sea water (Pita et al., 1985; Mantelatto and Martinelli, 1999; Negreiros-Fransozo et al., 1999; Mantelatto, 2000; Mantelatto and Fransozo, 2000; Baptista et al., 2003). Both species are reported to inhabit sandy bottoms, with differences in granulometry of preferred substrates (Pinheiro et al., 1997), from the intertidal region down to 75 m of depth (Melo, 1996). However, *C. danae* is distinctly reported to be more abundant in internal estuarine areas of lower salinity, with a frequently reported behavior of ovigerous females migrating to deeper areas for spawning (Pita et al., 1985; Pinheiro et al., 1997; Teixeira and Sá, 1998; Mantelatto and Fransozo, 2000; Baptista et al., 2003). *C. ornatus* occupies the same bays and estuarine areas, but is relatively more abundant in areas of higher salinity (above ~20‰), also with ovigerous females found more abundantly in open areas of full-strength salinity (Teixeira and Sá, 1998; Negreiros-Fransozo et al., 1999; Mantelatto, 2000; Mantelatto and Fransozo, 2000; Baptista et al., 2003; Keunecke et al., 2008).

The hypothesis tested in this study was that two closely-related species of crabs, of the same genus but with a distinct wider salinity range of distribution by one of them (*C. danae*), would display different patterns of response of their antioxidant system when facing saline stress. The more inner-estuarine species, *C. danae*, (from a more unstable environment) was expected to show higher levels of antioxidant enzymes when compared to its outer-estuarine "more marine" congener, *C. ornatus*, from a more stable environment. Oxygen stress (air exposure plus reimmersion) was offered as a putative "positive control" for redox imbalance to both species of crabs. The response of the antioxidant system investigated here involved the activities of the enzymes catalase, GPX, and GST, plus the quantification of oxidative damage to proteins (carbonyl proteins), and membrane lipids (TBARS), in four tissues: hepatopancreas, anterior and posterior gills, and muscle.

2. Material and methods

2.1. Experimental animals

Crabs of the genus *Callinectes* (*Callinectes danae* Smith, 1869 and *Callinectes ornatus* Ordway, 1863) were purchased from local fishermen of Ipanema beach in the coast of Paraná State (25°37′30″S, 48°25′08″W), in Southern Brazil. These crabs are a byproduct of commercial shrimp dredging. Trawl nets with 2 cm interknot distance were dragged for 1 h, along the coast (salinity ~33‰). Crabs were immediately placed in styrofoam boxes without water and were transported (~2 h travel by car) to the laboratory at the Department of Physiology, Federal University of Paraná. In the laboratory they were kept in a sea water tank (160 L) of salinity 33‰ (range: 32–34‰), water temperature of 21–23 °C, pH of 7.7–8.3, for 5–8 days. Crabs were obtained from June 2005 to December 2005. A total number of 46 *C. danae* (9.2 ± 1.1 cm of total carapace width, mean ± sd), 17 males and 29 females, and 50 *C. ornatus* (8.3 ± 0.9 cm), 47 males and 3 females, were used in this study. All crabs were adults in intermolt stage, and the sex ratio and size range was totally compatible with that reported by Keunecke et al. (2008) for those 2 species, for the size range of 7–11 cm of total carapace width.

After the acclimation period the crabs were divided into 6 groups: 1) air exposure (Ae), exposed to air for 3 h; 2) reimmersed (Ri), reimmersed in control sea water (33‰) for 1 h after 3 h of exposure to air; 3) hyposaline sea water (Ho), 2 h in 10‰ salinity; 4) return from hyposaline (RHo), 1 h in sea water of salinity 33‰ after 2 h in 10‰ salinity; 5) hypersaline sea water (He), 2 h in 40‰ salinity; and 6) return from hypersaline (RHe), 1 h in sea water of salinity 33‰ after 2 h in 40‰ salinity. Times of exposure have been chosen to simulate environments under tidal influence. Two to five crabs of each species were randomly placed in 30-liter aquaria according to the conditions/groups described above. Experiments were repeated until sufficient samples of all tissues for all the assays were produced. Control crabs were withdrawn from the stock tank, with sea water of salinity of 33‰. Salinity was always verified with a Shibuya S-28 refractometer–salinometer. Air temperature was of 22 ± 2 °C, relative humidity was of ~80%, and aquaria water temperature was of 23 ± 2 °C. Hyposaline sea water was prepared by appropriate dilution of full-strength SW with filtered (cellulose and activated charcoal filters) tap water. Hypersaline sea water of salinity 40‰ was prepared by the addition of 40 g of commercially available marine salt to 1 L of filtered tap water.

After the exposure to either control or experimental conditions, a sample of hemolymph (~500 μL) was withdrawn using a disposable insulin syringe through puncture of the arthrodial membrane of the base of the first or second pereiopod. The hemolymph sample was immediately frozen at −20 °C until thawing for osmolality and chloride determinations. Crabs were then quickly killed by quickly ablating the central nervous system between their ocular peduncles, using scissors.

The hepatopancreas (~1–2 g), the whole anterior (1st–3rd) and posterior (6th–8th) gills, and a fragment of skeletal muscle from the chelipod and from the insertion of the legs (~1–3 g) were dissected, and immediately frozen in liquid nitrogen. Tissues were later thawed and homogenized (whole tissues) for the assays of glutathione peroxidase, catalase, glutathione-S-transferase, levels of carbonylated proteins, and lipid peroxidation (TBARS). The experimental procedure adopted with the crabs has been approved by the Ethics Committee on Animal Experimentation of the Biological Sciences Sector of Federal University of Paraná (Certificate number 200).

2.2. Reagents

All chemicals used in buffers, solvents, and alcohols were of reagent grade and were obtained from Reagen Quimibrás (Rio de Janeiro, Brazil), and enzymes and enzyme substrates were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

2.3. Hemolymph osmolality and chloride concentrations

Hemolymph osmolality of the crabs *C. danae* and *C. ornatus* was determined in undiluted samples using the vapor pressure micro-osmometer Wescor VAPRO 5520 (Logan, UT, USA). Chloride concentrations were determined in samples appropriately diluted with deionized water, in duplicates, through a colorimetric assay, using commercially available kits (Labtest®, Lagoa Santa, Brazil). Briefly, chloride ions react with mercury thiocyanate in the presence of ferric nitrate, forming ferric thiocyanide, with absorbance read at 470 nm (Ultrospec 2100 Amersham Pharmacia biotech, Uppsala, Sweden).

2.4. Assays of the antioxidant enzymes

Samples of excised tissues stored at −70 °C were transferred to liquid nitrogen. Fragments were then weighed and diluted (1:15 for muscle and 1:20 for the other tissues) in potassium phosphate buffer

for homogenization. The potassium phosphate buffer (50 mM), pH 7.2, also contained 0.5 mM EDTA, and 1% phenylmethylsulphonyl fluoride. Whole-tissue homogenates were centrifuged at 4 °C for 15 min at 13,000 g, and the supernatants (enzymatic extracts) were then used in the several assays, and for the determination of total protein content (determined using the method of Bradford, 1976). Extracts were stored at −70 °C. The activities of glutathione peroxidase (GPX; EC 1.11.19), catalase (EC 1.11.1.6) and glutathione-S-transferase (GST; EC 2.5.1.18) in the extracts were determined as described by Ramos-Vasconcelos and Hermes-Lima (2003). The kinetics of each enzyme was obtained as the rate of change in the absorbance every 2 s, for 20–40 s.

2.5. Assays to evaluate oxidative damage

For determination of carbonyl proteins (by means of DNPH — dinitrophenylhydrazine-reaction with carbonyl groups), whole tissues stored at −70 °C were homogenized with sulphosalicylic acid 5%, diluted 1:20 (Furtado-Filho et al., 2007). Determination of lipid peroxidation, as TBARS (thiobarbituric acid reactive substances), from the frozen crab tissues was performed exactly as described by Furtado-Filho et al. (2007).

2.6. Statistical analysis

Data were statistically evaluated through one-way ANOVAs followed by the post hoc test of Tukey. Non-parametric ANOVAs (Kruskal–Wallis ANOVA on ranks) were performed whenever requirements for normality or homogeneity of variances were not met. Independent ANOVAs have been run for each species and each tissue, within a certain assayed parameter. Student's t-tests were employed to compare respective control values between both species. All tests had their limit of significance set at 0.05.

3. Results

3.1. Hemolymph osmolality and chloride concentrations

Hemolymph osmolality in control crabs was of 820 ± 46 mOsm/kg H_2O in *C. danae* and 847 ± 34 mOsm/kg H_2O in *C. ornatus*. Hemolymph osmolality of crabs of both species was respectively 27% (*C. danae*) and 47% (*C. ornatus*) lower in the Ho group when compared to the crabs submitted to the hyperosmotic (He) condition. For *C. ornatus*, hemolymph osmolality of crabs of the Ho group was also reduced with respect to the air exposure (Ae) group (Fig. 1A).

Exposure to hyposmotic conditions (Ho) led to reduced hemolymph chloride concentrations, when compared to control crabs (452 ± 24 mM in *C. danae* and 486 ± 10 mM in *C. ornatus*). Hemolymph chloride values of crabs that returned to control conditions for 1 h were still below those of control crabs (Fig. 1B).

3.2. Glutathione peroxidase (GPX) activity

C. danae crabs submitted to air exposure (Ae) and reimmersion (Ri) did not display any alteration in GPX activity, in any of the four tissues examined (Fig. 2). On the other hand, in *C. ornatus* there was a 4.7-fold increase in hepatopancreas GPX activity (Fig. 2A) and a 3.1-fold increase in anterior gills (Fig. 2C) in crabs from the Ri group when compared to controls. In muscle, a 2.4-fold increase in GPX activity was observed in the Ae group (Fig. 2B). No significant changes were observed in GPX activity in the posterior gills of *C. ornatus* during the air exposure experiment (although a trend towards an increase in Ae and Ri was noted — Fig. 2D).

In *C. danae*, the only effect observed on GPX activity in the salinity challenge experiments was a decrease in GPX activity in hepatopancreas of crabs submitted to hyposaline (Ho) sea water, to 25% of the

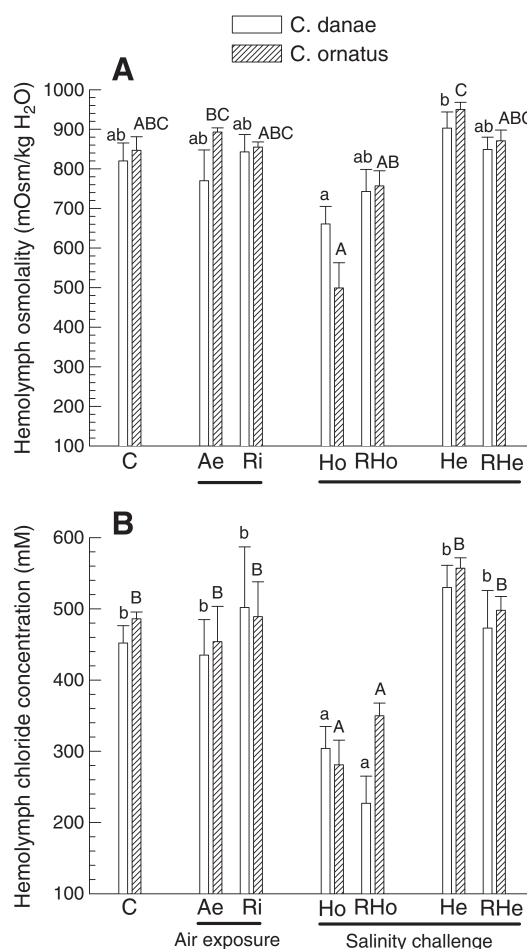

Fig. 1. Hemolymph osmolality (A, mOsm/kg H_2O) and hemolymph chloride concentration (B, mM) of *Callinectes danae* and *Callinectes ornatus*. Groups: control (C), air exposure (Ae), reimmersion following air exposure (Ri), hyposaline sea water (Ho), return to control sea water from hyposaline sea water (RHo), hypersaline sea water (He), return to control sea water from hypersaline sea water (RHe). Bars indicate the mean ± s.e.m. For both species, for osmolality and chloride determinations, the number of samples/crabs used for each group were: 10–11 (C), 7–9 (Ho), 7–8 (He), 5–6 (RHo), and 4–7 (Ae, Ri, RHe). Lower case (*C. danae*) or upper case (*C. ornatus*) letters above bars indicate statistically significant differences, within each species. Groups that do not share any letter in common are statistically different ($p < 0.05$).

control value (Fig. 3A). In a quite different pattern, all tissues examined in *C. ornatus*, except for the posterior gills, displayed increase in GPX activity upon exposure to hypersaline sea water (He), when compared to controls: 2.4-fold in hepatopancreas (Fig. 3A), 5.3-fold in muscle (Fig. 3B), and 1.9-fold in anterior gills (Fig. 3C). In the hepatopancreas of *C. ornatus* GPX activity did not return to control values after the return to full-strength sea water (RHe), whereas it did so in muscle and anterior gills (Fig. 3). No alterations in GPX activity were observed in *C. ornatus* during hyposaline challenge. The activity of GPX in the hepatopancreas and muscle of control *C. danae* was ~3-fold higher than the respective control values in the same tissues of *C. ornatus* (Figs. 2A, B, 3A and B).

3.3. Catalase activity

In the air exposure experiment, a decrease in catalase activity to 10% of the control value was observed for *C. danae* hepatopancreas submitted to air (Ae) (Fig. 4A). In this same species, catalase activity in muscle and anterior gills of the Ri group was 25% of control values (Fig. 4B,C). In *C. ornatus*, the only effect noted in the air exposure experiment was a 3.8-fold increase of catalase activity in muscle upon aerial exposure when compared to controls (Fig. 4B).

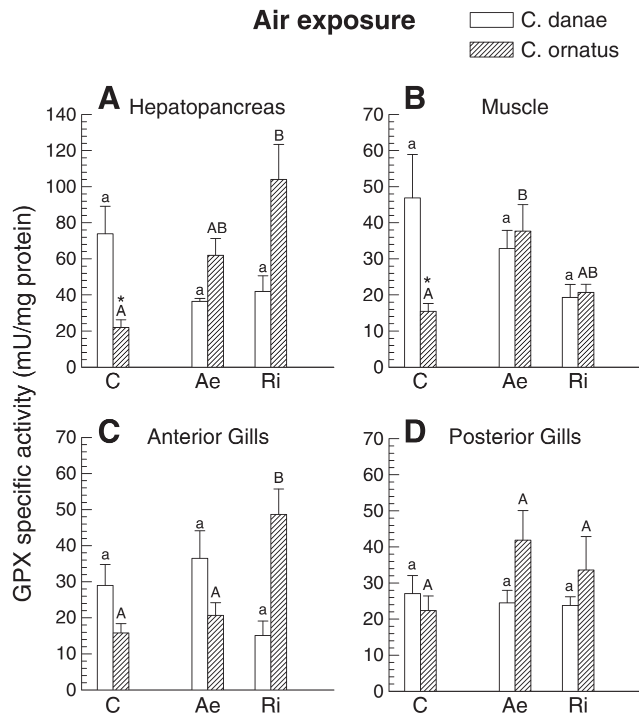

Fig. 2. Glutathione peroxidase (GPX) activity upon air exposure. GPX specific activity (mU/mg protein) in hepatopancreas (A), muscle (B), anterior gills (C), and posterior gills (D) of *Callinectes danae* and *C. ornatus* of the groups: control (C), air exposure (Ae), and reimmersion following air exposure (Ri). Bars indicate the mean ± s.e.m. For both species and the four tissues, the number of samples/crabs used for each group were: 8–11 (C), and 4–6 (Ae, Ri). Lower case (*C. danae*) or upper case (*C. ornatus*) letters above bars indicate statistically significant differences, within each tissue. Groups that do not share any letter in common are statistically different (p<0.05). Asterisk above *C.ornatus* control bar in A and B indicate that it is significantly different from the respective control group of *C. danae* (p<0.05).

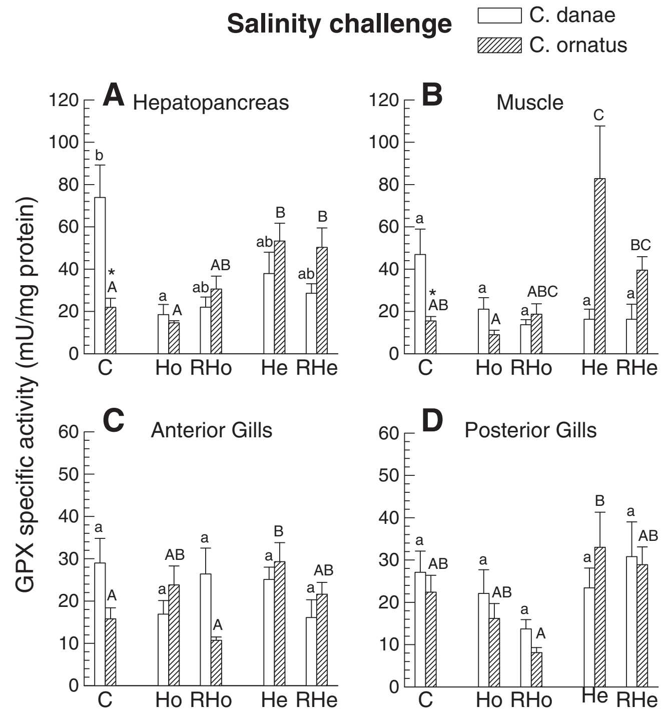

Fig. 3. Glutathione peroxidase (GPX) activity upon salinity challenge. GPX specific activity (mU/mg protein) in hepatopancreas (A), muscle (B), anterior gills (C), and posterior gills (D) of *Callinectes danae* and *C. ornatus* of the groups: control (C), hyposaline sea water (Ho), return to control sea water from hyposaline sea water (RHo), hypersaline sea water (He), return to control sea water from hypersaline sea water (RHe). Bars indicate the mean ± s.e.m. For both species and the four tissues, the number of samples/crabs used for each group were: 8–11 (C), 6–7 (Ho, He), and 4–6 (RHo, RHe). Lower case (*C. danae*) or upper case (*C. ornatus*) letters above bars indicate statistically significant differences, within each tissue. Groups that do not share any letter in common are statistically different (p<0.05). Asterisk above *C.ornatus* control bar in A and B indicate that it is significantly different from the respective control group of *C. danae* (p<0.05). Control data are the same as in Fig. 2.

The tissues of *C. danae* did not display any alteration in catalase activity during the salinity challenge experiments (Fig. 5). Again the picture was different for *C. ornatus*: there was a 2.3-fold increase in catalase activity in the hepatopancreas (Fig. 5A), a 3.1-fold increase in anterior gills (Fig. 5C), and a 2.4-fold increase in posterior gills (Fig. 5D) of crabs exposed to He sea water. In addition, there was a 3.6-fold increase also in the group that returned to control sea water after the hyposmotic shock (RHo) in the hepatopancreas (Fig. 5A). Catalase activity returned to control levels in the hepatopancreas, anterior and posterior gills of *C. ornatus* during recovery of the hypersaline challenge (RHe) (Fig. 5A). In muscle, the ANOVA revealed an effect of the treatment (P = 0.039 for *C. danae* and P = 0.034 for *C. ornatus*), but the *post hoc* test did not identify the differences (Fig. 5B). Control catalase activity in tissues of *C. danae* were higher (between 2.6 in posterior gills and 4.2-fold in muscle) than respective activities measured in the same tissues of *C. ornatus* (Figs. 4,5).

3.4. Glutathione S-transferase (GST) activity

For both crab species there were essentially no alterations in GST activity upon air exposure and reimmersion. The only exception was in muscle of *C. ornatus*, where the ANOVA indicated an effect of the treatment (P = 0.037), but differences were not localized (Table 1).

The salinity challenge imposed resulted in 2.4-fold increase in GST activity in the anterior gills of *C. danae* in the RHe group when compared to controls (Table 1). Still in *C. danae*, the salinity challenge was relevant to explain alterations in GST activity in muscle (P = 0.005) and posterior gills (P = 0.042), but in both tissues differences were not localized. Upon the salinity challenge offered to *C. ornatus*, GST activity in the hepatopancreas was reduced; in the RHe group it was 36% of the value measured in the He group (Table 1). Controls of both species were not different with respect to GST activity, for all four tissues.

3.5. Carbonyl protein levels

In both species of crabs there were no changes in the levels of carbonyl proteins in all tissues upon air exposure or reimmersion (Table 2). On the other hand, significant oxidative damage to proteins was detected *C. danae* muscle in the salinity challenge experiment, in which the RHe group displayed higher (5-fold) levels of carbonyl proteins than the He group (Table 2). No changes were observed in *C. ornatus* during salinity challenges (Table 2). Controls of both species were not different with respect to levels of carbonyl proteins, for all four tissues.

3.6. Lipid peroxidation determined as TBARS

In the hepatopancreas of *C. danae*, TBARS levels decreased upon Ae to 36% of control values. The air exposure experiment did not cause any change in TBARS levels in the tissues of *C. ornatus* (Table 3). Further, TBARS in *C. danae* were not affected by the salinity challenge. On the other hand, TBARS levels in the hepatopancreas of *C. ornatus* submitted to He was 2-fold higher than those of control crabs. In the case of anterior gills of *C. ornatus*, TBARS concentration in the He group was 3.2- to 6-fold higher than those measured in the other experimental groups, RHe, Ho, and RHo (Table 3). Controls of both species were not different with respect to levels of TBARS, for all four tissues.

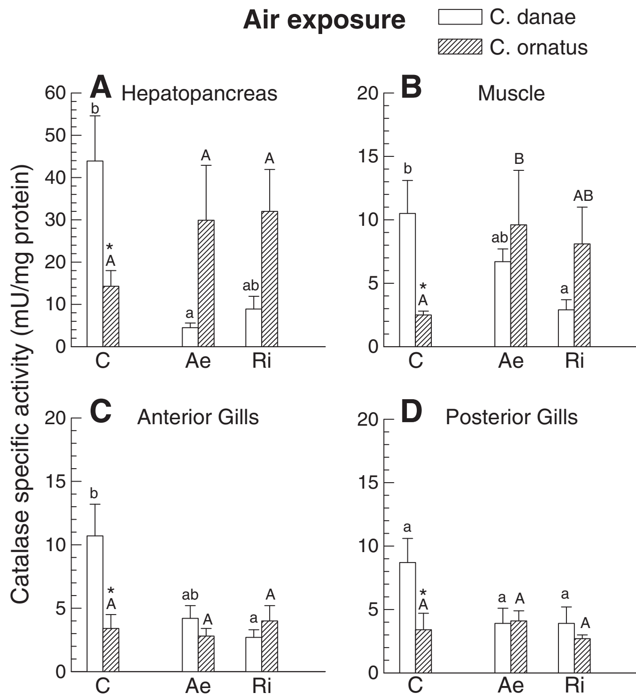

Fig. 4. Catalase activity upon air exposure. Catalase specific activity (mU/mg protein) in hepatopancreas (A), muscle (B), anterior gills (C), and posterior gills (D) of *Callinectes danae* and *C. ornatus* of the groups: control (C), air exposure (Ae), and reimmersion following air exposure (Ri). Bars indicate the mean ± s.e.m. For both species and the four tissues, the number of samples/crabs used for each group were: 9–11 (C), 6–7 (Ae), and 5–7 (Ri). Lower case (*C. danae*) or upper case (*C. ornatus*) letters above bars indicate statistically significant differences, within each tissue. Groups that do not share any letter in common are statistically different ($p<0.05$). Asterisk above *C.ornatus* control bar indicate that it is significantly different from the respective control group of *C. danae* ($p<0.05$).

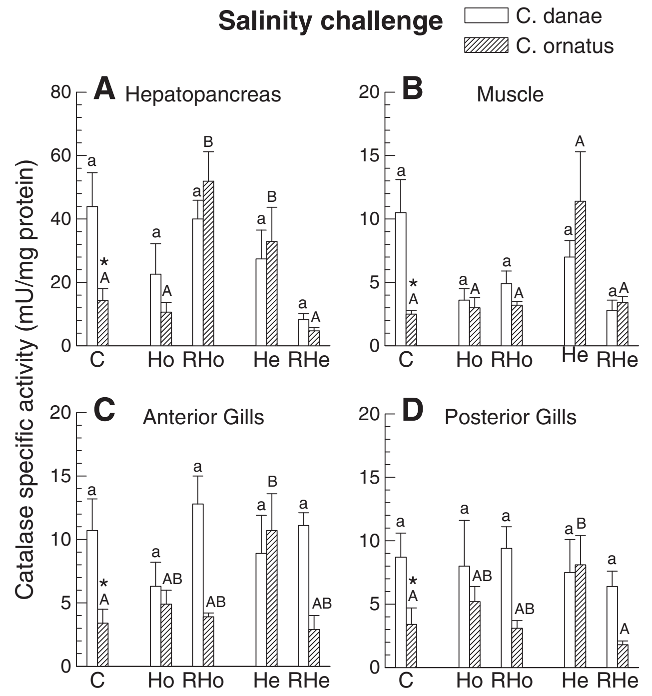

Fig. 5. Catalase activity upon salinity challenge. Catalase specific activity (mU/mg protein) in hepatopancreas (A), muscle (B), anterior gills (C), and posterior gills (D) of *Callinectes danae* and *C. ornatus* of the groups: control (C), hyposaline sea water (Ho), return to control sea water from hyposaline sea water (RHo), hypersaline sea water (He), return to control sea water from hypersaline sea water (RHe). Bars indicate the mean ± s.e.m. For both species and the four tissues, the number of samples/crabs used for each group were: 9–11 (C), 6–8 (Ho), 6–7 (He), and 4–6 (RHo, RHe). Lower case (*C. danae*) or upper case (*C. ornatus*) letters above bars indicate statistically significant differences, within each tissue. Groups that do not share any letter in common are statistically different ($p<0.05$). Asterisk above *C. ornatus* control bar indicate that it is significantly different from the respective control group of *C. danae* ($p<0.05$). Control data are the same as in Fig. 4.

4. Discussion

C. danae and *C. ornatus* live in estuaries and salt marshes, beaches and open sea. However, in estuaries and bays, *C. danae*'s range includes areas of very dilute sea water, while *C. ornatus* is more abundant in estuarine areas of higher salinities (>20‰), and coastal areas of full-strength sea water. Ovigerous females of both species migrate from estuarine brackish waters to areas of full-strength sea water for spawning. Thus, both species are euryhaline, but *C. ornatus* migrates over a shorter distance, and prefers higher salinities during its whole life cycle, being less euryhaline than *C. danae* (e.g., Pita et al., 1985; Pinheiro et al., 1997; Keunecke et al., 2008).

4.1. Crab osmoregulation

Upon the salinity challenge offered here, the two species behaved essentially the same in their extracellular fluid (ECF) homeostasis of osmolality and chloride. The hemolymph dilution noted when the two crabs were submitted to low salinity (Ho) was also very similar to that shown by *Callinectes sapidus* and *C. similis*, also ~600–700 mOsm/kg H_2O when in dilute SW of salinity 10‰ for 14–67 days (Piller et al., 1995; Guerin and Stickle, 1997). It is important to highlight that, despite this hemolymph dilution, all these crabs remain significantly hyper-osmotic to the water of salinity ~10‰ (~300 mOsm/kg H_2O). *C. danae* is more "inner-estuarine" such as *C. sapidus*, while *C. ornatus* is more "outer-estuarine" such as *C. similis* (e.g., Piller et al., 1995; Pinheiro et al., 1997; Mantelatto and Fransozo, 2000). Thus, a higher capacity for osmotic/chloride extracellular homeostasis upon salinity decrease would be expected for *C. danae*, when compared to *C. ornatus*. In addition, the gills of *C. danae* display higher activities of branchial Na,K-ATPase than the gills of *C. ornatus* (Masui et al., 2002; Leone et al., 2005; Garçon et al., 2007), and acclimation to sea water dilution (salinity 15‰) leads to higher expression of Na,K-ATPase in *C. danae* (Leone et al., 2005). Thus, the unexpected lack of difference in the response of both species is possibly explained by the short period of exposure to diluted seawater, although Piller et al. (1995) did not find differences in gill enzyme activities (Na,K-ATPase and carbonic anhydrase) between *C. sapidus* and *C. similis* even after 2 weeks of acclimation to diluted seawater. This hemolymph dilution in *Callinectes* submitted to hyposalinity is entirely compatible with the reports of effectively high permeabilities in low salinities in the also marine/estuarine crab *Carcinus maenas* (Spaargaren, 1989). Estuarine crabs are weak-osmoregulators, able to absorb salt, but not from freshwater. These estuarine crabs do not avoid water entry and hemolymph dilution, not only in order to facilitate oxygen uptake and metabolism for osmoregulation (Piller et al., 1995; McGaw, 2006; Paital and Chainy, 2010), but also because their tissues/cells can probably regulate their volume very well upon hemolymph dilution (Péqueux, 1995; Freire et al., 2008b; Foster et al., 2010).

Interestingly, upon increase in salinity (He), ECF homeostasis was preserved in both *C. danae* and *C. ornatus*. However, it should be noted that the increase in salinity (7 units, from 33 to 40‰ in He) in fact was of much lower magnitude than the decrease imposed (23 units, from 33 to 10‰ in Ho), because these crabs live in estuaries, of brackish water, and most probably do not encounter hypersaline waters in their lifetimes. The preserved extracellular osmotic (and chloride) homeostasis after 2 h in 40% SW possibly resulted from reduced apparent permeability from decreased branchial perfusion (possibly also from a reduced heart rate) in the He/RHe groups (Spaargaren, 1982). If this is true, a hypoxic state in the hemolymph could result,

Table 1
Glutathione S-transferase (GST) specific activity (mU/mg protein) in hepatopancreas, anterior (ant.) gills, posterior (post.) gills, and muscle of *Callinectes danae* and *Callinectes ornatus*, after the experiments of air exposure or saline challenge. Groups: control (C, same group for both experiments), air exposure (Ae), reimmersion (Ri), hyposaline sea water (Ho), return from hyposaline (RHo), hypersaline sea water (He), return from hypersaline (RHe).

Experiment	Species, group	GST specific activity (mU/mg protein)			
	C. danae	Hepatopancreas	Muscle	Ant. gills	Post. gills
Air exposure	C	$^{a}407 \pm 96.7$ (10)	$^{a}63.4 \pm 18.7$ (10)	$^{a}77.1 \pm 12.3$ (11)	$^{a}76.6 \pm 18.7$ (11)
	Ae	$^{a}322 \pm 157$ (5)	$^{a}26.9 \pm 8.9$ (6)	$^{a}48.3 \pm 10.8$ (6)	$^{a}55.7 \pm 13.3$ (6)
	Ri	$^{a}132 \pm 64.7$ (5)	$^{a}28.6 \pm 5.2$ (6)	$^{a}41.8 \pm 10.4$ (6)	$^{a}64.7 \pm 7.6$ (5)
Salinity challenge	C	$^{a}407 \pm 96.7$ (10)	$^{a}63.4 \pm 18.7$ (10)	$^{a}77.1 \pm 12.3$ (11)	$^{a}76.6 \pm 18.7$ (11)
	Ho	$^{a}292 \pm 70.1$ (7)	$^{a}18.7 \pm 3.5$ (8)	$^{ab}103 \pm 31.4$ (7)	$^{a}77.1 \pm 16.6$ (7)
	RHo	$^{a}425 \pm 104$ (5)	$^{a}15.2 \pm 2.1$ (6)	$^{a}71.2 \pm 24.7$ (6)	$^{a}46.0 \pm 11.3$ (6)
	He	$^{a}465 \pm 107$ (7)	$^{a}76.2 \pm 28.8$ (6)	$^{ab}123 \pm 14.7$ (7)	$^{a}120 \pm 26.8$ (7)
	RHe	$^{a}375 \pm 76.2$ (5)	$^{a}16.2 \pm 1.9$ (4)	$^{b}177 \pm 28.2$ (5)	$^{a}136 \pm 15.2$ (5)
	C. ornatus	Hepatopancreas	Muscle	Ant. gills	Post. gills
Air exposure	C	$^{a}329 \pm 78.6$ (11)	$^{a}31.1 \pm 7.0$ (10)	$^{a}139 \pm 47.3$ (10)	$^{a}126 \pm 20.0$ (11)
	Ae	$^{a}553 \pm 92.0$ (5)	$^{a}103 \pm 26.9$ (5)	$^{a}61.6 \pm 14.8$ (6)	$^{a}109 \pm 15.9$ (6)
	Ri	$^{a}332 \pm 68.5$ (5)	$^{a}28.7 \pm 8.1$ (6)	$^{a}78.1 \pm 5.8$ (5)	$^{a}72.4 \pm 15.5$ (6)
Salinity challenge	C	$^{ab}329 \pm 78.6$ (11)	$^{a}31.1 \pm 7.0$ (10)	$^{a}139 \pm 47.3$ (10)	$^{a}126 \pm 20.0$ (11)
	Ho	$^{ab}377 \pm 35.3$ (7)	$^{a}30.3 \pm 3.7$ (7)	$^{a}68.5 \pm 8.0$ (6)	$^{a}104 \pm 15.2$ (7)
	RHo	$^{ab}437 \pm 102$ (5)	$^{a}19.6 \pm 4.7$ (5)	$^{a}98.7 \pm 25.6$ (4)	$^{a}128 \pm 30.5$ (5)
	He	$^{b}606 \pm 63.0$ (6)	$^{a}49.6 \pm 11.3$ (6)	$^{a}133 \pm 11.8$ (6)	$^{a}213 \pm 31.3$ (6)
	RHe	$^{a}218 \pm 60.4$ (5)	$^{a}18.3 \pm 5.8$ (6)	$^{a}68.7 \pm 26.2$ (5)	$^{a}96.3 \pm 43.3$ (6)

Values are mean ± s.e.m., and (n, number of samples that yielded the mean and SEM). One ANOVA was performed for each experiment, each tissue and each species, separately. Lower case letters in superscript indicate statistically significant differences. Groups that do not share any letter in common are statistically different ($p<0.05$).

albeit with maintained hemolymph osmotic/chloride homeostasis, indeed expected given the short duration of the challenge. In conclusion, the osmoregulatory behavior of the two species is very similar, and compatible with the literature.

4.2. Redox metabolism versus salinity stress

Indeed, an activation of the antioxidant system (GPX and catalase activities) was detected, and caused either by increased salt in the external medium or by the functional hypoxia that potentially ensued from reduced branchial perfusion upon hypersalinity exposure, in the more outer-estuarine and less euryhaline crab species, *C. ornatus*. The response was even more widespread (in terms of tissues affected) upon air exposure (alterations in GPX and catalase in various tissues of both crabs). The hypersaline challenge offered represented a highly stressful condition for the crabs, much more than the hyposaline condition, which is actually a "real-life" challenge for estuarine or intertidal animals subject to freshwater runoffs from rivers or heavy rainfall during low tides (Freire et al., 2008a,b). This suggests that *C. ornatus*, despite being less euryhaline than *C. danae*, is more adapted to lowered salinities, than to hyperconcentrated sea water. Interestingly, *C. danae* presented 75% loss in GPX activity upon hyposalinity exposure.

Oxidative stress and/or activation of antioxidant enzymes have been previously related to salinity alterations in a few animal species, for variable periods of exposure. For example, high salinity stress (from 17 to 35‰) induced more oxidative injury (lipid peroxidation and protein carbonyls) than low salinity (10‰) in the estuarine mud crab *S. serrata* (Paital and Chainy, 2010). Moreover, salinity challenges affected the antioxidant systems of marine shrimps: muscle GPX and SOD activities decreased with either increase (30 to 50‰) or reduction (to 5‰) in salinity (Liu et al., 2007). In the marine flounder *Paralichthys olivaceus*, salinity reduction for 2 days, from 35 to 4‰, caused an increase in the expression of liver GPX and GST; further reduction in salinity (to 0‰) induced a decrease in enzyme expression (Choi et al., 2008). Moreover, hypersaline stress (52.5‰) in the Pacific oyster caused an increase in SOD and HSP90 expression in gills (Jo et al., 2008). Other studies indicated that hyposaline stress (10‰) prompted increases in both activity and expression of hepatic antioxidant enzymes (and increase in H_2O_2 production and lipid peroxidation) in black porgy (An et al., 2010). In a study with ark shells, An and Choi (2010) observed that exposure to either hypersaline (45‰) or hyposaline (25‰) waters caused oxidative stress, being more extensive under hyposalinity. Furthermore, hyposalinily stress (10‰) induced a 3-fold increase in 2-Cys peroxiredoxin expression in the gills of mud crabs (Van Horn et al., 2010).

Clearly the literature, so far, does not reveal a consistent pattern of effect of salinity on the enzymatic antioxidant capacity and levels of markers of oxidative stress. It appears that, depending on the animal species and its natural habitat, either hypo- or hyper-salinities may affect the redox balance by modulating ROS generation and the expression of antioxidant defenses. There are other studies evaluating the effect of hypoxia/anoxia or air exposure in estuarine species that could be of help to understand mechanisms of antioxidant regulation in short-term salt stress. For example, anoxia exposure of the estuarine crab *Chasmagnathus granulata* led to increased catalase and GST activities in the gills, and to decreased SOD activity in that organ (Oliveira et al., 2005). Moreover, air exposure of the intertidal mussel *Perna perna* resulted in increased SOD activity in digestive

Table 2
Measure of oxidative damage: carbonyl proteins content (nmol/mg tissue) in hepatopancreas, anterior (ant.) gills, posterior (post.) gills, and muscle of *Callinectes danae* and *Callinectes ornatus*, after the experiments of air exposure or salinity challenge. Groups: control (C, same group for both experiments), air exposure (Ae), reimersion (Ri), hyposaline sea water (Ho), return from hyposaline (RHo), hypersaline sea water (He), return from hypersaline (RHe).

Experiment	Species, group	Carbonyl proteins (nmol/mg tissue)			
	C. danae	Hepatopancreas	Muscle	Ant. gills	Post. Gills
Air exposure	C	$^{a}301 \pm 63.9$ (11)	$^{a}99.4 \pm 25.6$ (8)	$^{a}115 \pm 18.9$ (10)	$^{a}121 \pm 23.2$ (10)
	Ae	$^{a}116 \pm 41.1$ (5)	$^{a}48.8 \pm 16.5$ (4)	$^{a}104 \pm 21.9$ (5)	$^{a}114 \pm 31.4$ (5)
	Ri	$^{a}291 \pm 27.6$ (5)	$^{a}73.8 \pm 21.7$ (4)	$^{a}154 \pm 35.7$ (6)	$^{a}119 \pm 13.1$ (6)
Salinity challenge	C	$^{a}301 \pm 63.9$ (11)	$^{ab}99.4 \pm 25.6$ (8)	$^{a}115 \pm 18.9$ (10)	$^{a}121 \pm 23.2$ (10)
	Ho	$^{a}249 \pm 64.1$ (8)	$^{ab}34.2 \pm 5.6$ (6)	$^{a}82.8 \pm 41.1$ (7)	$^{a}115 \pm 34.4$ (6)
	RHo	$^{a}145 \pm 26.3$ (6)	$^{a}19.6 \pm 7.7$ (5)	$^{a}48.6 \pm 13.5$ (5)	$^{a}120 \pm 64.0$ (5)
	He	$^{a}152 \pm 25.3$ (7)	$^{a}27.3 \pm 7.9$ (6)	$^{a}134 \pm 30.6$ (6)	$^{a}105 \pm 19.5$ (7)
	RHe	$^{a}108 \pm 85.2$ (4)	$^{b}137 \pm 54.6$ (4)	$^{a}47.6 \pm 17.4$ (4)	$^{a}56.3 \pm 21.0$ (4)
	C. ornatus	Hepatopancreas	Muscle	Ant. gills	Post. Gills
Air exposure	C	$^{a}260 \pm 46.0$ (10)	$^{a}59.9 \pm 14.7$ (9)	$^{a}111 \pm 25.9$ (10)	$^{a}97.9 \pm 21.0$ (10)
	Ae	$^{a}229 \pm 72.8$ (6)	$^{a}51.8 \pm 32.2$ (5)	$^{a}189 \pm 48.4$ (6)	$^{a}184 \pm 34.4$ (6)
	Ri	$^{a}359 \pm 33.3$ (5)	$^{a}69.1 \pm 8.4$ (5)	$^{a}201 \pm 48.0$ (6)	$^{a}185 \pm 49.5$ (5)
Salinity challenge	C	$^{a}260 \pm 46.0$ (10)	$^{a}59.9 \pm 14.7$ (9)	$^{a}111 \pm 25.9$ (10)	$^{a}97.9 \pm 21.0$ (10)
	Ho	$^{a}196 \pm 41.8$ (8)	$^{a}39.8 \pm 13.0$ (7)	$^{a}83.1 \pm 23.4$ (7)	$^{a}70.7 \pm 10.1$ (8)
	RHo	$^{a}102 \pm 32.5$ (4)	$^{a}53.4 \pm 24.0$ (4)	$^{a}159 \pm 65.5$ (4)	$^{a}82.0 \pm 26.7$ (4)
	He	$^{a}100 \pm 33.3$ (8)	$^{a}48.4 \pm 24.3$ (7)	$^{a}164 \pm 39.5$ (7)	$^{a}83.8 \pm 19.0$ (8)
	RHe	$^{a}202 \pm 75.2$ (4)	$^{a}71.8 \pm 17.3$ (5)	$^{a}95.4 \pm 30.3$ (5)	$^{a}99.1 \pm 26.1$ (5)

Values are mean ± s.e.m., and (n, number of samples that yielded the mean and s.e.m.). One ANOVA was performed for each experiment, each tissue and each species, separately. Lower case letters in superscript indicate statistically significant differences. Groups that do not share any letter in common are statistically different ($p < 0.05$).

glands (Almeida and Bainy, 2006). When the subtidal crab *Paralomis granulosa* was air-exposed for up to 1 day, a tissue-specific peak in antioxidant enzyme activity, carbonyl protein and lipid peroxidation was observed after 6 h (Romero et al., 2007).

It is thus clear that i) variation in oxygen availability and ii) in salinity, both affect the redox metabolism and the antioxidant system of aquatic animals. The use of two species of the same genus (this study), with differences in the occupation of estuaries, has disclosed different constitutive activities of antioxidant enzymes (GPX and catalase), and different responses to air/salinity stress. The more euryhaline and inner-estuarine species, *C. danae*, displays high constitutive (control) activities of GPX and catalase. Even a certain reduction in enzyme activity was observed in *C. danae*: GPX in hepatopancreas, upon hyposalinity, catalase in hepatopancreas upon air exposure, and catalase in muscle and anterior gills upon reimmersion. Moreover, the constitutive activities (controls) of GPX (in hepatopancreas and muscle) and catalase (in four tissues) of *C. danae* were approximately 3-fold higher than those in *C. ornatus*. Thus, the more euryhaline species (*C. danae*) shows higher constitutive enzyme activities, while the less euryhaline crab (*C. ornatus*) displays activation of its antioxidant system when environmentally-challenged (as other animals do upon hypoxia exposure; Hermes-Lima and Zenteno-Savín, 2002): air and hypersaline exposures resulted in increases in GPX and catalase activities.

The higher constitutive activities of GPX and catalase in several tissues of the inner-estuarine *C. danae*, when compared to the outer-estuarine congener *C. ornatus*, was rather similar to the pattern exhibited by the intertidal limpet *Nacella* (*Patinigera*) *magellanica*, which displayed higher constitutive catalase and SOD activities in the digestive gland, when compared to its subtidal congener *N.* (*P.*) *deaurata* (Malanga et al., 2004). These results are also coherent with observations of higher activities of antioxidant enzymes in estuarine fishes caged (for 2 weeks) in estuaries with the widest variations in dissolved oxygen and other abiotic parameters (Ross et al., 2001). The pattern found here for the two crab species is also similar to the repeatedly reported high levels of expression of heat-shock proteins in intertidal species when compared to subtidal species (e.g., Lesser, 2006; Dong et al., 2008; Gracey et al., 2008). This same pattern of high constitutive activities in species from unstable environments also applies to goldfish from waters of wide circadian variation in oxygen levels (Lushchak et al., 2005), and also to freshwater turtles (see Hermes-Lima and Zenteno-Savín, 2002).

During hypersaline stress, hepatopancreas and anterior gills of *C. ornatus* shows increased levels of both lipid peroxidation and antioxidant enzymes (catalase and GPX). This indicates that hypersaline stress induces some degree of oxidative stress in this species, and this situation resulted in an activation of antioxidant enzymes. It should be pointed out, however, that, given the fact that hypersalinity exposure did not result in increased hemolymph salt content and osmolality, the oxidative disturbance detected could have been an indirect effect of hypersalinity. It could have been a direct effect of the functional hypoxia that resulted from a putative state of reduced branchial perfusion. As observed for the case of the black porgy (An et al., 2010), an augmentation in ROS formation during salinity challenge could induce lipid peroxidation and trigger activation of antioxidant defenses. That would prevent further oxidative

Table 3
Measure of oxidative damage: lipid peroxidation levels (TBARS, nmole/g tissue) in hepatopancreas, muscle, anterior (ant.) gills, and posterior (post.) gills of *C. danae* and *C. ornatus*, after the experiments of air exposure or saline challenge. Groups: control (C, same group for both experiments), air exposure (Ae), reimersion (Ri), hyposaline sea water (Ho), return from hyposaline (RHo), hypersaline sea water (He), return from hypersaline (RHe).

Experiment	Species, group	Lipid peroxidation (TBARS, nmole/g tissue)			
	C. danae	Hepatopancreas	Muscle	Ant. gills	Post. gills
Air exposure	C	$^{b}39.6 \pm 5.6$	$^{a}15.5 \pm 7.1$	$^{a}10.2 \pm 3.2$	$^{a}5.8 \pm 2.6$
	Ae	$^{a}14.2 \pm 3.0$	$^{a}3.1 \pm 0.9$	$^{a}2.8 \pm 0.6$	$^{a}5.3 \pm 1.4$
	Ri	$^{ab}21.0 \pm 1.4$	$^{a}5.2 \pm 1.9$	$^{a}7.1 \pm 2.1$	$^{a}10.5 \pm 1.3$
Salinity challenge	C	$^{a}39.6 \pm 5.6$	$^{a}15.5 \pm 7.1$	$^{a}10.2 \pm 3.2$	$^{a}5.8 \pm 2.6$
	Ho	$^{a}23.4 \pm 13.0$	$^{a}12.9 \pm 5.4$	$^{a}9.6 \pm 0.5$	$^{a}16.8 \pm 4.0$
	RHo	$^{a}10.0 \pm 0.3$	$^{a}4.2 \pm 0.5$	$^{a}5.3 \pm 1.4$	$^{a}9.7 \pm 2.3$
	He	$^{a}16.0 \pm 3.7$	$^{a}2.4 \pm 1.8$	$^{a}4.6 \pm 3.0$	$^{a}17.3 \pm 7.0$
	RHe	$^{a}35.6 \pm 2.1$	$^{a}4.3 \pm 0.4$	$^{a}5.4 \pm 2.0$	$^{a}6.4 \pm 1.4$
	C. ornatus	Hepatopancreas	Muscle	Ant. gills	Post. gills
Air exposure	C	$^{a}31.3 \pm 7.0$	$^{a}21.6 \pm 6.1$	$^{a}10.1 \pm 2.2$	$^{a}12.5 \pm 5.4$
	Ae	$^{a}45.8 \pm 9.3$	$^{a}8.5 \pm 1.9$	$^{a}22.4 \pm 8.9$	$^{a}11.5 \pm 1.3$
	Ri	$^{a}37.3 \pm 1.0$	$^{a}6.4 \pm 0.8$	$^{a}7.8 \pm 2.7$	$^{a}13.9 \pm 2.6$
Salinity challenge	C	$^{a}31.3 \pm 7.0$	$^{a}21.6 \pm 6.1$	$^{ab}10.1 \pm 2.2$	$^{a}12.5 \pm 5.4$
	Ho	$^{a}24.3 \pm 6.4$	$^{a}17.5 \pm 3.4$	$^{a}3.1 \pm 1.5$	$^{a}19.8 \pm 10.9$
	RHo	$^{a}17.1 \pm 1.4$	$^{a}4.2 \pm 1.1$	$^{a}5.4 \pm 1.6$	$^{a}3.0 \pm 1.2$
	He	$^{b}61.8 \pm 10.4$	$^{a}13.6 \pm 6.9$	$^{b}18.5 \pm 3.8$	$^{a}17.7 \pm 4.1$
	RHe	$^{a}18.1 \pm 2.5$	$^{a}3.0 \pm 0.5$	$^{a}5.7 \pm 1.0$	$^{a}2.3 \pm 0.4$

Values are mean ± s.e.m., n = 3 for all groups. One ANOVA was performed for each experiment, each tissue, and each species, separately. Lower case letters in superscript indicate statistically significant differences. Groups that do not share any letter in common are statistically different ($p < 0.05$).

damage, such as oxidation of proteins. In fact, carbonyl proteins did not change during hypersaline stress in *C. ornatus*. On the other hand, the activation of GPX and catalase in *C. ornatus* under aerial exposure was not followed by lipid peroxidation during either aerial exposure or recovery. This is in the same line of observations as of increased enzymatic antioxidant defenses (enzyme activity and/or expression) during anoxia/hypoxia/freezing exposure in several "lower" vertebrates and mollusks (Bickler and Buck, 2007; Gorr et al., 2010). In these cases, the increase in antioxidant enzymes during low oxygenation has been considered a form of "preventive" management of oxidative stress (originally coined "*preparation for oxidative stress*"; Hermes-Lima and Zenteno-Savín, 2002), that should strike the organisms during reoxygenation.

In *C. danae,* aerial exposure prompted a decrease in both catalase activity and TBARS (carbonyl protein was unchanged). Moreover, salinity stress induced a decrease in catalase and GPX activity without change in either TBARS or carbonyl protein. This type of response to aerial exposure and salinity stress is typical of animals displaying high constitutive levels of antioxidant defense (Hermes-Lima and Zenteno-Savín, 2002; Gorr et al., 2010), and thus, the loss of some of this antioxidant potential leaves enough enzyme activity to manage oxidative damage under stress conditions.

Independent of the strategy, a tissue-specific antioxidant response is in general observed to counteract environmental challenges (e.g., Paital and Chainy, 2010). A higher activity of antioxidant enzymes in the posterior gills when compared to anterior gills was noted in the estuarine crab *C. granulata* (Oliveira et al., 2005), but was not the case here with both *C. danae* and *C. ornatus*. In brachyuran crabs, anterior gills display a thin epithelium, specialized for gas transfer in respiration, whereas posterior gills have a much thicker epithelium, specialized for active ion transport in osmoregulation (reviewed in Péqueux, 1995 and Freire et al., 2008a). However, no increase in the activities of the antioxidant enzymes in the posterior gills was noted here, for both species, upon hypo-salinity challenge. Enhanced activities of these enzymes could perhaps be expected in posterior gills, given the probable increased metabolism for hyper-osmoregulation (e.g., Van Horn et al., 2010). In fact, considering that hemolymph concentrations decreased upon the short-term hypo-salinity challenge offered here, the time allowed (2 h) may not have been sufficient to elicit increased metabolism associated with hyper-osmoregulation. Thus, the lack of activation of the antioxidant system in the gills of *C. danae* and *C. ornatus* under low salinity is indeed compatible with the hemolymph data.

4.3. Salinity stress and environmental biomarkers

Catalase activity is frequently employed as a biomarker and biomonitoring tool for the evaluation of oxidative status of aquatic animals exposed to polluted waters (Geracitano et al., 2004; Pinho et al., 2005). Thus, it is very important to consider that it may be affected by salinity, especially in those animals that inhabit intertidal or estuarine habitats. The interacting effects between the abiotic factors (temperature, salinity, and light exposure) and polluting agents on catalase activity has been demonstrated in marine mollusks submitted to organochlorides (Khessiba et al., 2005) and in estuarine polichaetes exposed to pollution (Geracitano et al., 2004). The abiotic factors salinity and temperature should also be considered when evaluating the role of catalase and other antioxidant enzymes as biomarkers of pollution in estuarine and intertidal animals (Cailleaud et al., 2007; see review in Monserrat et al., 2007).

Increased activity of GST has also been frequently associated to pollutants, and this enzyme is also often employed as a biochemical biomarker of exposure to pollution, especially in estuaries (Monserrat et al., 2007). Cailleaud et al. (2007) reported that GST is affected by salinity in an estuarine copepod. We observed that salinity challenge induced GST activity in the anterior gills of *C. danae* and hepatopancreas of *C. ornatus*. Given its hepatic role in xenobiotic detoxification, substantial variability in GST activity was observed in the digestive gland of bivalves (*Mytilus edulis*), a fact which warrants caution in the use of hepatic GST for biomonitoring contaminated areas (Manduzio et al., 2004). The present results are consistent with this anti-xenobiotic/detoxifying function of GST, as the environmental challenges offered herein (air exposure and salinity variation) did not frequently affect GST activity of both crabs.

5. Conclusion and perspectives

In conclusion, air exposure or increased salinity for a few hours was a relevant inducer of GPX and catalase activities, in the more outer-estuarine *C. ornatus*. The more inner-estuarine and euryhaline *C. danae* displayed a distinct pattern of high constitutive activity of these same enzymes, even showing decreased activities upon the imposed challenges. Both the high constitutive activities in *C. danae* and the inducible activity in *C. ornatus* were effective in preventing significant oxidative damage to lipids and proteins during exposure to air or the salinity challenge. Interestingly, these two species have

shown a "horizontal" pattern similar to the "vertical" pattern displayed by related (also congeneric) intertidal and subtidal species in rocky shores. The most euryhaline species (intertidal, estuarine) show higher constitutive activities of antioxidant enzymes versus relatively reduced activities in the species that are restricted to more stable habitats. The crab species more restricted to the stable outer marine or subtidal habitats (*C. ornatus*) display activation of antioxidant defenses upon need.

The next logical step of these studies is to identify what cellular components are involved in the redox regulation under both salinity stress and air exposure. Putative candidates include the family NF-κB transcription factors and the transcription factor Nrf2, which are involved in the regulation of gene expression of several antioxidant enzymes (Lushchak, 2011). The hypoxia inducible factor (HIF) could also play a role in the up-regulation of target genes (Ju et al., 2007; Miller, 2009) in crabs under low-oxygen/high-salinity stress. Moreover, glutathione and SOD could also be implicated in antioxidant protection and redox regulation of crab tissues, as it has been reported for several cases of animals under stress conditions (Gorr et al., 2010). Finally, the inter-relationship between water salinity and a suite of cellular processes (including antioxidant protection) has been recently demonstrated in another crab of the *Callinectes* family, *Portunus trituberculatus* (Xu et al., 2010).

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References

Almeida, E.A., Bainy, A.C.D., 2006. Effects of aerial exposure on antioxidant defenses in the brown mussel *Perna perna*. Braz. Arch. Biol. Technol. 49, 225–229.
An, M.I., Choi, C.Y., 2010. Activity of antioxidant enzymes and physiological responses in ark shell, *Scapharca broughtonii*, exposed to thermal and osmotic stress: effects on hemolymph and biochemical parameters. Comp. Biochem. Physiol. B 155, 34–42.
An, K.W., Kima, N.N., Shin, H.S., Kil, G.-S., Choi, C.Y., 2010. Profiles of antioxidant gene expression and physiological changes by thermal and hypoosmotic stresses in black porgy (*Acanthopagrus schlegeli*). Comp. Biochem. Physiol. A 156, 262–268.
Baptista, C., Pinheiro, M.A.A., Blankensteyn, A., Borzone, C.A., 2003. Estrutura populacional de *Callinectes ornatus* Ordway (Crustacea, Portunidae) no Balneário Shangri-lá, Pontal do Paraná, Paraná. Brasil. Revta Bras. Zool. 20, 661–666.
Bickler, P.E., Buck, L.T., 2007. Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. Annu. Rev. Physiol. 69, 145–170.
Bradford, M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of Protein-Dye-Bonding. Anal. Biochem. 72, 248–254.
Cailleaud, K., Maillet, G., Budzinski, H., Souissi, S., Forget-Leray, L., 2007. Effects of salinity and temperature on the expression of enzymatic biomarkers in *Eurytemora affinis* (Calanoida, Copepoda). Comp. Biochem. Physiol. A 147, 841–849.
Choi, C.Y., An, K.W., An, M.I., 2008. Molecular characterization and mRNA expression of glutathione peroxidase and glutathione S-transferase during osmotic stress in olive flounder (*Paralichthys olivaceus*). Comp. Biochem. Physiol. A 149, 330–337.
Dong, Y., Miller, L.P., Sanders, J.G., Somero, G.N., 2008. Heat-shock protein 70 (Hsp70) expression in four limpets of the genus *Lottia*: interspecific variation in constitutive and inducible synthesis correlates with in situ exposure to heat stress. Biol. Bull. 215, 173–181.
Foster, C., Amado, E.M., Souza, M.M., Freire, C.A., 2010. Do osmoregulators have lower capacity of muscle water regulation than osmoconformers? A study on decapod crustaceans. J. Exp. Zool. 313A, 80–94.
Freire, C.A., Onken, H., McNamara, J.C., 2008a. A structure-function analysis of ion transport in crustacean gills and excretory organs. Comp. Biochem. Physiol. A 151, 272–304.
Freire, C.A., Amado, E.M., Souza, L.R., Veiga, M.P.T., Vitule, J.R.S., Souza, M.M., Prodocimo, V., 2008b. Muscle water control in crustaceans and fishes as a function of habitat, osmoregulatory capacity, and degree of euryhalinity. Comp. Biochem. Physiol. A 149, 435–446.
Freire, C.A., Welker, A., Storey, J.M., Storey, K.B., Hermes-Lima, M., 2011. Oxidative stress in estuarine and intertidal species. In: Abele, D., Zenteno-Savin, T., Vázquez-Medina, J.P. (Eds.), Oxidative Stress in Aquatic Ecosystems. Wiley-Blackwell, New York, pp. 41–57.
Furtado-Filho, O.V., Polcheira, C., Machado, D.P., Mourão, G., Hermes-Lima, M., 2007. Selected oxidative stress markers in a South American crocodilian species. Comp. Biochem. Physiol. C 146, 241–254.
Garçon, D.P., Masui, D.C., Mantelatto, F.L.M., McNamara, J.C., Furriel, R.P.M., Leone, F.A., 2007. K^+ and NH_4^+ modulate gill (Na^+, K^+)-ATPase activity in the blue crab, *Callinectes ornatus*: fine tuning of ammonia excretion. Comp. Biochem. Physiol. A 147, 145–155.
Geracitano, L.A., Monserrat, J.M., Bianchini, A., 2004. Oxidative stress in *Laeoneris acuta* (Polychaeta, Nereididae): environmental and seasonal effects. Mar. Environ. Res. 58, 625–630.
Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83, 733–752.
Gracey, A.Y., Chaney, M.L., Boomhower, J.P., Tyburczy, W.R., Connor, K., Somero, G.N., 2008. Rhythms of gene expression in a fluctuating intertidal environment. Curr. Biol. 18, 1501–1507.
Guerin, J.L., Stickle, W.B., 1997. A comparative study of two sympatric species within the genus *Callinectes*: osmoregulation, long-term acclimation to salinity and the effects of salinity on growth and moulting. J. Exp. Mar. Biol. Ecol. 218, 165–186.
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 & Sons, New York, pp. 319–368.
Hermes-Lima, M., Zenteno-Savín, T., 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp. Biochem. Physiol. C 133, 537–556.
Jo, P.G., An, K.W., Park, M.S., Choi, C.Y., 2008. mRNA expression of HSP90 and SOD, and physiological responses to thermal and osmotic stress in the Pacific oyster, *Crassostrea gigas*. Mollusc. Res. 28, 158–164.
Jones, D.P., 2006. Forum review, redefining oxidative stress. Antiox. Redox Signaling 8, 1865–1879.
Ju, Z., Wells, M.C., Heater, S.J., Walter, R.B., 2007. Multiple tissue gene expression analyses in Japanese medaka (*Oryzias latipes*) exposed to hypoxia. Comp. Biochem. Physiol. C 145, 134–144.
Keunecke, K.A., D'Incao, F., Moreira, F.N., Silva Jr., D.R., Verani, J.R., 2008. Idade e crescimento de *Callinectes danae* e *C. ornatus* (Crustacea, Decapoda) na Baía de Guanabara, Rio de Janeiro, Brasil. Iheringia. Ser. Zool. 98, 231–235.
Khessiba, A., Roméo, M., A ssa, P., 2005. Effects of some environmental parameters on catalase activity measured in mussel (*Mytilus galloprovincialis*) exposed to lindane. Environ. Pollut. 133, 275–281.
Leone, F.A., Furriel, R.P.M., McNamara, J.C., Mantelatto, F.L.M., Masui, D.C., Alves, L.R., Gonçalves, R.R., Garçon, D.P., 2005. (Na+, K+)-ATPase from crustacean gill microsomes: a molecular marker to evaluate adaptation to biotopes of different salinity. Trends Comp. Biochem. Physiol. 11, 1–15.
Lesser, M.P., 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annu. Rev. Physiol. 68, 253–278.
Liu, Y., Wang, W.-N., Wang, A.-L., Wang, J.-M., Sun, R.-Y., 2007. Effects of dietary vitamin E supplementation on antioxidant enzyme activities in *Litopenaeus vannamei* (Boone, 1931) exposed to acute salinity changes. Aquaculture 265, 351–358.
Lushchak, V.I., Bagnyukova, T.V., Husak, V.V., Luzhna, L.I., Lushchak, O.V., Storey, K.B., 2005. Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. Int. J. Biochem. Cell Biol. 37, 1670–1680.
Lushchak, V.I., 2011. Adaptive response to oxidative stress: bacteria, fungi, plants and animals. Comp. Biochem. Physiol. C 153, 175–190.
Malanga, G., Esteves, M.S., Calvo, J., Puntarulo, S., 2004. Oxidative stress in limpets exposed to different environmental conditions in Beagle Channel. Aquat. Toxicol. 69, 299–309.
Manduzio, M., Monsinjon, T., Galap, C., Leboulenger, F., Rocher, B., 2004. Seasonal variations in antioxidant defeses in blue mussels *Mytilus edulis* collected area: major contributions in gill of an inducible isoform of Cu/Zn-superoxide dismutase and glutathione s-transferase. Aquat. Toxicol. 70, 83–93.
Masui, D.C., Furriel, R.P.M., McNamara, J.C., Mantelatto, F.L.M., Leone, F.A., 2002. Modulation by ammonium ions of gill microsomal (Na+, K+)-ATPase in the swimming crab Callinectes danae: a possible mechanism for regulation of ammonia excretion. Comp. Biochem. Physiol. C 132, 471–482.
Mantelatto, F.L.M., 2000. Allocation of the portunid crab *Callinectes ornatus* (Decapoda: Brachyura) in the Ubatuba Bay, northern coast of São Paulo State, Brazil. In: Klein, J.C.V.V., Schram, F.R. (Eds.), The Biodiversity Crisis and Crustacea: Proceedings of the Fourth International Crustacean Congress, Amsterdam, Netherlands, 20–24 July 1998, vol. 12. A.A, Balkema, Rotterdam, pp. 431–443.
Mantelatto, F.L.M., Fransozo, A., 2000. Brachyuran community in Ubatuba Bay, northern coast of São Paulo state. Brazil. J. Shellfish Res. 19, 701–709.
Mantelatto, F.L.M., Martinelli, J.M., 1999. Carapace width-weight relationships of *Callinectes ornatus* (Brachyura, Portunidae) from Ubatuba Bay, Brazil. Iheringia. Sér. Zool. 87, 111–116.
McGaw, I.J., 2006. Prioritization or summation of events? Cardiovascular physiology of postprandial dungeness crabs in low salinity. Physiol. Biochem. Zool. 79, 169–177.
Melo, G.A.S., 1996. Manual de Identificação de Brachyura (Caranguejos e Siris) do Litoral Brasileiro. Ed. Plêiade/FAPESP, São Paulo.
Miller, W.A., 2009. Effects of molting and hyposalinity stress on the expression of HIF-α, molting, and immune response genes in juvenile *Cancer magister*. pp 49. Master of Science Thesis, University of Oregon, USA.
Monserrat, J.M., Martinez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M., Pinho, G.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp. Biochem. Physiol. C 146, 221–234.

Negreiros-Fransozo, M.L., Mantelatto, F.L.M., Fransozo, A., 1999. Population biology of *Callinectes ornatus* Ordway, 1863 (Decapoda, Portunidae) from Ubatuba (SP). Brazil. Sci. Mar. 63, 157–163.
Nybakken, J.W., 1988. Marine Biology: An Ecological Approach, 2nd Ed. Harper-Collins Publishers, New York.
Oliveira, U.O., Araújo, A.S.R., Belló-Klein, A., Silva, R.S.M., Kucharski, L.C., 2005. Effects of environmental anoxia and different periods of reoxygenation on oxidative balance in gills of the estuarine crab *Chasmagnathus granulata*. Comp. Biochem. Physiol. B 140, 51–57.
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.
Péqueux, A., 1995. Osmotic regulation in Crustacea. J. Crust. Biol. 15, 1–60.
Piller, S.C., Henry, R.P., Doeller, J.E., Kraus, D.W., 1995. A comparison of the gill physiology of two euryhaline crab species, *Callinectes sapidus* and *Callinectes similis*: energy production, transport-related enzymes and osmoregulation as a function of acclimation salinity. J. Exp. Biol. 198, 349–358.
Pinheiro, M.A.A., Fransozo, A., Negreiros-Fransozo, M.L., 1997. Dimensionamento e sobreposição de nichos dos portunídeos (Decapoda, Brachyura), na Enseada da Fortaleza, Ubatuba, São Paulo. Brasil. Revta Bras. Zool 14, 371–378.
Pinho, G.L.L., Rosa, C.M., Maciel, F.E., Bianchini, A., Yunes, J.S., Proença, L.A.O., Monserrat, J.M., 2005. Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. Ecotoxicol. Environ. Saf. 61, 353–360.
Pita, J.B., Rodrigues, E.S., Graça Lopes, R., Coelho, J.A.P., 1985. Levantamento preliminar da Família Portunidae (Crustacea, Decapoda, Brachyura) no complexo Baía estuário de Santos, São Paulo. Brasil. Bolm Inst. Pesca 12, 153–162.
Ramos-Vasconcelos, G.R., Hermes-Lima, M., 2003. Hypometabolism, antioxidant defenses and free radical metabolism in the pulmonate land snail *Helix aspersa*. J. Exp. Biol. 206, 675–685.
Romero, M.C., Ansaldo, M., Lovrich, G.A., 2007. Effect of aerial exposure on the antioxidant status in the subantarctic stone crab *Paralomis granulosa* (Decapoda: Anomura). Comp. Biochem. Physiol. C 146, 54–59.
Ross, S.W., Dalton, D.A., Kramer, S., Christensen, B.L., 2001. Physiological (antioxidant) responses of estuarine fishes to variability in dissolved oxygen. Comp. Biochem. Physiol. C 130, 289–303.
Spaargaren, D.H., 1982. Cardiac output in the shore crab, *Carcinus maenas* (L.) in relation to solute exchange and osmotic stress. Mar. Biol. Lett. 3, 231–240.
Spaargaren, D.H., 1989. Adaptation to estuarine conditions in shore crabs *Carcinus maenas* (L.) in relation to body size. J. Exp. Mar. Biol. Ecol. 129, 251–263.
Teixeira, R.L., Sá, H.S., 1998. Abundance of decapod macrocrustaceans occupying the shallow waters of a tropical estuary. Revta Bras. Biol. 58, 393–404.
Van Horn, J., Malhoe, V., Delvina, M., Thies, M., Tolley, S.G., Ueda, T., 2010. Molecular cloning and expression of a 2-Cys peroxiredoxin gene in the crustacean *Eurypanopeus depressus* induced by acute hypo-osmotic stress. Comp. Biochem. Physiol. B 155, 309–315.
Xu, Q., Liu, Y., Liu, R., 2010. Expressed sequence tags from cDNA library prepared from gills of the swimming crab, *Portunus trituberculatus*. J. Exp. Mar. Biol. Ecol. 394, 105–115.
Yamashita, Y., Tominaga, O., Takami, H., Yamada, H., 2003. Comparison of growth, feeding and cortisol level in *Platichthys bicoloratus* juveniles between estuarine and nearshore nursery grounds. J. Fish Biol. 63, 617–630.