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Biochemical responses and physiological status in the crab *Hemigrapsus crenulatus* (Crustacea, Varunidae) from high anthropogenically-impacted estuary (Lenga, south-central Chile)

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ABSTRACT

Estuarine environmental assessment by sub-individual responses is important in order to understand contaminant effects and to find suitable estuarine biomonitor species. Our study aimed to analyze oxidative stress responses, including glutathione-*S*-transferase (GST) activity, total antioxidant capacity (ACAP) and lipid peroxidation levels (TBARS) in estuarine crabs *Hemigrapsus crenulatus* from a high anthropogenically-impacted estuary (Lenga) compared to low and non-polluted estuaries (Tubul and Raqui), in a seasonal scale (winter–summer), tissue specific (hepatopancreas and gills) and sex related responses. Results showed that hepatopancreas in male crabs better reflected inter-estuary differences. Morpho-condition traits as Cephalothorax hepatopancreas index (CHI) could be used as an indicator of physiological status of estuarine crabs. Discriminant analysis also showed that GST and TBARS levels in summer are more suitable endpoints for establishing differences between polluted and non-polluted sites. These results suggest the importance of seasonality, target tissue, sex and physiological status of brachyuran crabs for estuarine biomonitoring assessment.

1. Introduction

Field monitoring of the estuaries represents a major challenge, as they represent areas of great importance in coastal areas with numerous pollution problems (Amiard-Triquet and Rainbow, 2009), where detecting anthropogenic stress in naturally stressed areas is difficult using conventional ecological tools (Elliott and Quintino, 2007).

Estuaries in south-central Chile are characterized by either both high industrial pollution or low anthropogenic impacts. Lenga is a heavily-polluted estuary which has both mercury (Hg) pollution from accidental spills from chlorine-soda industries in the past decades (Díaz et al., 2001; Díaz-Jaramillo et al., in press) and a historical deposition of polycyclic aromatic hydrocarbons (PAHs) in sediments due to many crude oil spills and the settlement of nearby oil refinery and steel industries (Pozo et al., 2011). Both chemicals above mentioned are described as priority pollutants because of their toxic properties (Leonard and Hellou, 2001; UNEP, 2008). In contrast, Tubul and Raqui estuaries located in the Arauco Gulf, are associated with a lower anthropogenic pressure (Díaz-Jaramillo et al., 2011).

The use of biomarkers has been proposed as sensitive tools for biological effects monitoring, playing an important role in the risk assessment of complex ecosystems, such as estuaries (Monserrat et al., 2007). Oxidative stress responses such as activity of the enzyme glutathione-*S*-transferase (GST), total antioxidant capacity against peroxyl radical (ACAP) and reactive substances to thiobarbituric acid (TBARS) have been used as non-specific biomarkers in high anthropogenically-impacted estuaries, given the ability of certain pollutants to generate reactive oxygen species (ROS), altering the balance of pro-oxidants and antioxidants at the molecular and cellular level (Monserrat et al., 2007).

GST is a family of enzymes involved in the detoxification of both xenobiotics and endogenous substances, also acting in the first line of defense against pro-oxidants (Cnubben et al., 2001).

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Additionally, some authors consider the measurements of total antioxidant capacity, related to both enzymatic and non-enzymatic antioxidants as a general state of antioxidant defenses in the organism (Bocchetti and Regoli, 2006). The evaluation of oxidative damage includes the measurement of lipid peroxidation by-products (TBARS), being considered an index of peroxidation of membrane phospholipids and cited as an effect biomarker (Gorbi et al., 2008).

Additionally, morpho-condition traits such as cephalothorax hepatopancreas index (CHI), based on classic hepatosomatic index in fishes (Kime, 1995) with differences in the use of length despite the total weight of individual, could be used as a reliable physiological index, due to the potential variability of water content and loss of some chelipeds and/or legs in some invertebrate species (i.e. crustaceans) that affect the final total weight in some species.

Despite the prevalent use of some crustaceans as bioindicators of pollution (Rinderhagen et al., 2000), the use of oxidative stress responses for field monitoring in estuarine crabs is scarce, and mainly focused in Northern hemisphere species (Orbea et al., 2002; Pereira et al., 2009; Maria et al., 2009; Martín-Díaz et al., 2005). The estuarine crab *Hemigrapsus crenulatus* (Brachyura, Grapsoidea, Varunidae) (Milne Edwards, 1837) is one of the most abundant epibenthic species from Chilean estuarine habitats and widely distributed along the Chilean and New Zealand coasts (Retamal, 2000; Urbina et al., 2010). Several characteristics such as, wide distribution, great densities (10 individuals per m^2; Pulgar et al., 1995), their role in the detrital cycle and estuarine food web (Elumalai et al., 2007; Licandeo et al., 2006) as well its sexual dimorphism, make this species suitable for biomonitoring anthropogenic impacts in estuaries. Additionally *H. crenulatus* represents an advantage in order to assess eventual gender-specific responses.

Considering the influence of natural factors (i.e. temperature, salinity) is necessary to establish the seasonal variability of sub-individual responses (Orbea et al., 2002; Bocchetti and Regoli, 2006; Dissanayake et al., 2011). Moreover gender, reproductive and physiological status might affect some biochemical endpoints in addition to differentiated oxidative responses among tissues (Maria et al., 2009; Dissanayake et al., 2011; Paital and Chainy, 2010). The objectives of this study were to, therefore, determine seasonal levels of hepatopancreas and gills GST activity, total antioxidant capacity against peroxyl radicals, levels of TBARS in males and females of *H. crenulatus* in conjunction with some biometric measurements (cephalothorax width, cephalothorax/hepasomatic index) and female gonadal weight from estuaries under different anthropogenic pressure. The variability of these biochemical/physiological endpoints according to organ, season, and contaminant status of the estuaries allow us to evaluate the suitability of the estuarine crab *H. crenulatus* as a good bioindicator species in environmental monitoring programmes by using oxidative stress responses.

2. Materials and methods

2.1. Study area

Lenga, Tubul and Raqui estuaries are located in central-southern Chile (39° S, 73° W, Fig. 1). Lenga is a small estuary (3.2 km^2) located

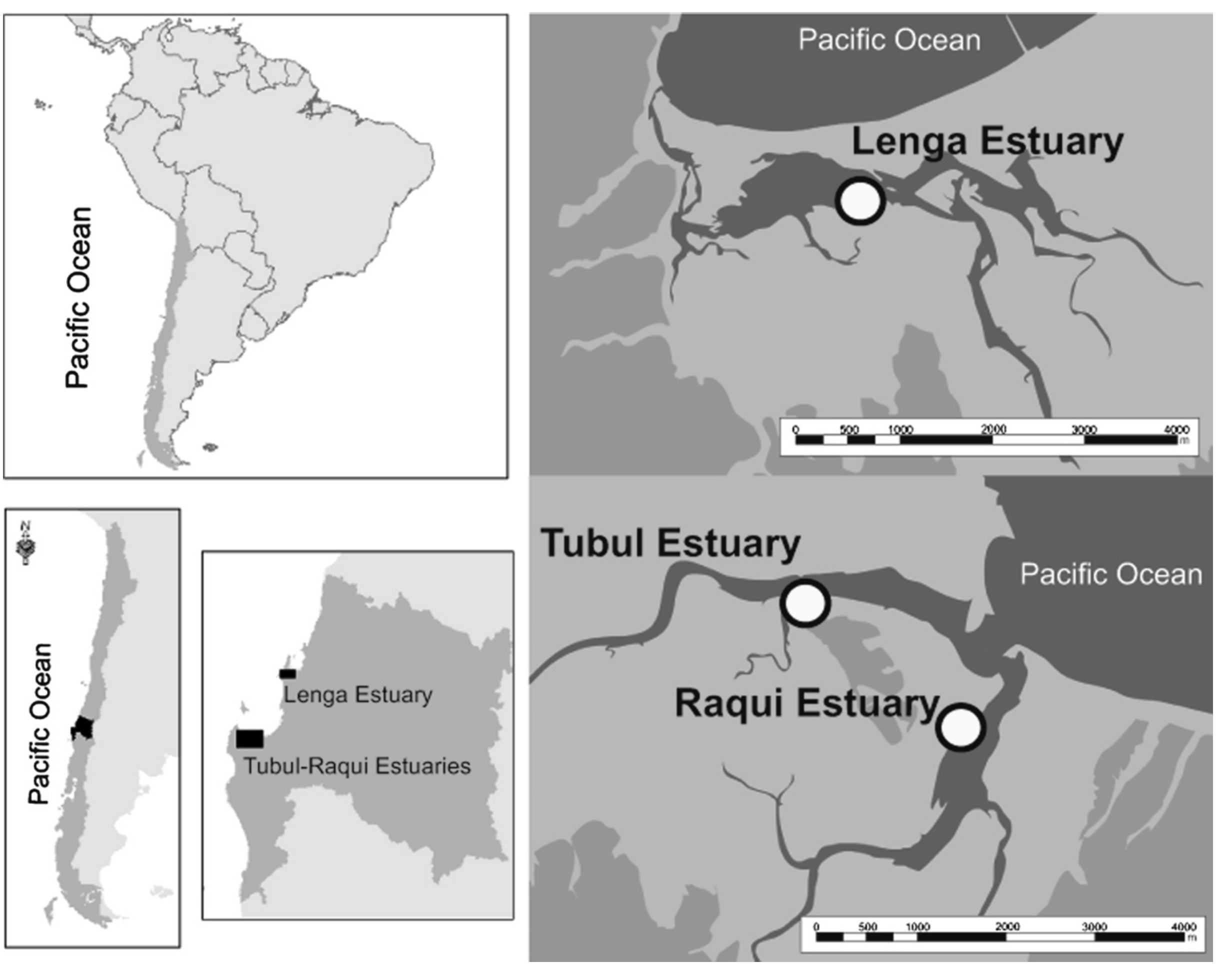

Fig. 1. *Hemigrapsus crenulatus* sampling sites from Lenga and Tubul-Raqui estuaries.

in San Vicente Bay, Biobio Region, Chile (36°45′ S; 73°10′ W). This estuary is heavily impacted by an adjacent chemical industrial complex including an oil refinery, steel mill, and other chemical industries (Ahumada et al., 2000). Tubul and Raqui estuaries (37°14′S; 73°26′W; Fig. 1) correspond with two respective rivers running into a small coastal-type basin catchment area, with an important salt marsh area (Díaz-Jaramillo et al., 2011). In terms of water and sediment physico-chemical variables, these estuaries present: (1) marked seasonal differences due to variables such as salinity related to the seasonal rainfall patterns (Díaz-Jaramillo et al., 2011); (2) predominance of a sandy fraction in sediments (Díaz-Jaramillo et al., 2010, 2011); (3) important organic enrichment in Lenga estuary, according to previous studies (Moscoso et al., 2006; Pozo et al., 2011); (4) high levels of Hg (0.4–12.6 mg/kg D.W) and PAHs (290–6118 ng/g D.W) in Lenga sediment layers compared with Raqui and Tubul estuaries, in some cases above of some marine sediments quality guidelines (MacDonald et al., 2000; Díaz-Jaramillo et al., in press; Pozo et al., 2011); (5) seasonal increment of sediment PAHs levels in Tubul in summer (Díaz-Jaramillo et al., 2011). For the purposes of the present study, the following estuaries were categorized a priori, from lowest anthropogenic impact to highest, thus, Raqui (lowest) < Tubul (intermediate) < Lenga (highest; Díaz-Jaramillo et al., 2011).

2.2. *H. crenulatus* sampling

Three estuarine sites, Lenga (36°46′11.48″S; 73°10′06.29″W), Tubul (37°13′54.01″S; 73°27′28.92″W), and Raqui (37°14′32.59″S; 73°26′21.86″W; Fig. 1), were sampled once each during the cold (August 2008) and warm (January 2009) seasons considering the same time and sampling sites from previous work on biomarker studies (Díaz-Jaramillo et al., 2011). On this basis, water physico-chemical characteristics from sampling sites exhibited the same seasonal pattern in terms of salinity (3.0–6.1 PSU in winter; 24.2–26.1 PSU in summer), temperature (9.3–14.0 °C in winter; 18.6–24.0 °C in summer), oxygen (8.9–10.2 mg/L in winter; 10.6–13.6 mg/L in summer) and pH (8.0–8.4 in winter; 8.2–8.3 in summer; Díaz-Jaramillo et al., 2011). In terms of Hg and PAHs concentration in their sediments, Lenga estuary was classified as polluted estuary (1.6–12.6 mg/kg Hg; 303.1–830.1 ng/g Total PAHs) and Tubul (0.02–0.18 mg/kg Hg; 18.1–346.5 ng/g Total PAHs) and Raqui (0.009–0.08 mg/kg Hg; 28.6–122.5 ng/g Total PAHs) as a low and non-polluted estuaries respectively (Díaz-Jaramillo et al., 2011). Oxidative stress responses and biometric index were assessed based on the larger-sized individuals observed in the sampling sites, presumably, thus, ensuring only adult individuals used in the study (Table 1). Male and female crabs in inter-molt stage were collected manually at low tide (n of males: 5–11; *n* of females: 8–10). Crabs were transported to the laboratory in areated water from each respective sampling sites, where upon arrival (1–3 h) the crabs were measured (cephalothorax width, CW) dissected and the organs extracted (hepatopancreas, gills and gonads (only females)), weighed and frozen at at −80 °C.

2.3. *H. crenulatus* cephalothorax hepatopancreas index (CHI)

CHI was determined using the relationships of the cephalothorax width (cm) (CW) with the hepatopancreas weight (g) by the following equation:

$$\text{CHI} = \text{hepatopancreas weight}/\text{cephalothorax width (CW)}*100.$$

2.4. *H. crenulatus* tissue homogenization and oxidative stress responses

For the biochemical measurements of protein content, GST and total antioxidant capacity, hepatopancreas and gills tissues were homogenized (1:3 w/v) in ice-cold buffer (20 mM Tris-base, 1 mM EDTA, 1 mM DL-dithiothreitol, 500 mM sucrose and 150 mM KCl) with pH adjusted to 7.60 (Geracitano et al., 2002). Homogenates were centrifuged at 9000 × *g* for 45 min (4 °C) and the supernatants were collected and stored at −80 °C for later use. For TBARS measurements, target tissues were homogenized in 1.15% KCl (1:5 w/v) containing 35 μM butylated hydroxytoluene (BHT), and stored at −80 °C until analysis (Oakes and Van Der Kraak, 2003). The activity of glutathione-*S*-transferase (GST) and thiobarbituric acid reactive substances (TBARS) were evaluated with methods fully described elsewhere (Habig and Jakoby, 1981; Oakes and Van Der Kraak, 2003). Total antioxidant capacity against peroxyl radicals (ACAP) was measured according to Amado et al. (2009) in a fluorescence microplate reader (Victor 2, Perkin Elmer), in wavelengths of 488 and 525 nm, for excitation and emission, respectively. Briefly, the relative difference between ROS area with and without pro oxidant 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) was considered a measure of antioxidant capacity, with high area difference meaning low antioxidant capacity, since high fluorescence levels were obtaining after adding

Table 1
Cephalotorax width (CW); cephalotorax/hepatopancreas index (CHI) and gonad weight (mean ± S.E) of *Hemigrapsus crenulatus* individuals used for oxidative stress responses from Lenga and Tubul-Raqui estuaries in winter and summer season (2008–2009). Different letters indicate significant differences among sampling sites and asterisk indicates seasonal significant differences respect winter ($p < 0.05$).

Sex/Morpho-condition traits	Season	Sites		
		Lenga	Tubul	Raqui
Male				
Cephalotorax width (cm) (CW)	Winter	3.47 ± 0.08^{a}	2.74 ± 0.09^{b}	3.25 ± 0.08^{a}
	Summer	3.40 ± 0.05^{a}	3.30 ± 0.04^{ab*}	3.05 ± 0.09^{b}
Cephalotorax/hepatopancreas index (CHI)	Winter	17.78 ± 1.23^{a}	12.48 ± 0.89^{b}	14.83 ± 1.78^{ab}
	Summer	16.16 ± 1.12^{a}	16.12 ± 1.46^{a}	14.39 ± 1.16^{a}
Female				
Cephalotorax width (cm) (CW)	Winter	3.11 ± 0.10^{a}	2.87 ± 0.05^{b}	2.78 ± 0.08^{b}
	Summer	3.04 ± 0.08^{a}	2.84 ± 0.07^{a}	2.87 ± 0.07^{a}
Cephalotorax/hepatopancreas index (CHI)	Winter	15.93 ± 1.20^{a}	11.48 ± 0.75^{b}	11.87 ± 1.43^{b}
	Summer	13.83 ± 0.85^{a}	11.60 ± 1.34^{a}	12.25 ± 1.12^{a}
Gonadal weight (g)	Winter	0.29 ± 0.03^{a}	0.24 ± 0.05^{a}	0.21 ± 0.02^{a}
	Summer	0.02 ± 0.00^{a*}	0.03 ± 0.01^{a*}	0.02 ± 0.00^{a*}

ABAP, meaning low competence to neutralize peroxyl radicals, conversely a low difference area meaning high antioxidant capacity (Amado et al., 2009).

2.5. Statistical analysis

Significant changes in oxidative stress responses and biometric measurements in male and female tissue crabs were tested by analysis of variance (ANOVA) using Neuman–Keuls test for post-hoc comparisons ($\alpha = 0.05$). Data were checked to meet the assumptions of normality and homogeneity of variances prior to analysis. Principal components analysis (PCA) was performed on both sexes in order to visualize in an integral way the behavior of the oxidative stress responses due to combinations of factors and their relationship with polluted and non-polluted sites. PCA was performed using the software PRIMER V.6 (Clarke and Gorley, 2005). Additionally, discriminant analyses (DAs) were performed in order to determine whether sites with different level of pollution (high, low and non-polluted) could be differentiated based on oxidative stress responses. In order to evaluate the season in which oxidative stress responses matrix could better discriminate between polluted and non-polluted sites, discriminant functions were obtained for each season by estimating the Wilks' lambda value. Wilks's lambda values ranging from 0 to 1 and represent the proportion of the total variance in the discriminant scores not explained by differences among the groups. In order to assess the differences between sites, the values of each site along the first two discriminant (canonical) axes were represented with confidence limits at 95%. We then performed a stepwise discriminant analysis to select the subset of the quantitative variables (biochemical responses) that better discriminate between groups. A forward-stepwise method with a tolerance value of 0.15 was used.

3. Results

GST activity in male crabs varies significantly between hepatopancreas (h-) and gill (g-) tissues ($p < 0.05$; Fig. 2a). Inter-estuary differences were observed only in hepatopancreas, where crabs

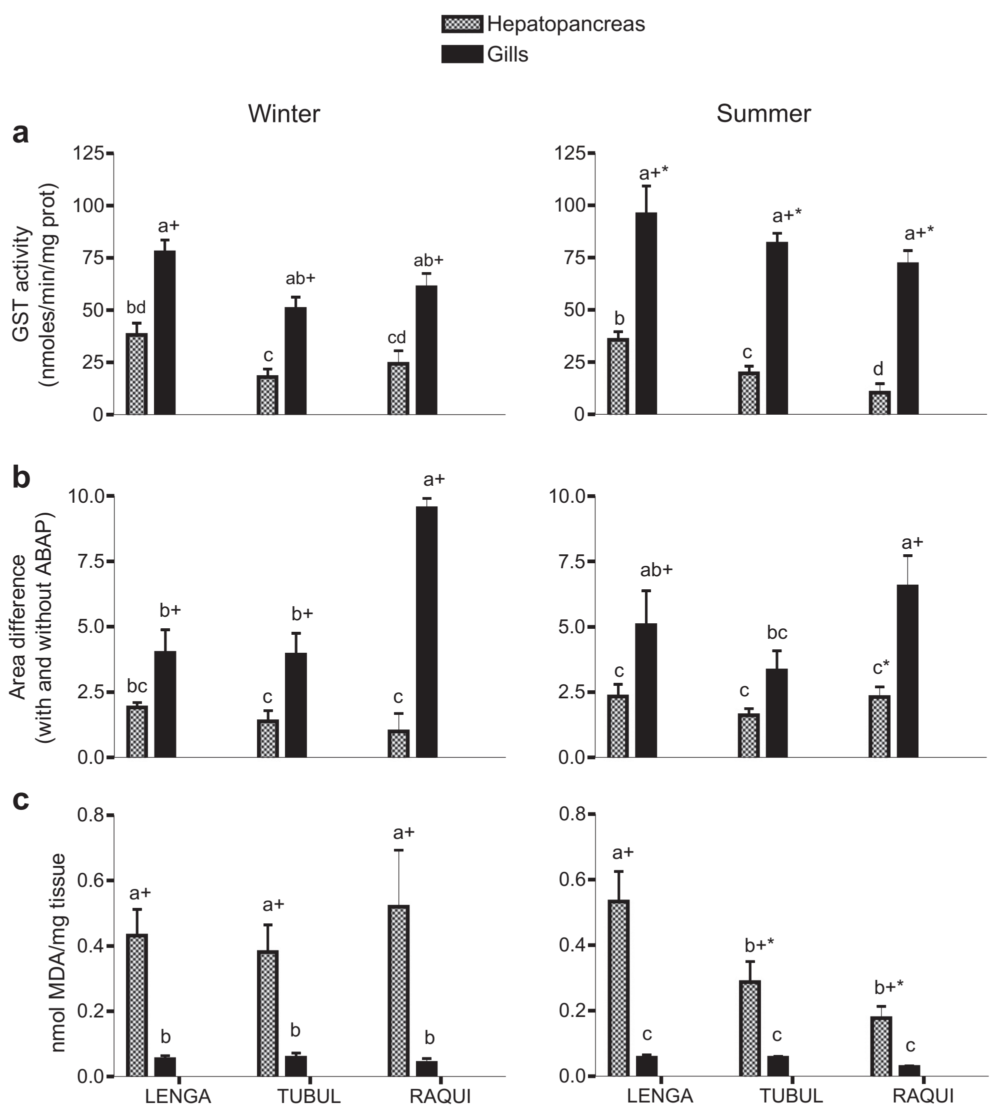

Fig. 2. Mean values (±1 SE) for: (a) glutathione-*S*-transferase (GST) activity, (b) total antioxidant capacity against peroxyl radicals and (c) thiobarbituric reactive substances (TBARS) levels in hepatopancreas and gills of male *Hemigrapsus crenulatus* from Lenga and Tubul-Raqui estuaries in winter and summer seasons. Lower values of relative area in ACAP data indicate higher antioxidant capacity. Different letters indicate significant differences among sampling sites, plus symbols indicate tissue significant differences and asterisks indicate seasonal significant differences respect winter ($p < 0.05$).

from Lenga show a significant increase respect other sites in summer ($p < 0.05$; Fig. 2a). Male crabs ACAP show only significant differences between organs, a significant increase was observed in hepatopancreas tissues compared to gills ($p < 0.05$; Fig. 2b). Although we found a significant decrease in h-ACAP in males from Raqui compared to other sampling sites ($p < 0.05$; Fig. 2b), no consistent inter-estuary differences patterns were observed in h-ACAP from male organs (Fig. 2b). In contrast, a significant increase was observed in h-TBARS male crabs compared to g-TBARS ($p < 0.05$; Fig. 2c). No inter-estuary differences in TBARS male crabs were observed in winter in both organs ($p < 0.05$; Fig. 2b). However, h-TBARS levels from male crabs show inter-estuary differences in summer, where Lenga male crabs show a significant increase compared to Tubul and Raqui h-TBARS ($p < 0.05$; Fig. 2b). Seasonal significant differences in male crabs organs were observed in g-GST activity in all sampling sites, h-ACAP from Raqui male crabs and h-TBARS from Tubul and Raqui estuaries ($p < 0.05$; Fig. 2).

Female crabs did not show significant GST and ACAP inter-estuary differences in both seasons ($p < 0.05$; Fig. 3a and b). Significant differences in female GST activity and ACAP were only organ specific, with a higher g-GST activity and lower g-ACAP ($p < 0.05$; Fig. 3a and b). TBARS levels on female crabs also show organ specific differences, with significant low levels on gills respect hepatopancreas ($p < 0.05$; Fig. 3c). Intersite differences were observed in Raqui h-TBARS respect other sites on winter ($p < 0.05$; Fig. 3c). Furthermore, a significant increase of h-TBARS in summer was observed in Lenga and Tubul compared to Raqui ($p < 0.05$; Fig. 3c). Seasonal differences in female crabs were only observed for g-GST in all sites as well as h-TBARS levels from Raqui ($p < 0.05$; Fig. 3).

Inter-estuary differences in male cephalothorax width (CW) were observed in Tubul in winter, a significant decrease was observed in Tubul crabs respect both sites ($p < 0.05$; Table 1). In the warm season, male crabs from Raqui show a significant decrease compared to Lenga ($p < 0.05$; Table 1). Seasonal differences regarding CW were observed only in males from Tubul estuary ($p < 0.05$; Table 1). Females CW only show inter-estuary differences in Lenga respect Tubul and Raqui crabs on winter without seasonal differences in all sampling sites ($p < 0.05$; Table 1).

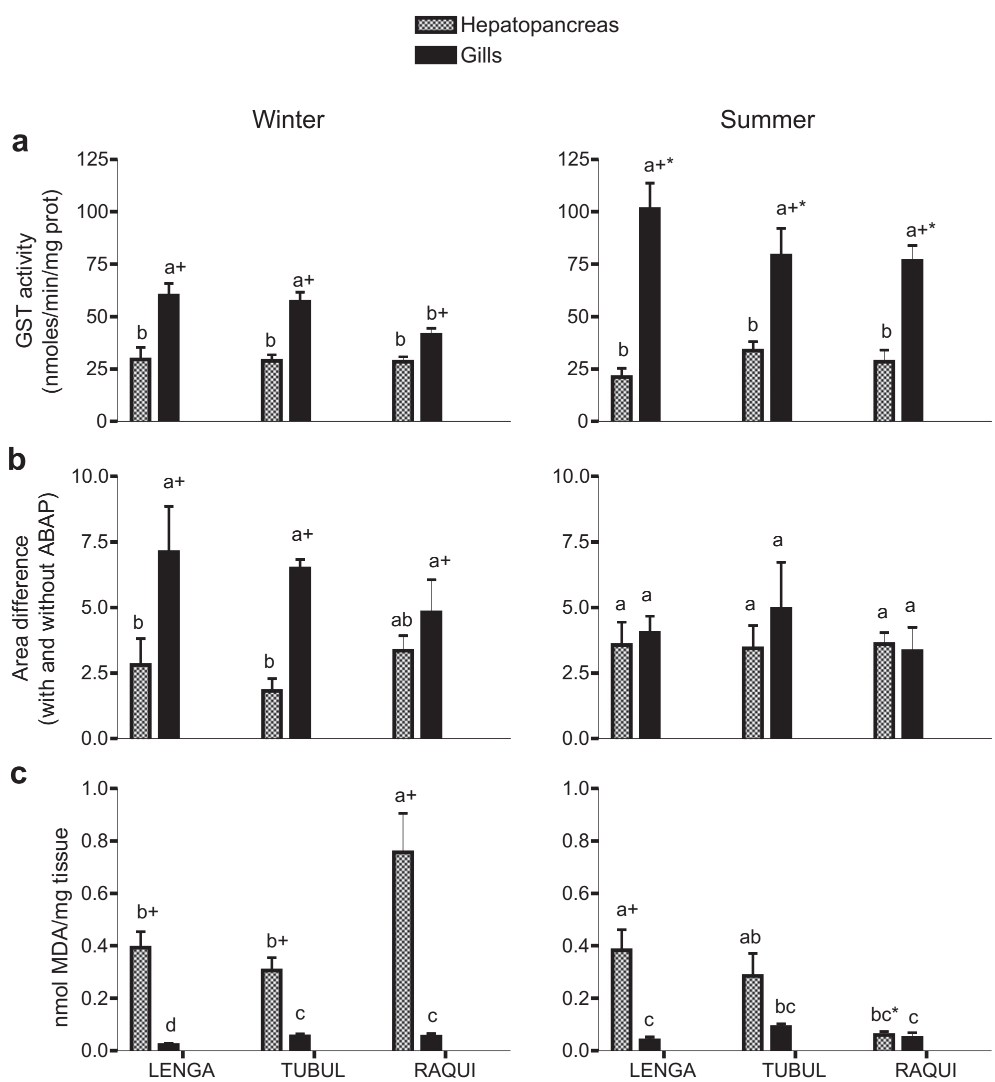

Fig. 3. Mean values (±1 SE) for: (a) glutathione-*S*-transferase (GST) activity, (b) total antioxidant capacity against peroxyl radicals and (c) thiobarbituric reactive substances (TBARS) levels in hepatopancreas and gills of female *Hemigrapsus crenulatus* from Lenga and Tubul-Raqui estuaries in winter and summer seasons. Lower values of relative area in ACAP data indicate higher antioxidant capacity. Different letters indicate significant differences among sampling sites, plus symbols indicate tissue significant differences and asterisks indicate seasonal significant differences respect winter ($p < 0.05$).

Cephalothorax hepatopancreas index (CHI) show inter-estuary differences only in the cold season ($p < 0.05$; Table 1), where a significant increase was observed in Lenga in both sexes compared to Tubul and Raqui ($p < 0.05$; Table 1). No inter-estuary differences were observed in summer ($p < 0.05$; Table 1). Female gonad weights exhibit a significant seasonal decrease in all sites during summer ($p < 0.05$; Table 1).

In order to compare the combinations of variables in both sexes, a Principal Component Analysis (PCA) was applied, using seasonal oxidative stress responses plus biometric measurements made in male hepatopancreas (the most responsive organ). The results indicated that the first two components explain 60% of the variation and indicated that male crabs from Lenga show differences with the other sites, in terms of highest GST activity, highest TBARS levels together with a long cephalothorax size (CW) (Fig. 4a). In contrast, PCA made with significant responses found in female crabs (Fig. 4b), did not show clear differences between sites where the first two components explain more than 59% of the variation (Fig. 4b).

Discriminant analysis (DA) indicated that oxidative stress responses matrix in summer can discriminate between polluted, low and non-polluted sites both in male (wilks λ: 0.30; $p = 0.001$) and female crabs (wilks λ: 0.39; $p = 0.02$) respectively (Table 2). Contrastingly, oxidative stress responses in winter, did not contribute to discrimination between sites with varying levels of pollution, either in male or female crabs (Table 2). In order to evaluate the importance of each predictor variable we estimated the standardized canonical coefficients. Table 3 shows the discriminant power of each oxidative response in males and females crabs during summer (the most responsive season). In the first canonical axis the variables GST and TBARS exhibited the highest discriminant power both in males and females ($p < 0.05$), while the variable ACAP was the lowest predictor ($p > 0.05$; Table 3). Consequently, GST and TBARS stand out as the variables that strongly contribute to the allocation of samples to the polluted or non-polluted groups.

Table 2
Hemigrapsus crenulatus discriminant analysis (DA). Wilks' lambda for seasonal/sex discriminant function performed with hepatopancreas biochemical responses. Wilks' lambda value varies from 0 to 1, with 0 meaning group means differ and highly contributes to the discriminant functions, and 1 meaning all group means are the same.

Season/sex	Wilks' Lambda	*F*	*p*
Winter males	0.55	1.71	0.15
Winter females	0.55	1.77	0.14
Summer males	0.30	5.16	**<0.01**
Winter females	0.39	3.03	**0.02**

Bold values indicate significant differences.

4. Discussion

The organ specific patterns, presently observed in antioxidant responses are similar to other related crab species, where gills tissue distribution of antioxidant enzymes are different to other target organs (Maciel et al., 2004; Paital and Chainy, 2010). On this basis, the higher GST activity on gills from both sex of *H. crenulatus* are similar from that reported for other estuarine species e.g. *Carcinus maenas* and *Neohelice* (*Chasmagnathus*) *granulata*, that showed higher gills activity of GST and another antioxidant enzymes such as catalase and superoxide dismutase than hepatopancreas (Elumalai et al., 2007; Maciel et al., 2004). Despite the high activity of gill GST, total antioxidant capacity against peroxyl radicals was lower than hepatopancreas. Furthermore, low levels of lipid peroxidation compared to hepatopancreas and the absence of inter-estuary differences in gill tissues suggest the importance of GST in both detoxification and lipid peroxidation processes in organs directly exposed to water and surface sediment layers (Elumalai et al., 2007). However, due to the differences in gill morphology according to distinct physiological process related to respiration, acid–base balance and osmotic–ionic regulation in some estuarine crabs (Bianchini et al., 2008), future studies must be focused in the assessment of differences in ROS production according to potential physiological differences in *H. crenulatus* gills.

Inter-estuary differences were evident in oxidative stress responses from hepatopancreas, which is associated with diverse metabolic activities ranging from supply of nutrition to ovary, to digestion and absorption of nutrients (Paital and Chainy, 2010). Differences were reflected by high GST activity in males and high levels of TBARS in both sexes from Lenga estuary. Furthermore, these differences were enhanced during the warm season. GST induction by Hg and some PAHs has been described in some aquatic species as effective inducer of this phase II detoxification enzyme (Elumalai et al., 2007; Lu et al., 2009). Also, the bioaccumulation capacity of Hg (both total and organic) of *H. crenulatus* hepatopancreas has been shown previously in Lenga (1.50–1.59 mg/kg

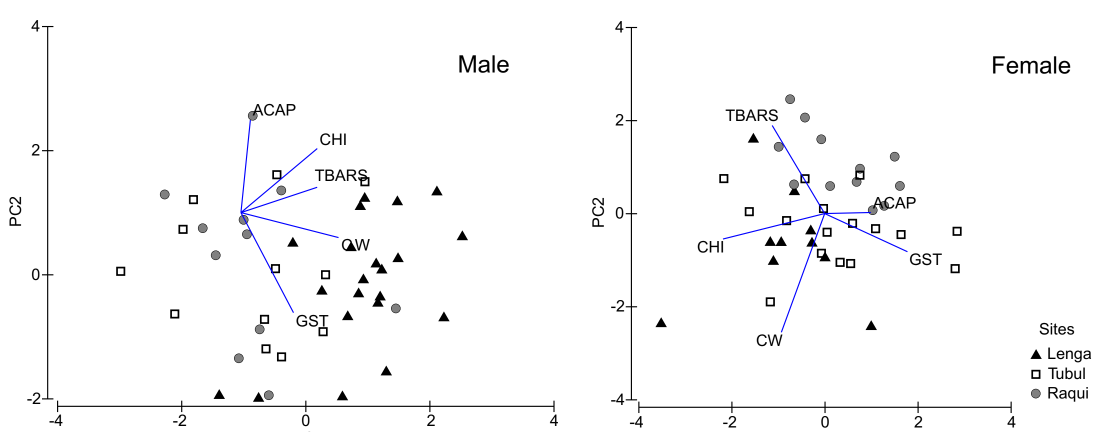

Fig. 4. Principal component analysis (PCA) performed with morpho-condition traits and biochemical responses obtained from hepatopancreas of male and female *Hemigrapsus crenulatus* individuals from Lenga and Tubul-Raqui estuaries in winter and summer seasons. Glutathione-*S*-transferase (GST) activity, total antioxidant capacity (ACAP), lipid peroxidation levels (TBARS), cephalothorax/hepatopancreas index (CHI) and cephalothorax width (CW).

Table 3
Hemigrapsus crenulatus discriminant analysis (DA). Standardized canonical discriminant function coefficients (CAN1 and CAN2) performed with hepatopancreas biochemical responses of male and female individuals from summer.

Sex/biochemical responses	Canonical axes		
	CAN1	CAN2	*p*
Female			
GST	1.27	0.87	**0.006**
ACAP	−0.25	−0.74	ns
TBARS	1.34	−0.15	**0.06**
Male			
GST	0.98	0.44	**0.002**
ACAP	0.09	0.66	ns
TBARS	0.85	−0.52	**0.001**

Bold values indicate significant differences.

T-Hg; 0.75–1.11 mg/kg Org-Hg) comparing to Tubul (0.14–0.21 mg/kg THg; <detection limits mg/kg Org-Hg) specimens (Díaz-Jaramillo et al., in press). Furthermore GST activity is not enough to compensate high hepatopancreas TBARS levels from Lenga in summer for both sexes, indicating a non-successful ROS neutralization by antioxidants from this organ. Seasonal increment of PAHs levels from Tubul are also reflected in TBARS levels on female and GST activity on males, similar to those reported in some biochemical endpoints in other estuarine species (Díaz-Jaramillo et al., 2011).

Seasonal differences in oxidative stress responses were mainly related by the enhanced activity in certain endpoints in summer (i.e. gills GST) and the absence of clear pattern between polluted and non-polluted sites on winter. On this basis, high water temperature and salinity could increase the susceptibility to contaminants by the reduced ability to sequester ROS in summer (Bocchetti and Regoli, 2006; Paital and Chainy, 2010). A possible mechanism could be related to high temperature and salinity act as additive stressor, enhanced ROS production and consequently increasing the oxidative pressure (Regoli et al., 2004; Paital and Chainy, 2010). These results inferred that the warm season is adequate to establish oxidative stress responses related to pollution problems in *H. crenulatus*.

In contrast, winter season indicates that not all results in oxidative stress responses fit the "expected" pattern of contaminant-related responses, where some confounding factors seem to play an important role. A maximum "peak" in reproductive activity, reported in *H. crenulatus* from Lenga and other south-central Chilean estuaries at the end of winter season (August) (Retamal, 1969; Pardo pers. con.) could play an important factor of "biological noise" when set polluted related responses by biochemical endpoints in aquatic species (Chiang et al., 2011). On the other hand, important decrease of salinity on winter due by the high seasonal rainfall pattern observed in these coastal-type basin estuaries (Díaz-Jaramillo et al., 2011) is a critical factor in terms of osmotic tolerance (Kang et al., 2008) and in terms of high energetic cost. The increased energy losses at low salinity due to respiration and excretion process were reported in *H. crenulatus* affected the physiological status of these species (Urbina et al., 2010). Seasonal differences in terms of depressed physiological and antioxidant status of other estuarine crabs on winter from low polluted estuaries were also reported (Dissanayake et al., 2011), supporting our results from some oxidative stress endpoints of Raqui crabs in the cold season.

Cephalothorax hepatopancreas index (CHI) could be used as an indicator of physiological status of estuarine crabs. Thus, in summer when CHI values where similar in between sites, antioxidant responses were clearly related with the pollution condition of these estuaries allowing the determination if there is no evidence of an altered physiological status between sites. On this physiological status antioxidant and oxidative damage responses are adequate pollution biomarkers in these decapod species.

The influence of sex in the oxidative stress responses reported here were evident and based on the gender-specific PCA, it can be inferred that males are more suitable for establish differences between polluted and non-polluted sites through these responses. However gender-specific sensitivity of oxidative responses varies among crabs species and specific oxidative stress responses (Maria et al., 2009). Differences in behavior and feeding habits between males and females reported in *H. crenulatus* (Retamal, 1969) could influence distinct bioaccumulation pattern of xenobiotics, which is known between sexes (Newman and Unger, 2003). Moreover, based on discriminant analysis (DA) that also reaffirms the seasonal differences and summer months as suitable season for discriminant between polluted and non-polluted sites. DA also showed that GST and TBARS levels appear to be an effective endpoint to discriminate polluted and non-polluted sites in decapod crustaceans in certain seasons (Koenig et al., 2009).

From an ecotoxicological point of view to establish the "health" status of coastal water bodies based on endemic invertebrates and biological effects monitoring, the holistic view only based in the multiple physiological techniques for assessment the chemical impacts on organisms (i.e. Multibiomarker approach, Galloway et al., 2004) is not enough in these environments. The strong seasonality of estuarine environments, suggest the variability of these responses and the difficulty to establish anthropogenic impact. A gap in knowledge is the assessment of seasonality of endpoints in order to estimate "windows of sensitivity" (Dissanayake et al., 2011) in this case according to find periods with the same or related physiological status in organisms when compared different estuarine areas. On this basis, sampling design of studies with adequate seasonal replication (e.g. monthly) are necessary in the future to better aid site discrimination considering the level at which provides the most variability (Schmitt and Osenberg, 1996). In contrast to infaunal benthic organism like *Perinereis gualpenesis*, which showed more consistent, polluted related oxidative stress responses in Lenga sampling site during the year (Díaz-Jaramillo et al., 2011). Epibenthic crabs like *H. crenulatus*, is also subjected to pronounced natural stressors (e.g. salinity), which in addition to mobility into the estuary diminish the ability to gives sites-specific responses. Finally, an important point of discussion in future studies is the influence of parasitism in biomarker responses and some reproductive endpoints (Marcogliese and Pietrock, 2011) since estudies in *H. crenulatus* from Lenga estuary have reported high parasitism rates by acantocephalans (Haye and Ojeda, 1998) that could be affecting sub-individual/reproductive responses and finally the reallocation of energy.

4.1. Conclusions

The present study provides information about the tissue-gender-specific and seasonal responses in some oxidative stress endpoints in *H. crenulatus*, first indicating that hepatopancreas best reflect differences among high and low polluted sites. Seasonal influence was evident on these endpoints, whereas the warm season, when individuals had the same physiological condition (CHI), seems to be adequate for the assessment of differences on estuarine pollution status. Therefore, CHI index constitute an easy and non-expensive tool for screening the physiological status of crabs helping in choose the sampling time as well to avoid data misinterpretation when assessing inter-estuary differences by biochemical endpoints. Oxidative stress responses in *H. crenulatus* are a good indicator of estuarine sediment pollution. However, seasonality, gender and tissue-type are critical factors to distinguish between polluted and non-polluted sites. On this basis, hepatopancreas male crabs from warm season and biochemical responses like GST and TBARS emerge as adequate variables and potential biomarkers in estuarine environmental monitoring survey.

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