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Between-habitat comparison of digestive enzymes activities and energy reserves in the SW Atlantic euryhaline burrowing crab *Neohelice granulata*

Silvina A. Pinoni, Oscar Iribarne, Alejandra A. López Mañanes *

Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina CONICET, Argentina

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A B S T R A C T

The digestive and metabolic characteristics at the biochemical level underlying between-habitat dietary shift of the SW Atlantic euryhaline burrowing crab *Neohelice granulata* under natural conditions are unknown. We made studies on adult males of *N. granulata* from the open mudflat and the vegetated saltmarsh in a SW Atlantic costal lagoon (Mar Chiquita, 37°32′–37°45′S; 57°19′–57°26′W, Argentina). We determined and compared amylase, maltase, sucrase, proteolytic, lipase and alkaline phosphatases activities in the hepatopancreas; glycemia, and glycogen, free glucose, triglycerides and protein concentrations in hepatopancreas, chela muscle, and anterior and posterior gills. The results show that *N. granulata* exhibits characteristics and between-habitat differences at the biochemical level (i.e. high amylase and disaccharidase activities, differences in total proteolytic, lipase and levamisole-insensitive AP activities in the hepatopancreas, and in the concentrations of glycogen in the gills, triglycerides in the hepatopancreas and of protein in the chela muscle) which could represent adaptive digestive and metabolic strategies to face the differences in environmental conditions (i.e. food availability). The possible relationship between digestive and metabolic characteristics and feeding patterns, type of food available and environmental conditions in each habitat is discussed.

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1. Introduction

The ability to balance the acquisition, storage and use of energy in the natural ambient is critical for survival, growth, maintenance and reproductive success of animals (Secor, 2001, 2005; Karasov and Martinez del Rio, 2007). Thus, to know the digestive and metabolic characteristics (i.e. at the biochemical level) and possible adjustments in the natural ambient constitutes an important clue to evaluate the performance of an individual, particularly in and/or between heterogeneous habitats concerning food quality and/or availability. The dietary habit of an animal is linked to specific behavioral, physiological and morphological features as well as with the life style. The extent to which digestive features allow or constrain shifts in diet constitutes a central issue in studies of foraging ecology and digestive physiology (Weiner, 1992; Starck, 1999; Karasov and McWilliams, 2005). Phenotypic flexibility, one category of phenotypic plasticity, implies reversible within-individual variations in phenotypic traits which can increase the chances of survival for animals facing spatio-temporal changes in environmental conditions (De Witt et al., 1998; Piersman and Drent, 2003; Hollander et al., 2006; Pfenning et al., 2010). Species showing a dietary shift in the natural ambient upon temporal and/or spatial variations in food quality and/or availability are expected to exhibit underlying digestive characteristics and/or adjustments supporting the differences in food intake (del Valle and López Mañanes, 2008; Naya, et al., 2008). A differential modulation of key digestive enzymes activity appears to constitute an important strategy at the biochemical level in the natural ambient to face temporal variations in food availability and/or energy demands in various animals (del Valle and López Mañanes, 2008; Figueiredo and Anderson, 2009; Iglesias et al., 2009). Animals successfully occupying contrasting and differential habitats (i.e. in the type of available food) within the same geographical area would be expected to exhibit digestive and metabolic strategies allowing the maintenance of energy balance. However, within-species studies on spatial variations in digestive and metabolic characteristics and/or adjustments at the biochemical level in the field, particularly in some ecologically important groups such as estuarine intertidal crabs are lacking. Moreover, the knowledge about the digestive characteristics at the biochemical level and the possible adjustments in key digestive enzymes in the natural ambient in estuarine crabs is quite scarce. To our knowledge, the works of McClintock et al. (1991) and Johnston and Freeman (2005) are the only one done on the occurrence of key digestive enzymes activities in the hepatopancreas (the main site of

* Corresponding author. Funes 3250 (7600) Mar del Plata, Argentina. Tel.: +54 223 4753554.
E-mail address: mananes@mdp.edu.ar (A.A.L. Mañanes).

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digestive enzymes production in decapod crustaceans) of estuarine crabs under natural conditions. However, these studies were done in crabs living in the same habitat.

Neohelice (*Chasmagnathus*) *granulata* is a semiterrestrial burrowing euryhaline crab distributed on intertidal mudflats and saltmarshes of the South Western Atlantic from southern Brazil to the northern Argentinean Patagonia (Boschi, 1964; Botto and Irigoyen, 1979; Spivak et al., 1994; Spivak, 1997; Iribarne et al., 1997, 2003) in which it has a key ecological role by controlling and/or affecting different physico-chemical and biological aspects in these areas (Iribarne et al., 2003; Bianchini et al., 2008). *N. granulata* is one of the dominant crabs in Mar Chiquita coastal lagoon (37°32′–37°45′S; 57°19′–57°26′W, Argentina) in which inhabits the whole intertidal area from bare mudflat to vegetated saltmarsh habitats covered mainly by the cordgrass *Spartina densiflora* (Spivak et al., 1994; Bortolus and Iribarne 1999; Anger et al., 2008; Fanjul et al., 2008). In the field, *N. granulata* changes its dietary behavior from omnivorous–detritivorous in the mudflat to herbivorous–detritivorous in the saltmarsh in relation to the between-habitats differences in food availability, reflecting an opportunistic and flexible feeding nature in relation to food items available (Iribarne et al., 1997; Bortolus and Iribarne, 1999; Bortolus et al., 2004; Botto et al., 2005). Digestive enzymes are a complementary tool useful for determining which dietary components are most effectively metabolized (Brethes et al., 1994; Johnston and Freeman, 2005). Preliminary works under laboratory conditions, show that *N. granulata* from both the open mudflat and the saltmarsh exhibits high activity of key digestive enzymes in the hepatopancreas suggesting its ability to utilize a broad range of dietary items (Asaro, 2009; Asaro et al., 2009; Pinoni, 2009; Michiels, 2010; Michiels et al., 2010; Pinoni and López Mañanes, 2010). However, no studies have been done addressing the digestive and/or metabolic characteristics at the biochemical level in the natural ambient and, furthermore, the possible between-habitats adjustments underlying the changes in dietary behavior.

The aim of this work was to determine the digestive and metabolic characteristics at the biochemical level of *N. granulata* in their natural ambient and the possible between-habitat differences. We determined and compared the amylase, maltase, sucrase, proteolytic, lipase and alkaline phosphatases activities in the hepatopancreas; glycemia, and the concentration of glycogen, free glucose, triglycerides and protein in the hepatopancreas, chela muscle, and anterior and posterior gills of adult males of *N. granulata* from the mudflat and the saltmarsh of Mar Chiquita lagoon. We hypothesized that *N. granulata* exhibits digestive and metabolic strategies at the biochemical level in the natural ambient which represent adaptive strategies to face the between-habitats differences in environmental conditions upon dietary shift in Mar Chiquita coastal lagoon. The possible relationship between digestive and metabolic characteristics and feeding patterns, type of food available and environmental conditions in each habitat is discussed.

2. Materials and methods

2.1. Study sites and animal collection

Mar Chiquita coastal lagoon (37°32′–37°45′S, 57°19′–57°26′W) (Argentina) is a body of brackish water (approximately 46 km^2) affected by low amplitude (≤1 m) tides (Spivak et al., 1994) and characterized by mudflat surrounded by a large cordgrass *S. densiflora* area (Olivier et al., 1972; Fasano et al., 1982). Crabs were live trapped during April–May 2009 (autumn) in burrows of two sites: the open mudflat and the saltmarsh dominated by cordgrasses. Only adult male crabs with a carapace width greater than 2.5 cm were caught. All individuals collected were in intermolt (Drach and Tchernigovtzeff, 1967). Captures were made at midday. In autumn, between-habitat movement of individuals of *N. granulata* is highly reduced; individuals remained inside burrows most of the time. Furthermore, in the mudflats the tidal movement of individuals is restricted even upon changes in environmental conditions (Luppi, personal communication). The amplitude of salinity and temperature variations in both habitats and between-habitats is lower in this time of the year. The values of salinity in burrows water in the sites of collection ranged from 21 to 37‰ in the mudflats and 24 to 37‰ in the saltmarshes while temperature in the burrows ranged from 3 to 17 °C in the mudflat and 7 to 11 °C in the saltmarsh. The air temperature values ranged from 3 to 20 and 3 to 17 °C in the mudflat and the saltmarsh, respectively (Luppi, personal communication; Pinoni, personal observations). Animals were transported to the laboratory on the day of collection in the water of the site of collection under continuous aeration.

2.2. Chemicals

Maleic acid, azocasein, (3,5)-dinitrosalicylic acid (DNS), pNPpalmitate (*p*-nitrophenylpalmitate), Tris-(hydroxymethylamino-methane) (Tris), ethyleneglicol *N*,*N*′,*N*′-tetraacetic acid (EGTA), bovine serum albumin and levamisole (L [−]-2, 3, 5, 6-Tetrahydro-6-phenylimidazol [2, 1-b] thiazole) (CAS No. 16595-80-5) were from Sigma (St. Louis, MO, USA); sucrose was obtained from Merck (Darmstadt, Germany); α-amyloglucosidase from *Aspergillus niger*, pNPP (*p*-nitrophenylphosphate), magnesium sulphate and Coomassie blue G250 were from Fluka (Germany); maltose was from ICN (Ohio, USA). All solutions were prepared in glass-distilled water.

2.3. Sampling procedures

Crabs were weighed and cryoanesthesized by putting them on ice for about 20 min. A sample of hemolymph was withdrawn for assaying of the concentration of glucose as described below. Both chelae were cut off and carapaces were removed. The hepatopancreas, chela muscle, anterior (1–5) and posterior (6–8) gills (López Mañanes et al., 2000) were immediately excised and weighed. Wet mass was measured to the nearest 0.01 g. After weighing, the hepatopancreas, chela muscle, anterior and posterior gills were immediately used to prepare the corresponding homogenates and enzyme extracts as follows. The hepatopancreas was homogenized in 0.1 M Tris/HCl pH 7.4 (4 mL g^{-1} of tissue) (CAT homogenizer ×120, tool T10) and centrifuged at 10,000×*g* for 15 min (Sorval, rotor SS34, refrigerated). Before centrifugation, an aliquot of homogenate was separated to be used for the determination of glycogen, triglycerides and proteins content. The supernatant was separated into 200 μL aliquots and stored at −20 °C for enzymatic assays. Glycerol (1.3% v/v) was added to supernatant samples before freezing (Ljungström et al., 1984). The chela muscles were mixed with homogenizing medium (0.25 M sucrose/0.5 mM EGTA–Tris, pH 7.4) (8 mL g^{-1} of muscle tissue) and homogenized with CAT homogenizer ×120, tool T10 on ice. The homogenate was fractionated into 400 μL aliquots and stored at −20 °C. The muscles from both chelae of one individual were pooled and used for each preparation of homogenate. The anterior and posterior gills were mixed with homogenizing medium (0.25 M sucrose/0.5 mM EGTA–Tris, pH 7.4) (4 mL g^{-1} of tissue) and homogenized on ice with 20 strokes in a motor-driven hand-operated Teflon-glass homogenizer (Potter-Elvehjem, 1700 rpm). The homogenate was separated into 200 μL aliquots and stored at −20 °C.

2.4. Biochemical assays

The determination of enzyme activities was always performed with samples without any previous thawing. The freezing procedure did not alter the activity values, activities being stable at least for up to eight months of freezing. The assay conditions used were the optimal for the corresponding activity in the hepatopancreas of *N. granulata* as determined in previous works in our laboratory (Asaro, 2009; Asaro et al., 2009; Pinoni, 2009; Michiels, 2010; Michiels et al., 2010).

Amylase activity (EC 3.2.1.1) was determined using the method described by Biesiot and Capuzzo (1990) with some modifications. Amylase activity was assayed in a reaction medium containing 15 mg mL^{-1} starch in 50 mM phosphate buffer (pH 5.2) at 30 °C (Asaro, 2009). The reaction was initiated by the addition of an aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot). The reaction was stopped by addition of 1.5 mL of dinitrosalicylic acid reagent (DNS) (Miller, 1959) and after a further incubation for 10 min at 100 °C, assay tubes were immediately cooled in ice. The amount of released maltose was determined by reading the absorbance at 540 nm. Amylase activity was expressed as μg maltose × min^{-1} × mg $protein^{-1}$.

Maltase and sucrase (EC 3.2.1) activities were assayed by measuring the glucose released from the hydrolysis of the corresponding substrate (maltose and sucrose, respectively) (Asaro, 2009). The reaction was initiated by adding an aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) to a reaction mixture containing 28 mM of the corresponding substrate (sucrose or maltose) in 0.1 M maleate–NaOH buffer (pH 5.2) at 37 °C. After incubation for 10 min, the reaction was stopped by addition of 1.5 mL of the combined enzyme color glucose reagent solution (10 kU/L glucose oxidase, 1 kU peroxidase, 0.5 mmol/L 4-aminophenazone, 100 mmol/L phosphate buffer pH 7.0, 12 mmol/L hydroxybenzoate) (Wiener-Lab AA Kit cod. 1400107). After 5 min at 37 °C, the amount of released glucose was determined by reading the absorbance at 505 nm of the colored quinonimine. The disaccharidase activities were expressed as μg glucose × min^{-1} × mg $protein^{-1}$.

Total proteolytic activity (EC 3.4) was assayed by adding an aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) to a reaction mixture containing 1% w/v azocasein in 0.1 M Tris–HCl buffer (pH 7.5). After incubation at 45 °C for 30 min, the reaction was arrested by adding 0.75 mL of cold trichloroacetic acid (TCA) (10% w/v) (Pinoni, 2009). Overnight absorbance was measured at 440 nm (A_{440}) in the supernatant resulting after centrifuging at 1800 × *g* for 20 min (IEC-Centra 7R, refrigerated). One unit activity (U) was defined as the amount of enzyme extract that produced an increase of 1 in A_{440}. The proteolytic activity was expressed as U × h^{-1} × mg $protein^{-1}$.

Lipase activity (EC 3.1.1) was determined by measuring pNPpalmitate hydrolysis (Markweg et al., 1995) with some modifications (Michiels et al., 2010). The reaction was initiated by the addition of pNPpalmitate (final concentration 0.7 mM) to a reaction mixture containing a adequate aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) in 0.1 M Tris–HCl buffer (pH 8.5)/4 μL of Tween 80. Incubation was carried out at 37 °C for 3 min. The reaction was stopped by addition of 0.75 mL of 0.2% w/v of TCA. The amount of released *p*-nitrophenol (pNP) was determined by reading the absorbance at 410 nm.

Levamisole-insensitive AP activity (EC 3.1.3.1) was determined by measuring pNPP hydrolysis in a reaction medium containing 4 mM $MgSO_4$ in 0.1 M Tris–HCl buffer (pH 7.7) in the presence of 16 mM levamisole. Levamisole-sensitive activity was determined as the difference between the pNPP hydrolysis in a reaction medium containing 4 mM $MgSO_4$ in 100 mM Tris–HCl buffer (pH 8.5) in the absence (total AP activity) and in the presence of 16 mM levamisole (Pinoni, 2009; Pinoni et al., 2005). An aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) was added to the reaction mixture and pre-incubated for 5 min at 37 °C. The reaction was initiated by the addition of pNPP (final concentration 9.5 mM). Incubation was carried out at 37 °C for 10 min. The reaction was stopped by addition of 2 mL of 0.1 M KOH. The amount of released pNP was determined by reading the absorbance at 410 nm (Pinoni, 2009; Pinoni et al., 2005).

The concentration of glucose was measured immediately after hemolymph extraction. Hemolymph (about 500 μL) was sampled from the infrabranchial sinus by mean of a syringe previously rinsed with sodium citrate buffer 10% w/v pH 7.4, at the base of the cheliped, and transferred to an iced centrifuge tube. Plasma was separated by centrifugation at 2000 × *g* for 3 min (IEC-Centra 7R, refrigerated). An adequate aliquot of hemolymph was incubated with 1.5 mL of the glycemia reagent (Wiener-Lab AA Kit). After 5 min at 37 °C, the amount of glucose was determined by reading the absorbance at 505 nm of the colored quinonimine complex.

Glycogen was determined as glucose equivalent, after hydrolysis, according to Schmitt and Santos (1993). The corresponding sample was boiled for 4 min and then incubated in acetate buffer (pH 4.8) (1:2) in the absence and in the presence of 0.2 mg mL^{-1} of α-amyloglucosidase for 2.5 h at 55 °C. After the incubation, it was centrifuge at 2000 × *g* for 30 min (IEC-Centra 7R, refrigerated). Glucose was quantified in the supernatant using glycemia commercial kit (Wiener-Lab AA). Free glucose was determined from assay in the absence of α-amiloglucosidase. Released glucose from glycogen was determined as the difference between the assays with and without α-amiloglucosidase. Results are presented as μmol × g $tissue^{-1}$.

Triglycerides (TG) were measured by the colorimetric method of glycerol phosphate oxidase with a commercial Kit (TAG Wiener-Lab AA cod. 178105). The corresponding sample was incubated with this reactive for 5 min at 37 °C. The amount of released glycerol was determined by reading the absorbance at 505 nm of the colored quinonimine complex. Results are presented as mg × g $tissue^{-1}$.

Protein was assayed according to Bradford (1976). Bovine serum albumin was used as standard. Results are presented as mg × g $tissue^{-1}$.

2.5. Statistical analysis

Statistical analyses were performed using the Sigma-Stat 3.0 statistical package for Windows operating system, which automatically performs a previous test for equal variance and normality. A parametric (*t*-test or One Way ANOVA) or non-parametric (Mann–Whitney or Kruskal–Wallis, respectively) analysis of variance was used to estimate the statistical significance of the differences and $p<0.05$ was considered to be significant. A posteriori ANOVA or Kruskal–Wallis test (the Holm–Sidak or the Dunn's method, respectively) was used to identify differences (Zar, 1999).

3. Results

Individuals of *N. granulata* from the mudflat and saltmarsh exhibited a high and similar amylase activity in the hepatopancreas (Fig. 1A). Crabs from both habitats also exhibited a similar sucrase and maltase activities in the hepatopancreas (Fig. 1B and C).

Individuals from the saltmarsh exhibited about two fold higher total proteolytic activity in the hepatopancreas than crabs from the mudflat (Fig. 2A). Lipase activity was also higher (about 1.5 fold) in the hepatopancreas of crabs from the saltmarsh than mudflat (Fig. 2B).

Levamisole-insensitive AP activity in the hepatopancreas was about fivefold higher in crabs from the saltmarsh than mudflat, whereas no differences occurred in levamisole-sensitive AP activity (Fig. 2C).

No between-habitats differences were found in the concentration of glucose in the hemolymph (mudflat: 0.79 ± 0.17 mmol × L^{-1}; saltmarsh: 0.88 ± 0.10 mmol × L^{-1}, $p>0.05$). In individuals from both sites, the hepatopancreas exhibited the lower concentration of glycogen among tissues studied (Fig. 3A). In crabs from the mudflat, the concentration of glycogen in the chela muscle was higher than those in the anterior and posterior gills. No differences were found between anterior and posterior gills. In individuals from the saltmarsh, no differences were found among the concentration of glycogen in the chela muscle, anterior or posterior gills (Fig. 3A). No between-habitat differences were found in the concentration of glycogen in the hepatopancreas or chela muscle. Crabs from the mudflat exhibited a lower concentration of glycogen (about 1.5 fold) in anterior and

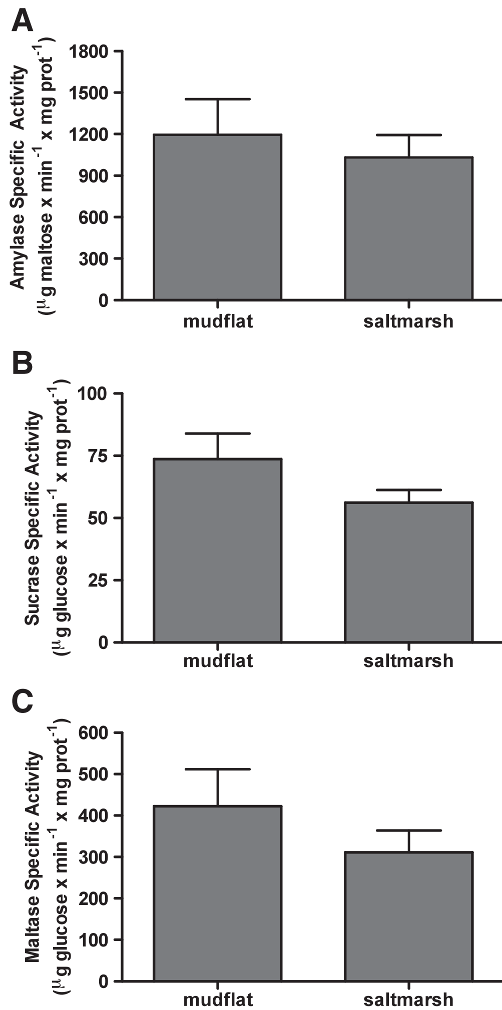

Fig. 1. Amylase (A), sucrase (B) and maltase (C) specific activities in hepatopancreas of individuals of *N. granulata* from the mudflat and the saltmarsh. Data are the mean ± S.E. for ten crabs from each habitat.

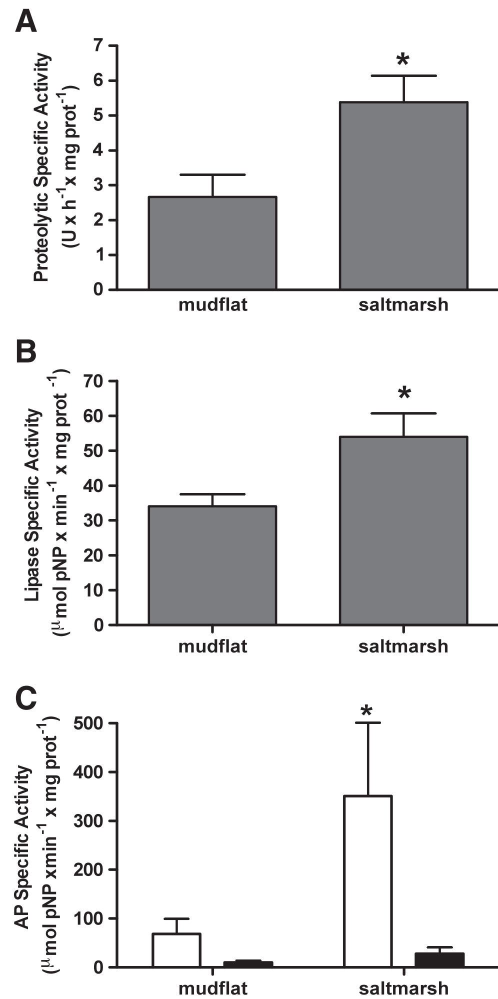

Fig. 2. Proteolytic (A), lipase (B) and AP (C) specific activities in hepatopancreas of individuals of *N. granulata* from the mudflat and the saltmarsh. Data are the mean ± S.E. for ten crabs from each habitat. Open bars: levamisole-insensitive AP activity; black bars: levamisole-sensitive AP activity. *Significantly different from the corresponding activity of individuals from mudflat (*t*-test, $p<0.05$).

posterior gills compared to those in saltmarsh (Fig. 3A). In both habitats, the chela muscle exhibited a lower free-glucose concentration than those in the hepatopancreas, anterior and posterior gills (Fig. 3B). No differences between-habitats were found in free-glucose concentration in any tissue tested (Fig. 3B).

In both habitats, the concentration of triglycerides in the hepatopancreas was higher than in the chela muscle whereas triglycerides were not detected in either anterior or posterior gills. Individuals from the saltmarsh exhibited a higher concentration of triglycerides in the hepatopancreas than crabs from the mudflat (Fig. 4).

In individuals from the mudflat, the concentration of proteins in the chela muscle was about 75% higher than in the hepatopancreas, anterior and posterior gills (Fig. 5). In crabs from the saltmarsh, the concentration of proteins in the chela muscle was higher than in the hepatopancreas but no different from those in the anterior and posterior gills. Crabs from the saltmarsh exhibited a lower concentration of proteins in the chela muscle than individuals from the mudflat whereas no differences occurred in the concentration of proteins in the hepatopancreas, anterior or posterior gills (Fig. 5).

4. Discussion

The results of this work show that *N. granulata* exhibits digestive and metabolic characteristics at the biochemical level that could support the successful occupancy of differential habitats. *N. granulata* in Mar Chiquita coastal lagoon successfully occupy the whole intertidal area in which excavates semi-permanent burrows in various types of habitats, including bare mudflats and vegetated areas covered by cordgrass *S. densiflora* (saltmarsh) (Spivak et al., 1994; Iribarne et al., 1997, 2003; Bortolus et al., 2002; Anger et al., 2008). The between-habitat dietary shift appears to be a strategy of this crab in the natural ambient in relation to available food behaving as omnivorous–detritivorous in the mudflat and herbivorous–detritivorous in the saltmarsh (Iribarne et al., 1997; Bortolus et al., 2002). In this work, we determined the activity of key digestive enzymes in the hepatopancreas and energy reserves content in individuals from both sites feeding in the natural habitat as an important tool to evaluate the digestive capabilities and metabolic characteristics and/or adjustments at the biochemical level underlying the dietary shift in relation to habitat.

In decapod crustaceans, the maintenance of adequate concentrations of glucose in the hemolymph is essential for supporting the regular functions of various organs, such as brain and muscle, and for the response to different environmental stress (Verri et al., 2001; Lorenzon et al., 2005, 2007). The fact that individuals of *N. granulata* from the mudflat and the saltmarsh habitats exhibited similar values of glycemia suggests that availability of glucose from the hemolymph would not be a

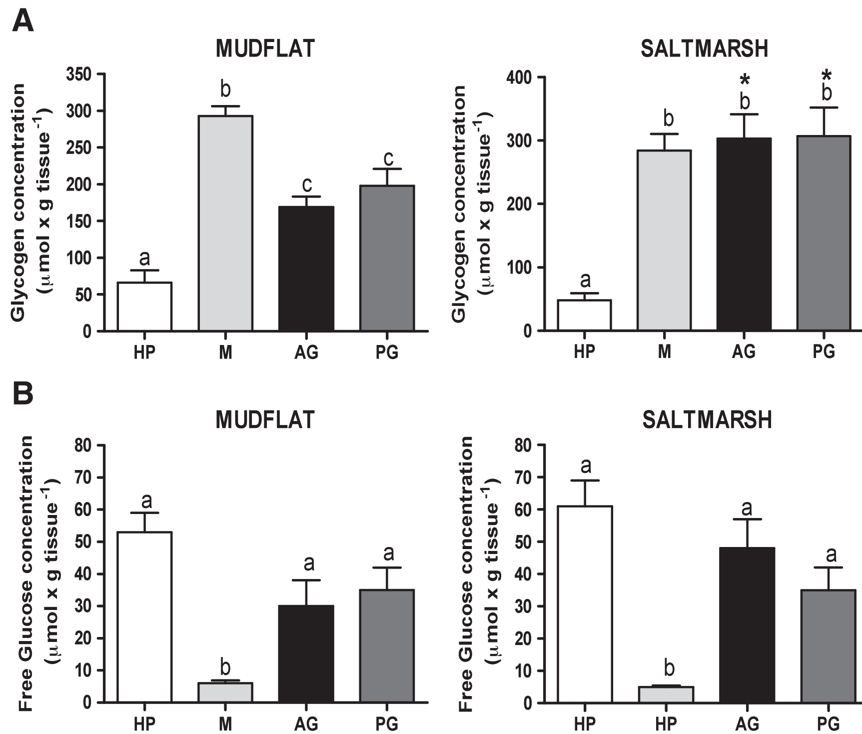

Fig. 3. Glycogen (A) and free-glucose (B) concentration in hepatopancreas (HP), muscle (M), anterior gills (AG) and posterior gills (PG) of individuals of *N. granulata* from the mudflat and the saltmarsh. Data are the mean ± S.E. for ten crabs from each habitat. Different letters indicate significant differences between organs (one-way ANOVA, $p<0.05$). *Significantly different from the corresponding organ of individuals from mudflat (*t*-test, $p<0.05$).

constraint in relation to habitat. Since one of the main sources of hemolymphatic glucose is the absorption through the hepatopancreas and intestine of the glucose extracted from the diet, an adequate capability for the digestion of dietary glycogenic carbohydrates (i.e. starch) is crucial for the maintenance of a balanced dietary glucose supply (Verri et al., 2001). The ability for digesting starch will depend on the occurrence and/or level of activity in the hepatopancreas of the key enzymes intervening in its degradation (i.e. amylase and maltase) (Verri et al., 2001; Gaxiola et al., 2005). An adequate level of maltase and sucrase activities in the hepatopancreas would further allow the use of dietary glycogenic disaccharides such as maltose and sucrose. The high and similar amylase, maltase and sucrase specific activities in the hepatopancreas of individuals of *N. granulata* from the mudflat and saltmarsh suggest the same potential capability for the complete degradation of dietary starch and for the use of dietary maltose and sucrose in both habitats. The similar amylase, maltase and sucrase activities could indicate that the corresponding substrate concentration does not decay between the mudflat and the saltmarsh habitats. Although diet composition of *N. granulata* changes in relation to food availability, consume of detritus is sustained in both habitats (Iribarne et al., 1997; Bortolus et al., 2002; Botto et al., 2005). *Spartina* marshes have a strong influence in the accumulation of organic matter in the intertidal sediments of our study site and *Spartina* debris are important as a food source for *N. granulata* in the whole intertidal area (Botto et al., 2005). Starch appears to be a common component of detritus within estuaries and based on the content of *Spartina* debris in organic matter, this could be also the case in Mar Chiquita coastal lagoon. Modulation of digestive enzyme activities would not be necessary if constitute levels of enzyme were enough for an efficient hydrolysis of any potential diet and the corresponding substrate does not decay below a threshold (Levey et al., 1999; Sabat et al., 1999; del Valle and López Mañanes, 2008). In juveniles of the estuarine crab *Callinectes sapidus*, the occurrence of maltase activity in the hepatopancreas has been related to the capacity of this crab to use detritus (McClintock et al., 1991).

Contrary to amylase and disaccharidase activities, differences in total proteolytic, lipase and levamisole-insensitive AP specific activities were found (Fig. 2), indicating the occurrence of a differential modulation of enzyme activities in the hepatopancreas in relation to habitat. The

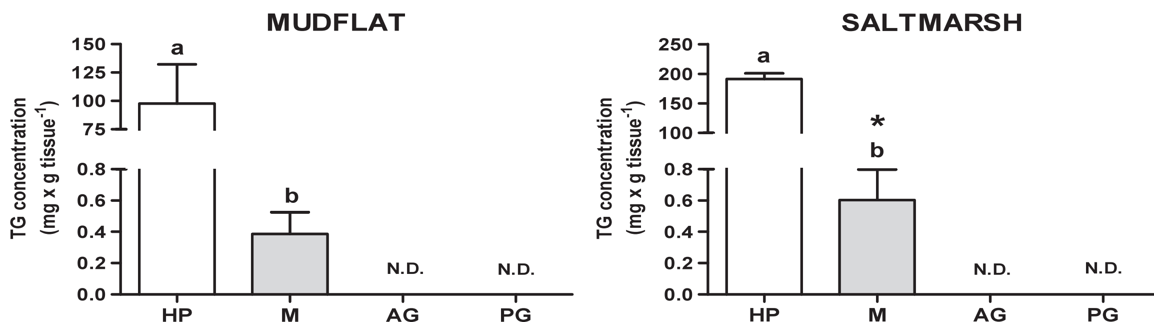

Fig. 4. Triglyceride (TG) concentration in hepatopancreas (HP), muscle (M), anterior gills (AG) and posterior gills (PG) of individuals of *N. granulata* from the mudflat and the saltmarsh Data are the mean ± S.E. for ten crabs from each habitat. Different letters indicate significant differences between organs (one-way ANOVA, $p<0.05$). *Significantly different from the corresponding organ of individuals from mudflat (*t*-test, $p<0.05$). N.D.: not detected.

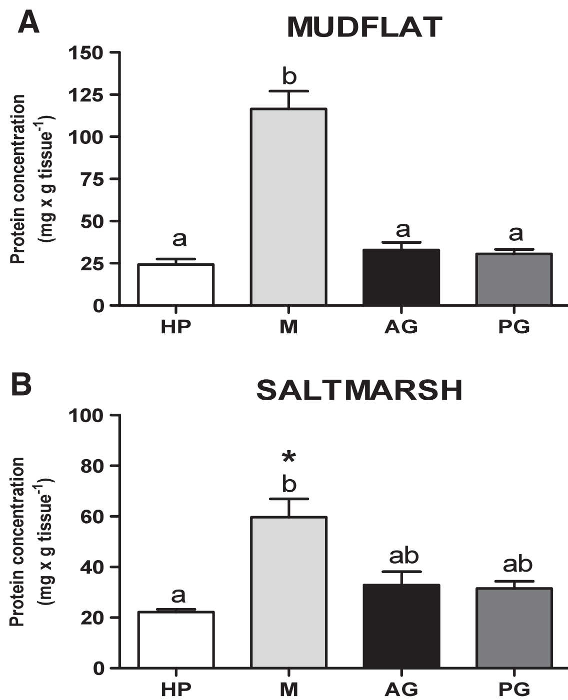

Fig. 5. Protein concentration in hepatopancreas (HP), muscle (M), anterior gills (AG) and posterior gills (PG) of individuals of *N. granulata* from the mudflat (A) and the saltmarsh (B). Data are the mean ± S.E. for ten crabs from each habitat. Different letters indicate significant differences between organs (one-way ANOVA, $p<0.05$). *Significantly different from the corresponding organ of individuals from mudflat (*t*-test, $p<0.05$).

higher total proteolytic and lipase activities suggest a potential higher capability for the digestion of proteins and lipids in individuals from the saltmarsh. Since *S. densiflora*, the main cordgrass consumed by crabs in the saltmarsh, is characterized by a low protein content (approximately 5% dry weight) and a low consumption of dietary items of animal origin (i.e. sediments with polychaetes, diatoms, ostracods, and nematodes) occurred in the saltmarsh (Iribarne et al., 1997; Bortolus et al., 2002; Alberti et al., 2007), proteins and lipids could be limited dietary components for individuals of this habitat. Thus, an enhanced proteolytic and lipase activity could lead to an optimization of the limited dietary components in the saltmarsh. Under laboratory conditions, an increase of digestive enzymes activities upon reduced quantity in the diet of the corresponding substrate occurred in various crustaceans (Sánchez-Paz et al., 2006).

The crustacean hepatopancreas is also the major site of nutrient absorption from digestive products. Nutrients are transferred across the epithelial lining from the hepatopancreas to the hemolymph for tissue distribution (Wright and Ahearn, 1997; Verri et al., 2001). Lipids ingested are degraded to di- and monoglycerides for absorption and stored mainly as triglycerides (over 80–90%), being the hepatopancreas the main site of storage in most species studied (Allen et al., 2000; Muldford and Villena, 2000; García et al., 2002; Sánchez-Paz et al., 2006; Latyshev et al., 2009). AP activity in the hepatopancreas of crustaceans has been suggested to be involved in several stages in the digestive cycle, including enzyme synthesis and secretion, and the absorption of digestive products (Barker and Gibson, 1977; Gibson and Barker, 1979; Verri et al., 2001). In mammals, AP activities of the digestive tract are involved in the digestion, absorption and intracellular accumulation of lipids (Hansen et al., 2007; Kaur et al., 2007; Mahmood et al., 1994, 2003). Preliminary studies in our laboratory suggested the role of AP activities in the hepatopancreas of *N. granulata* in digestion process in this crab (Pinoni, 2009). The concomitant higher levamisole-insensitive AP activity and lipase activity in the hepatopancreas of individuals of *N. granulata* from the saltmarsh (Fig. 2) suggest the occurrence of a coordinated modulation of these activities which could lead to an optimization of digestion and absorption of lipids upon a diet implying a potential reduced intake of this nutrients in this habitat. However, further studies (i.e. at tissue and cellular level) are needed to establish the possible physiological link between levamisole-insensitive AP and lipase activities detected in the hepatopancreas of *N. granulata* in the natural ambient, as well as the exact role of AP activities in the hepatopancreas. The fact that levamisole-sensitive AP activity was similar in the hepatopancreas of crabs from the mudflat and the saltmarsh suggests a specific modulation of levamisole-insensitive AP activity in relation to habitat. We have previously suggested the differential role of both AP activities of the chela muscle of *N. granulata* in biochemical adaptations of this crab (Pinoni et al., 2005; Pinoni, 2009). The results of this work suggest that this could be also the case for these AP activities in the hepatopancreas of *N. granulata* in the natural ambient.

The levels and types of energy reserves are an expression of the metabolic characteristics and adjustments of an animal in relation to environmental conditions and habitat (del Valle et al., 2006; Sánchez-Paz et al., 2006, 2007). In the natural ambient, food quality and/or availability is one of the main factors affecting the type and levels of reserves stored and further utilized upon energy-demanding conditions (del Valle et al., 2006; Buckup et al., 2008). The occurrence of intraspecific seasonal variations in the metabolic profile of several crustaceans in the field has been shown, which has been attributed mainly to changes in environmental and/or energy-demanding conditions (Kucharski and da Silva, 1991; da Silva-Castiglioni et al., 2007; Oliveira et al., 2007; Buckup et al., 2008; Dutra et al., 2008). A differential seasonal metabolic pattern was recently found in the ghost crab *Ocypode quadrata* from different geographical areas of south Brazil which is suggested to be a consequence of the differential environmental conditions in both areas (Freitas-Antunes et al., 2010). We have shown, under laboratory conditions, that in individuals of *N. granulata* from both the mudflat and the saltmarsh of Mar Chiquita lagoon, the chela muscle, anterior and posterior gills are major glycogen storage sites, whereas the hepatopancreas and the chela muscle appear to be main site of triglycerides and protein storage, respectively (Artillo et al., 2008; Pinoni, 2009; Pinoni and López Mañanes, 2010; Pinoni and López Mañanes, unpublished results). Thus, we expected that a between-habitat differences in the natural ambient could occur in the storage and/or utilization of the main energy reserves in these tissues of *N. granulata* in Mar Chiquita lagoon. Based on the dietary availability and capability for the utilization of the glycogenic substrates in both habitats as we discussed above, we expected not to find between-habitat differences concerning glycogen stores. This appears to be the case for the chela muscle and the hepatopancreas (Fig. 3). However, individuals from the mudflat exhibited a lower glycogen concentration in the anterior and posterior gills (Fig. 3). Sudden short-term changes in salinity more often occurred in the mudflat than in the saltmarsh and, since the tidal movement of crabs in this habitat in autumn is quite limited even upon change in environmental conditions (Luppi, personal communication), individuals in this habitat have to cope with these abrupt variations in this key environmental factor. We have shown that *N. granulata* from Mar Chiquita lagoon exhibits a strong short-term (2–4 h) osmoregulatory capacity after abrupt change of salinity and that individual anterior and posterior gills are involved in the energy-demanding osmoregulatory responses at the biochemical level (López Mañanes et al., 2000; Schleich et al., 2001; Pinoni, 2009; Pinoni and López Mañanes, 2009). The lower content of glycogen in the anterior and posterior gills of individuals of *N. granulata* from the mudflat in the natural ambient could suggest its utilization as a local rapid energy source to support the branchial osmoregulatory machine upon short-term abrupt changes of salinity in this habitat.

The hepatopancreas of *N. granulata* from Mar Chiquita lagoon exhibited a low glycogen content under differential laboratory conditions (Artillo et al., 2008; Pinoni, 2009; Pinoni and López Mañanes, unpublished results). The low glycogen concentration in crabs from the mudflat and the saltmarsh suggests that this is also the case for

N. granulata in the natural ambient (Fig. 3). The high and similar levels of free glucose in the hepatopancreas (Fig. 3) could suggest a similar role in the metabolism of carbohydrates in both habitats probably in the maintenance of an adequate and sustained glucose supply. As discussed above, the availability of glycogenic substrates (i.e. starch) appears not to be a constraint for individuals of *N. granulata* from the mudflat and the saltmarsh. Furthermore, the similar activities of the main degrading enzymes suggest a potential same capability for the degradation of starch which could in turn lead to a similar glucose availability for storage or use as an energy source. The similar glycemia levels in crabs from both habitats further support this idea. A similar physiological link between glucose intake from the diet, absorption and posterior distribution to main storage sites (i.e. chela muscle and gills) could be operating in individuals from both habitats. Further studies about mechanisms of regulation involved are needed to test this hypothesis.

The fact that a higher concentration of triglycerides in the hepatopancreas of individuals from the saltmarsh was found along with a higher lipase and levamisole-insensitive AP activity (Figs. 2 and 4) suggests a higher capability for the digestion, absorption and storage of lipids upon limited dietary supply as we discussed above. Since, triglycerides are known to be a major source of energy in various decapod crustaceans in several essential processes for their growth, molting and reproduction (Sánchez-Paz et al., 2006; Dima et al., 2009), the utilization as energy source of triglycerides stored in the hepatopancreas could be diminished in individuals of the saltmarsh during autumn in order to support the energy-demanding conditions (i.e. molting, reproduction) during the outcoming seasons (Luppi, personal communication).

An adequate protein intake is essential to support aminoacids provision necessary for the maintenance of key essential functions (i.e. osmoregulation, growth, reproduction) (Helland et al., 2003; Sánchez-Paz et al., 2006; Bianchini et al., 2008). The chela muscle constitutes a main site of protein storage in *N. granulata* from Mar Chiquita lagoon, in which an increase in protein concentration occurs after feeding, suggesting a post-ingesta *de novo* protein synthesis in this tissue (Pinoni, 2009; Pinoni and López Mañanes, unpublished results). As we pointed out above, proteins appear to be a limited dietary component for individuals in the saltmarsh. The higher proteolytic activity found in the hepatopancreas (Fig. 2A) could constitute a digestive adjustment at the biochemical level which could lead to an optimization of protein digestion in this habitat. Modulation of digestive proteolytic activities under varying and/or stressful conditions appears to be a digestive strategy in several animals under natural conditions (del Valle and López Mañanes, 2008; Figueiredo and Anderson, 2009; Iglesias et al., 2009). However, the lower concentration of protein in the chela muscle of crabs from the saltmarsh could suggest a further adjustment (i.e. differential utilization of protein reserve in this tissue) in relation to habitat. In several decapod crustaceans, the utilization of proteins in muscle appears to be a primary adjustment upon a deficiency in proteins supply (Rosa and Nunes, 2004; Sánchez-Paz et al., 2006). We cannot discard that the lower concentration of protein in the chela muscle in the saltmarsh could be due to a modulation of protein synthesis. The possible links among dietary protein availability, proteolytic activity (i.e. identification of proteases involved) in the hepatopancreas and the utilization and/or building of protein storage in the chela muscle must be investigated in future experiments**.**

In conclusion, the results of this study show that in the natural ambient *N. granulata* from the mudflat and the saltmarsh of Mar Chiquita coastal lagoon exhibits characteristics at the biochemical level in the hepatopancreas (i.e. high amylase and disaccharidase activities; and between-habitat differences in total proteolytic, lipase and levamisole-insensitive AP activities in the hepatopancreas) and differences in the concentrations of glycogen in the anterior and posterior gills, triglycerides in the hepatopancreas and protein in the chela muscle which could represent adaptive digestive and metabolic strategies to face the habitat variations in environmental conditions. Since the links between digestive process at the biochemical level (i.e. activity of key digestive enzymes) and metabolic pathways in *N. granulata* have not been yet elucidated, more studies are needed to establish the possible relationships between the intake of a dietary substrate, absorption of the resulting digestion products and the storage and/or utilization as energy reserves as well as the possible differential mechanisms of regulation operating in crabs from both habitats. Future studies focused on laboratory experiments with individuals from both open mudflat and saltmarsh under differential ambient factors as independent variables would allow a better understanding of the complex adaptive responses at the biochemical level of *N. granulata* in relation to habitat.

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