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ORIGINAL PAPER

Phenotypic flexibility in response to environmental salinity in the euryhaline crab *Neohelice granulata* from the mudflat and the saltmarsh of a SW coastal lagoon

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Abstract This study constitutes a first attempt to investigate intraspecific differences in osmoregulatory capacity and digestive and metabolic responses at the biochemical level in relation to hyper- and hypo-regulation in a single species of estuarine crab inhabiting contrasting habitats within a same intertidal area. We compared hemolymph osmolality, key digestive enzymes, glycemia and energy reserves in *Neohelice granulata* (Dana in Proc Acad Nat Sci Philadelphia 5:247–254, 1851) from the mudflat and saltmarsh of Mar Chiquita coastal lagoon (37°32′/37°45′S-57°19′/57°26′W) under a wide range of salinities (6–60 psu). Individuals from both sites exhibited high and similar osmoregulatory capacity, but while in individuals from mudflat low and high salinities affected lipase activity in hepatopancreas and triglycerides in muscle, in crabs from saltmarsh, high salinities affected glycogen in anterior gills. Low salinity differentially affected free glucose in anterior gills. The results suggest the occurrence of intraspecific distinct digestive and metabolic adjustments in relation to osmoregulatory responses and habitat.

Introduction

Estuaries constitute extremely challenged environments in which abiotic factors, particular salinity, vary abruptly both spatially and temporally. 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 spatiotemporal changes in environmental conditions (Piersman and Drent 2003; Kelly et al. 2012). Reversible changes allow individuals to adapt favorably to the prevailing environmental regime (Piersman and Drent 2003; Pfenning et al. 2010; Kelly et al. 2012). Intertidal estuarine crabs have to cope with a variety of challenges such as abrupt spatial and temporal changes in environmental salinity, therefore requiring strategies at different levels for controlling movements of water and ions between the individuals and their medium (Anger 2001; Kirschner 2004; McNamara and Faria 2012; Romano and Zeng 2012). Species successfully inhabiting differential habitats (i.e., mudflats and saltmarshes) within the intertidal area of a same estuary are expected to exhibit phenotypic flexibility at the biochemical and physiological level in key parameters. The differential patterns of salinity changes along with other factors such as food availability in the mudflat and the saltmarsh may impose differential challenges to individuals of a same species (Iribarne et al. 1997; Méndez-Casariego et al. 2011; Spivak et al. 2012). This could result in intraspecific differences concerning regulation of osmotic and ionic balances and the possible associated use and/or distribution of energy resources (Spivak et al. 2012). Intraspecific comparisons in key parameters at the physiological and biochemical level would allow testing the phenotypic flexibility in euryhaline crab species successfully living in both habitats. Strikingly, in spite of the great importance for the understanding of ecophysiology of intertidal estuarine crabs, few studies relate ecological aspects of phenotypic flexibility of individuals of a single species of hyper-/hypo-regulating crabs living in

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differential habitats of the intertidal area with internal mechanisms involved (Pinoni et al. 2011; Spivak et al. 2012).

Hyper-/hypo-osmoregulator crabs are able to maintain the osmotic concentration of the hemolymph within a stable range, above or below that of the external medium in low and high salinities, respectively (Péqueux 1995; Lucu and Towle 2003; Freire et al. 2008; McNamara and Faria 2012; Romano and Zeng 2012). Flexibility in osmoregulatory behavior is essential for survival and successful occupancy of estuarine habitats. Osmoregulatory capacity, which is defined as the difference between the osmotic pressures of the hemolymph and that of the external medium at a given salinity, is commonly used to evaluate osmoregulatory performance (Charmantier et al. 1989; Lignot et al. 2000). Comparisons of osmoregulatory capacity values of an individual subjected to a wide range of salinities allow to evaluate possible differential performance depending on the type and degree of osmotic challenge (i.e., higher value indicates a greater osmoregulatory performance for that condition). Molecular and biochemical changes involved in osmoregulatory responses (i.e., in ion-transport systems in gills) have been studied extensively in various species of osmoregulating crabs; however, some mechanisms are not fully understood, particularly not from a metabolic perspective (McNamara and Faria 2012; Romano and Zeng 2012). Biochemical adaptation to environmental salinity is a complex process involving also the participation of different enzymes and transport systems in extrabranchial tissues such as hepatopancreas and muscle (Jahn et al. 2006; Pinoni and López Mañanes 2004, 2008, 2009; Martins et al. 2011; Athamena et al. 2011; Michiels et al. 2013). We have recently shown that differential modulation of the activity of key digestive enzymes in the hepatopancreas (the major site of digestive enzyme production and absorption of nutrients) is one component of the complex responses to low and high salinity in the euryhaline crab *Cyrtograpsus angulatus* (Michiels et al. 2013). In the hepatopancreas of the euryhaline pacific white shrimp *Litopenaeus vannamei*, the responses of digestive enzymes activity to extreme environmental salinity would be related to deriving extra energy for osmoregulation (Li et al. 2008). Osmoregulatory adaptation to salinity may imply mobilization of energy reserves involving distinct type of reserves and storage organs (Luvizotto-Santos et al. 2003; Bianchini et al. 2008; Romano and Zeng 2012). Concomitant measurements of several parameters in various organs appear then necessary for achieving an integrative evaluation of responses underlying biochemical adaptation to salinity.

Neohelice (*Chasmagnathus*) *granulata* (the South American rainbow crab) is a euryhaline burrowing crab considered as an emergent animal model for biochemical, physiological and ecological research (Spivak 2010). This crab is distributed on intertidal areas of the southwestern Atlantic from southern Brazil to the northern Argentinean Patagonia (Boschi 1964; Botto and Irigoyen 1979; Spivak 1997) where it has a key ecological role (Iribarne et al. 2003; Fanjul et al. 2008; Daleo and Iribarne 2009; Méndez-Casariego et al. 2011; Luppi et al. 2012). *N. granulata* is found in extremely heterogeneous habitats concerning salinity conditions ranging from near 0 to about 63 psu (Luppi et al. 2012). Interpopulation differences exist in biochemical (i.e., ion-transport systems in gills) and physiological adaptations to salinity (Luquet et al. 1992, 2002a, b, 2005; López Mañanes et al. 2000; Schleich et al. 2001; Genovese et al. 2004; Novo et al. 2005; Pinoni et al. 2005; Bianchini et al. 2008; Pinoni and López Mañanes 2009), suggesting the influence of habitat. A single population of *N. granulata* may inhabit two contrasting types of habitats (mudflats and saltmarshes) (Spivak 1997). This is the case in Mar Chiquita coastal lagoon (37°32′–37°45′S, 57°19′–57°26′W) (Argentina) in which *N. granulata* is one of the dominant crabs inhabiting the whole intertidal area from open mudflat to vegetated saltmarsh (Spivak et al. 1994; Iribarne et al. 1997, 2003; Bortolus and Iribarne 1999; Anger et al. 2008; Fanjul et al. 2008). Adult males are the more active and numerous group in these habitats (Spivak et al. 1994; Luppi et al. 2012). The modulation of key digestive enzymes activities and/or energy reserves is an expression of the digestive and metabolic adjustments in relation to environmental conditions and habitat (del Valle et al. 2006; Sánchez-Paz et al. 2006, 2007; Buckup et al. 2008; Sjoboen et al. 2010; del Valle and López Mañanes 2008, 2011). We have found that, in the natural environment, adult males of *N. granulata* exhibit phenotypic flexibility at the biochemical level (digestive and metabolic) between habitats (Pinoni et al. 2011). Intraspecific between-habitat differences in key digestive enzymes (i.e., lipase and proteolytic activities in the hepatopancreas) and in key energy reserves (i.e., triglycerides content) were found suggesting a differential utilization of energy sources to face environmental conditions. Lipase and proteolytic activity in the hepatopancreas and triglycerides content appears to be under differential modulation being components of the biochemical adaptation to habitat (Pinoni et al. 2011). In the saltmarsh, the lower consumption of dietary items of animal origin compared to the mudflat could lead to a lower ingestion of lipids and proteins and a decreased utilization of triglycerides content (Iribarne et al. 1997; Bortolus et al. 2002; Alberti et al. 2007; Pinoni et al. 2011). As we pointed out above, biochemical and molecular changes in response to salinity imply differential energy needs and adjustments in digestive and metabolic parameters, which may involve different energy sources and tissues (Romano and Zeng 2012). We speculate that the

differential digestive and metabolic pattern that we found to be exhibited by males of *N. granulata* in the natural environment (Pinoni et al. 2011) could lead to differential adjustments, if any, in digestive enzymes activities and/or in the utilization of distinct energy storages to face low and high salinity conditions. Previous works of our laboratory show that *N. granulata* from both the mudflat and the saltmarsh behave as hyper- and hypo-regulators (López Mañanes et al. 2000; Schleich et al. 2001; Pinoni et al. 2005; Pinoni and López Mañanes 2009; González et al. 2012); however, between-habitat comparison of osmoregulatory pattern and capacity over a wider range of salinities faced by this crab in the field and further of the possible digestive and metabolic adjustments at the biochemical level upon salinity acclimation to low and high salinities are lacking. To increase the knowledge of different aspects of the biology of *N. granulata,* and as part of our integrative studies on the biochemical adaptations to salinity in hyper-/hypo-regulating crabs, the aims of this work were to study and compare the hemolymph osmoregulatory pattern and capacity of individuals from the mudflat and the saltmarsh, and the effect of low and high salinities on key digestive enzyme activities in hepatopancreas and energy reserves content. We determined the hemolymph osmolality; lipase, total proteolytic, amylase, maltase and sucrase activities in the hepatopancreas; glycemia, and the concentration of triglycerides, protein, glycogen and free glucose in the hepatopancreas, chela muscle, and anterior and posterior gills of adult males from both the mudflat and the saltmarsh acclimated to salinities from 6 to 60 psu. We have previously shown that the chela muscle and anterior and posterior gills are major glycogen storage sites, while the hepatopancreas and the chela muscle are main sites of triglycerides and protein storage, respectively (Artillo et al. 2008; Pinoni 2009; Pinoni et al. 2011). This work will increase the knowledge about the complex responses at the physiological and biochemical level to environmental salinity of hyper-/hypo-osmoregulating crabs and the intraspecific variability in relation to habitat.

Materials and methods

Animal collection and maintenance

Crabs were live-trapped during autumn (April–May 2009) in burrows of two sites from the Mar Chiquita coastal lagoon (37°32′–37°45′S, 57°19′–57°26′W) (Argentina): the open mudflat and the saltmarsh dominated by cordgrass (Pinoni et al. 2011). 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 when between-habitat movement of individuals of *Neohelice granulata* is highly reduced, crabs remaining inside burrows most of the time (Luppi et al. 2012; pers observations). Both sites exhibited a complex and extremely variable pattern of salinity variations. The values of salinity in burrows water in the sites of collection range from 6 to 47 psu although frequently higher values can be reached (up to 60 psu). Daily changes in salinity are higher and more abrupt in the mudflat area (amplitude reaching differences up to 30 psu) depending on weather and tidal conditions. Daily changes in salinity are usually less pronounced in the saltmarsh, and high and sudden variations may also occur, but mostly in terms of various days (up to a week) (Spivak et al. 1994; Iribarne et al. 1997; Bortolus et al. 2002; Luppi et al. 2012; pers observations). In autumn, the amplitude of salinity variations in both habitats and between habitats is lower (Luppi et al. 2012; pers observations). At time of collection, the values of salinity in burrows water ranged from 21 to 37 psu in the mudflats and 24 to 37 psu in the saltmarshes. Animals were transported to the laboratory on the day of collection in the water of the site of collection under continuous aeration. A group of crabs from both sites was used immediately for sampling and measurement of the different parameters tested without any acclimation (Pinoni et al. 2011). Another group of crabs from both sites (this work) was maintained in natural seawater (35 psu = 919 mOsm kg^{-1}), low salinities (6 and 10 psu = 114 and 316 mOsm kg^{-1}, respectively, diluted seawater) or high salinities (45 and 60 psu = 1,192 and 1,707 mOsm kg^{-1}, respectively, concentrated seawater) for at least 10 days prior to use. Diluted seawater was obtained by dilution of natural seawater with distilled water. Concentrated seawater was obtained by addition of commercial marine salt (Red Sea Salt, Israel) to natural seawater. Salinity was measured with a refractometer and the corresponding osmotic pressure with a cryoscopic osmometer (Osmomat 030, Gonotec). For all the experiments, salinity was measured in practical salinity units (psu). The aquaria contained 15 crabs in 36 L of water continuously aerated and filtered and partially changed every second day to maintain constant physicochemical parameters. A regime of 12-h light/12-h dark was applied, and the temperature and pH were kept at 20 ± 2 °C and 7.4, respectively. Crabs were fed three times a week with commercial food (Wardley T.E.N., USA: 48 % carbohydrates, 30 % proteins, 1.5 % lipids, 9 % fiber) (about 0.07 g $individual^{-1}$), but they were starved 24 h prior to experiments. No differences in the feeding behavior occurred in the experimental conditions used. No mortality of individuals occurred at any salinity throughout the experimental period.

Chemicals

Maleic acid, (3,5)-dinitrosalicylic acid (DNS), azocasein, pNP-palmitate (*p*-nitrophenylpalmitate), Tris-(hydroxymethylamino-methane) (Tris), ethyleneglycol *N,N',N'*-tetraacetic acid (EGTA) and bovine serum albumin were from Sigma (St. Louis, MO, USA); sucrose was obtained from Merck (Darmstadt, Germany); maltose was from ICN (Ohio, USA); α-amyloglucosidase was from *Aspergillus niger*, and magnesium sulfate and Coomasie blue G250 were from Fluka (Germany). All solutions were prepared in glass-distilled water.

Sampling procedures

Crabs were weighed and cold-anaesthetized by putting them on ice for about 25 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 were immediately excised, shortly and gently dried by putting them on paper towel and weighed. Wet mass was measured to the nearest 0.01 g. Immediately after weighing, the hepatopancreas, chela muscle, and anterior and posterior gills were put on the corresponding buffer used to prepare the corresponding homogenates and enzyme extracts as follows. Previous works provide physiological, biochemical and ultrastructural evidence for considering gill 6 of *N. granulata* as a posterior gill (López Mañanes et al. 2000; Genovese et al. 2004).The hepatopancreas was homogenized in 0.1 M Tris/HCl pH 7.4 (4 ml g tissue^{-1}) (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 triglycerides content. The supernatant was separated into 200-µl aliquots and stored at −20 °C until to be used for enzymatic assays. Glycerol (1.3 % v v^{-1}) 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-muscle tissue^{-1}) and homogenized with CAT homogenizer × 120, tool T10 on ice. The homogenate was fractionated into 400-µL aliquots and stored at −20 °C until use. 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 tissue^{-1}) and homogenized on ice with 20 strokes in a motor-driven hand-operated Teflon-glass homogenizer (Potter–Elvehjem, 1,700 rpm). The homogenate was separated into 200-µL aliquots and stored at −20 °C until use.

Measurement of hemolymph osmolality

Hemolymph (about 500 µL) was sampled from the intrabranchial sinus by means of a syringe previously rinsed with sodium citrate buffer 10 % w v^{-1} pH 7.4, at the base of the cheliped, and transferred to an iced centrifuge tube. Plasma was separated by centrifugation at 2,000×*g* for 3 min (IEC-Centra 7R, refrigerated). Osmolality (mOsm kg^{-1}) was measured in an aliquot of 50 µL of hemolymph and medium with a cryoscopic osmometer (Osmomat 030, Gonotec). The results were expressed as hemolymph osmolality (measurement of solutes concentration) defined as the number of osmoles (Osm) of solutes per kilogram of solvent and as osmoregulatory capacity, which is calculated as the difference between the value of osmolality of the hemolymph and that of the medium (Charmantier and Anger 2011).

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. The assay conditions (temperature, pH, substrate concentration) used for all the enzymes activities tested were optimal for the corresponding activity in the hepatopancreas of *N. granulata* as determined in previous works in our laboratory (Pinoni 2009; Michiels 2010; Asaro et al. 2011; Michiels et al. 2011; Pinoni et al. 2011).

Lipase activity was determined by measuring pNP-palmitate hydrolysis (Markweg et al. 1995) with some modifications. The reaction was initiated by the addition of pNP-palmitate (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^{-1} of trichloroacetic acid (TCA) (Michiels et al. 2011). The amount of released *p*-nitrophenol (pNP) was determined by reading the absorbance at 410 nm. Lipase activity was expressed as µmol pNP min^{-1} mg protein^{-1}.

Total proteolytic activity 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^{-1} 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 TCA (10 % w v^{-1}) and standing overnight at 4 °C. Absorbance was measured at 440 nm (A_{440}) in the supernatant resulting after centrifuging at 2,500 rpm 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} (Pinoni 2009; Pinoni et al. 2011). The proteolytic activity was expressed as U h^{-1} mg $protein^{-1}$.

Amylase activity was determined using the method described by Biesiot and Capuzzo (1990) with some modifications. Amylase activity was determined in a reaction medium containing 15 mg ml^{-1} starch in 50 mM phosphate buffer (pH 5.2) at 30 °C (Asaro et al. 2011). 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 activities were assayed by measuring the glucose released from the hydrolysis of the corresponding substrate (maltose and sucrose, respectively). 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 (Asaro et al. 2011). After incubation for 10 min, the reaction was stopped by the addition of 1.5 ml of the combined enzyme color glucose reagent solution (oxidate glucose 10 kU L^{-1}; peroxidase 1 kU; 1,4-aminophenazone 0.5 mmol L^{-1}; phosphates pH 7.0100 mmol L^{-1}, hydroxybenzoate 12 mmol L^{-1}) (Wiener Lab AA Kit cod. 1400101). After 5 min at 37 °C, the amount of released glucose was determined by reading the absorbance at 505 nm of the colored quinone complex. The disaccharidases activities were expressed as µg glucose min^{-1} mg $protein^{-1}$.

Triglycerides (TG) were measured by the colorimetric method of glycerol phosphate oxidase with a commercial kit (TAG Wiener Lab AA cod. 861110001). The corresponding sample was incubated with this reagent for 5 min at 37 °C (Pinoni et al. 2011). The amount of released glucose was determined by reading the absorbance at 505 nm of the colored quinone complex. Results are presented as mg g $tissue^{-1}$.

The concentration of glucose was measured in the plasma immediately after hemolymph extraction and centrifugation. 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 quinone complex. Results are presented as mmol glucose L^{-1}.

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 (Pinoni et al. 2011). After the incubation, it was centrifuged at 3,000 rpm for 30 min (IEC-Centra 7R, refrigerated). Glucose was quantified in the supernatant using a commercial kit for measuring glycemia (Wiener Lab AA). Free glucose was determined from assay in the absence of α-amyloglucosidase. Released glucose from glycogen was determined as the difference between the assays with and without α-amyloglucosidase. Results are presented as µmol glucose g $tissue^{-1}$.

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

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 nonparametric (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).

Results

Hemolymph osmolality of individuals from the mudflat

In crabs acclimated to 6 and 10 psu, the hemolymph osmolality was about four- and twofold higher from the external medium, respectively. No differences were found at 35 psu. In 45 and 60 psu, hemolymph osmolality was 27 and 32 % lower from the external medium, respectively (Fig. 1). The difference between the hemolymph osmolality and that of the external medium was higher in 6 psu than in 10 psu and in 60 psu compared to 45 psu (Fig. 1, inset).

Hemolymph osmolality of individuals from the saltmarsh

In individuals acclimated to 6 and 10 psu, the hemolymph osmolality was about five- and twofold higher from the external medium. Similarly to individuals from the mudflat, no differences were found between the hemolymph and medium osmolality in 35 psu. In 45 and 60 psu, the hemolymph osmolality was about 33 and 35 % lower from

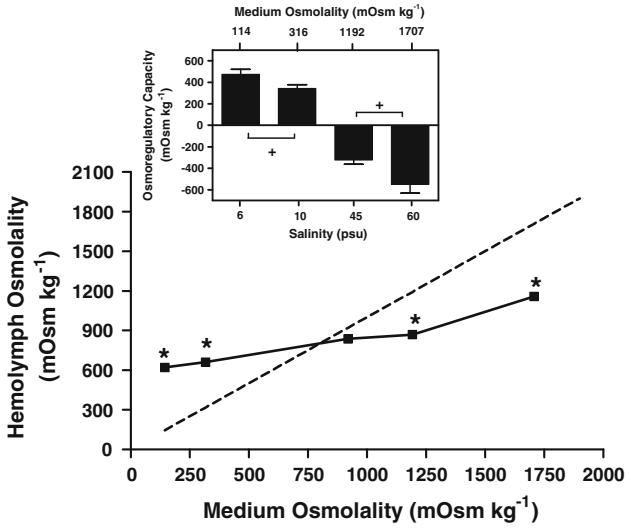

Fig. 1 Hemolymph osmolality of individuals of *N. granulata* from the mudflat acclimated to 6 (114 mOsm × kg^{-1}), 10 (316 mOsm × kg^{-1}), 35 (919 mOsm × kg^{-1}), 45 (1,192 mOsm × kg^{-1}) and 60 (1,707 mOsm × kg^{-1}) psu. *Dashed line*: isoosmotic line. Isoosmotic point: 798 mOsm × kg^{-1} (GraphPad Prism 4.0). *Asterisk* significantly different from the medium osmolality (*t* test, $p < 0.05$). *Inset*: osmoregulatory capacity (difference between hemolymph and medium osmolality); *plus symbol* significantly different (*t* test, $p < 0.05$). Data are the mean ± SEM for six individuals

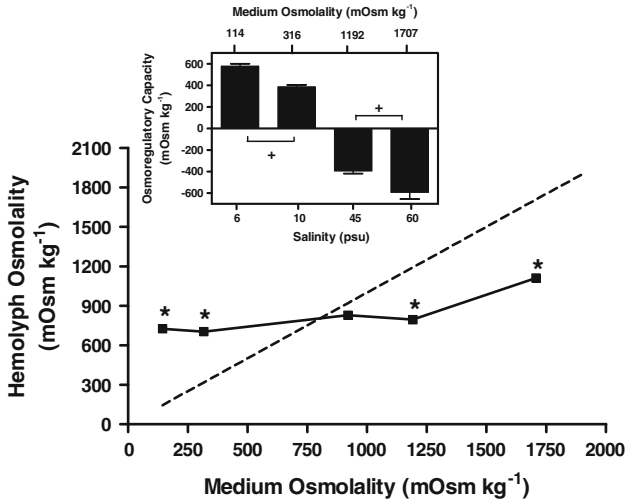

Fig. 2 Hemolymph osmolality of individuals of *N. granulata* from the saltmarsh acclimated to 6 (114 mOsm × kg^{-1}), 10 (316 mOsm × kg^{-1}), 35 (919 mOsm × kg^{-1}), 45 (1,192 mOsm × kg^{-1}) and 60 (1,707 mOsm × kg^{-1}) psu. *Dashed line*: isoosmotic line. Isoosmotic point: 798 mOsm × kg^{-1} (GraphPad Prism 4.0). *Asterisk* significantly different from the medium osmolality (*t* test, $p < 0.05$). *Inset*: osmoregulatory capacity (difference between hemolymph and medium osmolality); *plus symbol* significantly different (*t* test, $p < 0.05$). *Data* are the mean ± SEM for six individuals

the external medium (Fig. 2). The difference between the hemolymph osmolality and that of the external medium was higher in 6 psu than in 10 psu and in 60 than in 45 psu (Fig. 2, inset).

Effect of acclimation to low and high salinity on digestive enzyme activities in hepatopancreas and energy reserves concentration in storage tissues of individuals from the mudflat

In crabs acclimated to low (6 and 10 psu) and high (45 and 60 psu) salinities, lipase activity in the hepatopancreas was lower (76, 47, 38 and 68 %, respectively) than the activity in 35 psu (Fig. 3a). No differences were found between total proteolytic activity (Fig. 3b), amylase, maltase and sucrase activities (Fig. 4) in hepatopancreas of crabs acclimated to low or high salinities and 35 psu.

In 6 and 10 psu, triglyceride concentrations in the chela muscle were lower (about 60 %) than in 35 psu. In 45 psu, triglyceride concentration was also lower than in 35 psu (about 60 %). Triglyceride concentration in the hepatopancreas was not affected (Fig. 5). No triglycerides were detected in anterior or posterior gills.

The concentration of glucose in the hemolymph of individuals acclimated to low and high salinities was similar to 35 psu (about 0.7 mmol L^{-1}; $p > 0.05$) (not shown). The concentration of glycogen in the chela muscle, hepatopancreas, and anterior and posterior gills was not affected by acclimation to low or high salinity (Fig. 6a). Free glucose concentration appeared to vary only in anterior gills of individuals acclimated to 10 psu being higher (twofold) than in 35 psu (Fig. 6b).

Protein concentration in the chela muscle, hepatopancreas, and anterior and posterior gills was not affected by environmental salinity (Fig. 6c).

Effect of acclimation to low and high salinity on digestive enzymes activities in hepatopancreas and energy reserves concentration in storage tissues of individuals from the saltmarsh

No differences in lipase, total proteolytic, amylase, maltase and sucrase activities in hepatopancreas of individuals from saltmarsh were found at any salinity (Figs. 3 and 4).

Triglyceride concentrations in chela muscle or in hepatopancreas of crabs from the saltmarsh acclimated to low and high salinities were similar to that in 35 psu (Fig. 5). Triglycerides were not detected in either anterior or posterior gills.

The concentration of glucose in the hemolymph of individuals from saltmarsh was not affected by environmental salinity being about 0.43 mmol L^{-1} ($p > 0.05$) (not shown). In 6 and 10 psu, the concentration of glycogen in the chela muscle, hepatopancreas, and anterior and

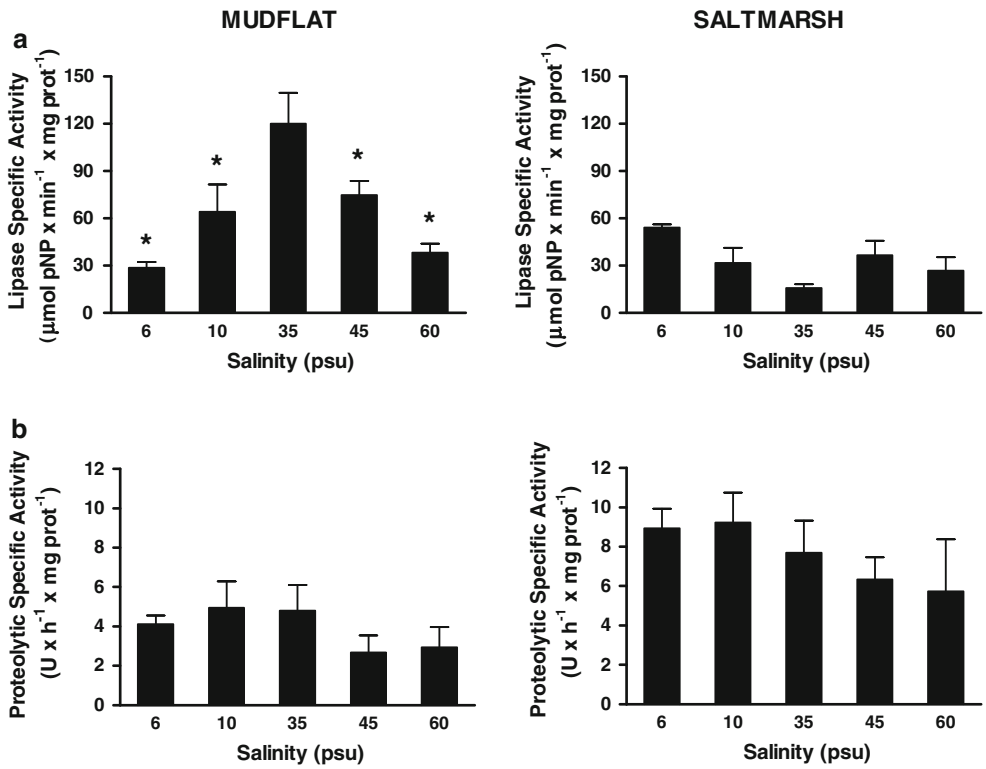

Fig. 3 Lipase (**a**) and proteolytic specific (**b**) activities in hepatopancreas of individuals of *N. granulata* from the mudflat (*left*) and the saltmarsh (*right*) acclimated to different salinities (6–60 psu). Data are the mean ± SEM for five crabs. *Asterisk* significantly different from the corresponding activity in 35 psu (one-way ANOVA, $p < 0.05$)

posterior gills was similar to the corresponding concentration in 35 psu. In 45 and 60 psu, the concentration of glycogen in anterior gills was lower (about 40 %) than in 35 psu. No differences occurred in the chela muscle, hepatopancreas or posterior gills (Fig. 6a). Free glucose concentration of anterior gills was affected by low and high salinities, being lower (about 45 %) than in 35 psu. Free glucose concentration in chela muscle, hepatopancreas and posterior gills was not affected by environmental salinity (Fig. 6b).

No differences in the protein concentration of chela muscle, hepatopancreas, and anterior and posterior gills were found at any salinity (Fig. 6c).

When overall values were considered (by pooling them without taking into account the salinity of acclimation), differences were found in lipase and proteolytic activity in the hepatopancreas and in triglycerides content in chela muscle compared with the corresponding values in crabs from the mudflat (Figs. 3 and 5).

Discussion

The results of this work show that individuals of *Neohelice granulata* from the mudflat and the saltmarsh exhibit high and similar hyper- and hypo-regulatory capacity and differential responses of digestive enzymes activities and contents of energy reserves to low and high salinity, suggesting the occurrence of distinct digestive and metabolic adjustments at the biochemical level in relation to osmoregulatory responses and habitat. In autumn, between-habitat movement of adult male individuals in Mar Chiquita coastal lagoon is highly reduced and crabs remained inside burrows most of the time (Pinoni et al. 2011; Luppi et al. 2012), which suggest the occurrence of physiological mechanisms to tolerate extreme variable salinity. We determined the hemolymph osmolality of crabs acclimated to salinities from 6 to 60 psu as a tool to evaluate and compare the osmoregulatory pattern and capacity over a wide range of salinities. Crabs from both habitats behave as hyper-/hypo-regulators since they exhibited hemolymph osmolality values higher and lower than those of the corresponding external medium upon acclimation to 6–10 and 45–60 psu, respectively, while osmoconforming at 35 psu (Figs. 1 and 2). As expected, based on the successful occupancy of the whole intertidal area, individuals from both sites exhibited high and similar hyper- and hypo-osmoregulatory capacity (Figs. 1 and 2, insets). The higher hyper- and hypo-osmoregulatory capacities at 6 and 60 psu, respectively, suggest the occurrence of biochemical

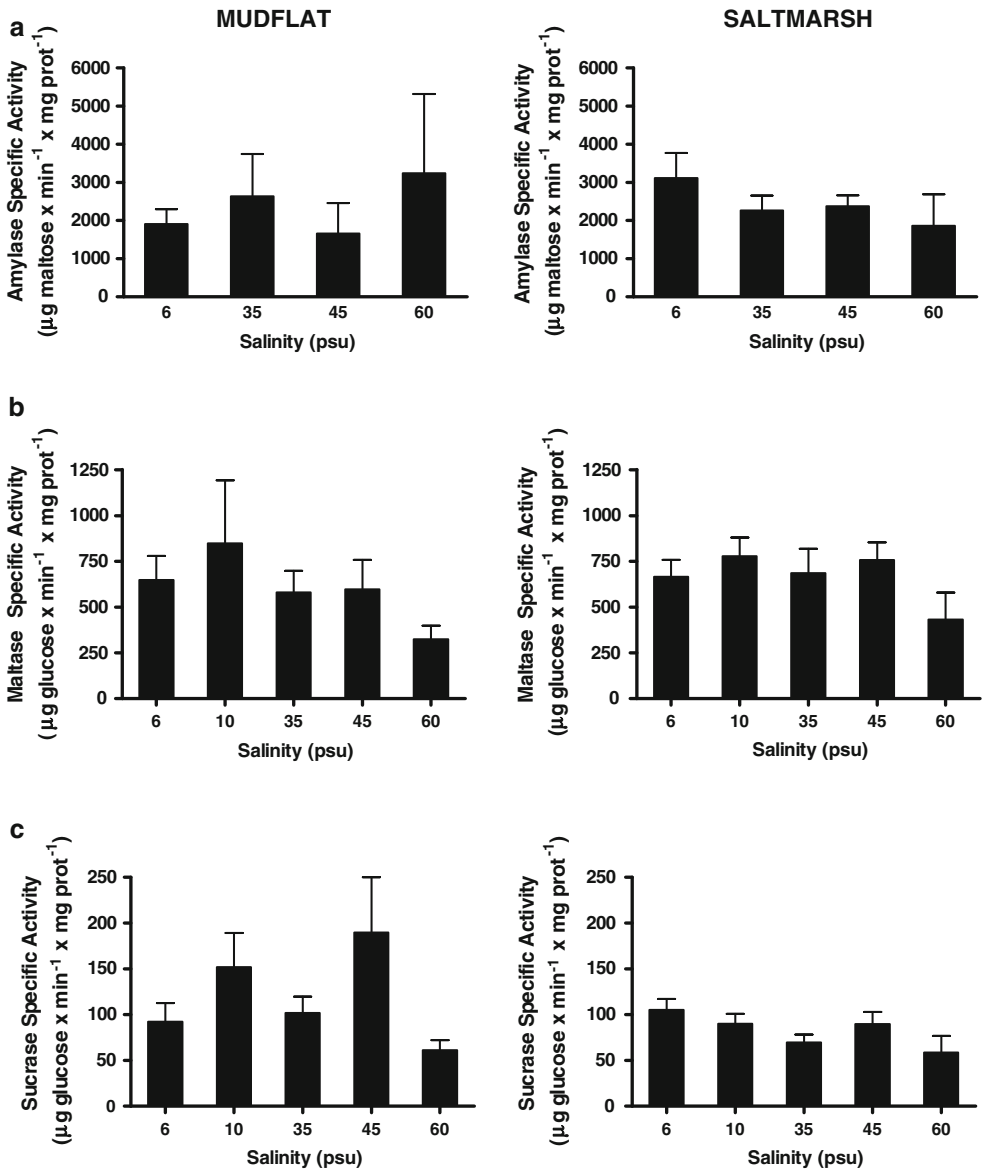

Fig. 4 Amylase (**a**), maltase (**b**) and sucrase (**c**) specific activities in hepatopancreas of individuals of *N. granulata* from the mudflat and the saltmarsh acclimated to different salinities (6–60 psu). Data are the mean ± SE for five crabs

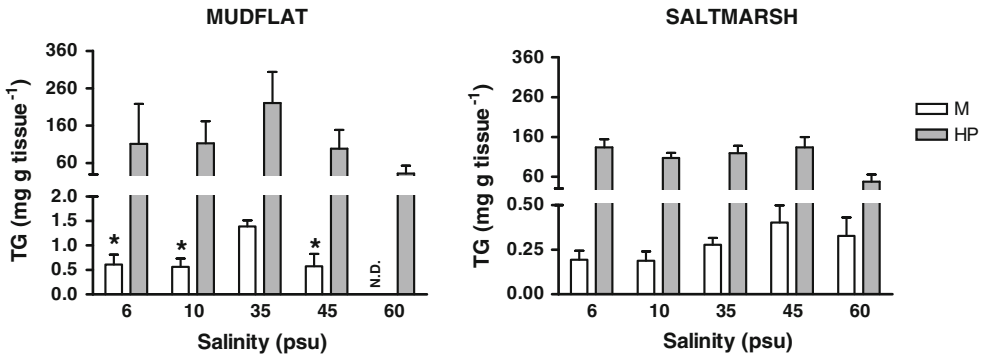

Fig. 5 Triglyceride (TG) concentration in muscle (M) and hepatopancreas (HP) of individuals of *N. granulata* from the mudflat (*left*) and the saltmarsh (*right*) acclimated to different salinities (6–60 psu). Data are the mean ± SEM for five crabs. *Asterisk* significantly different from the corresponding values in 35 psu (one-way ANOVA, $p < 0.05$), *ND* not determined

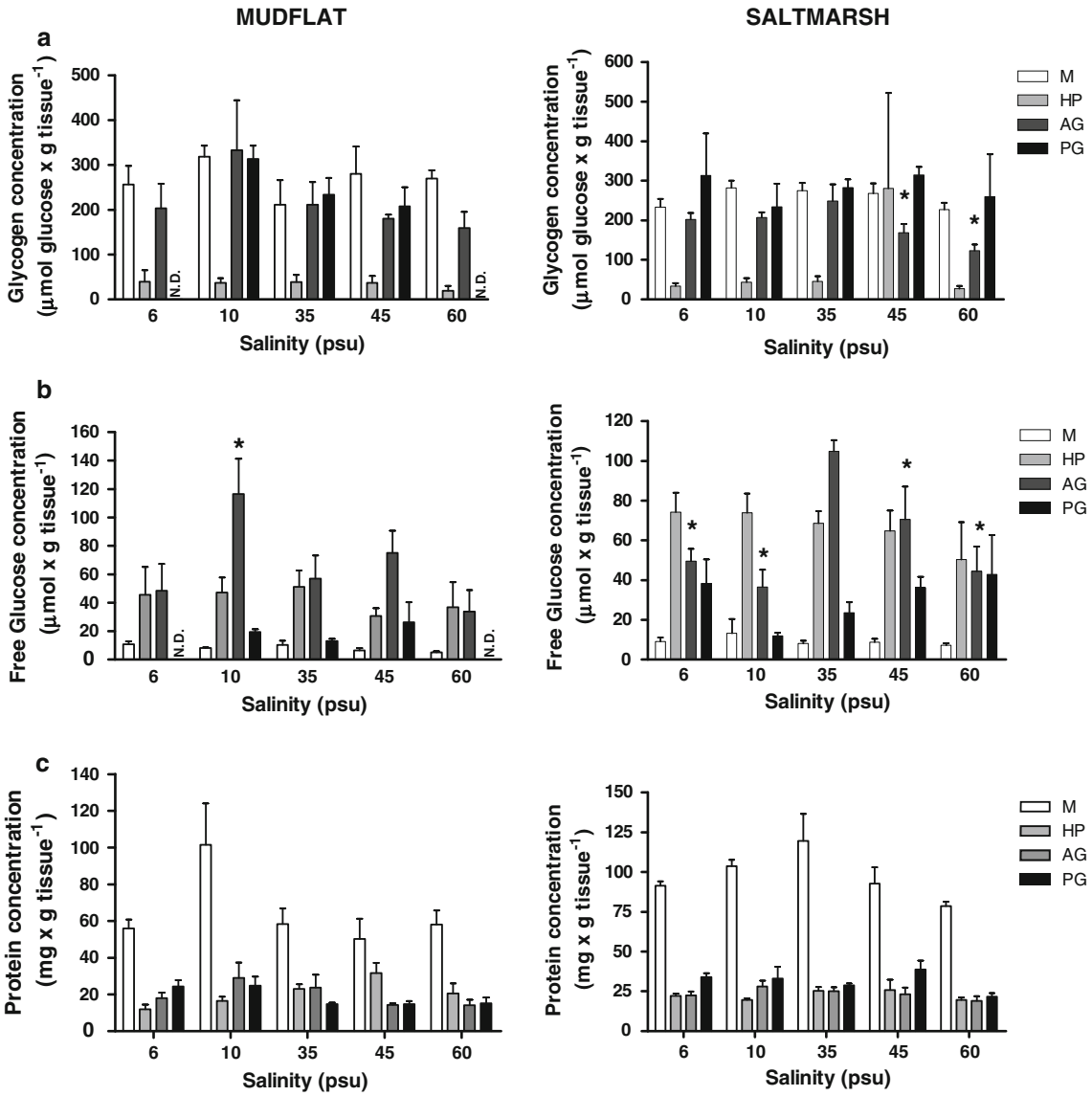

Fig. 6 Glycogen (**a**), free glucose (**b**) and protein (**c**) concentrations in muscle (M), hepatopancreas (HP), anterior gills (AG) and posterior gills (PG) of individuals of *N. granulata* from the mudflat (*left*) and the saltmarsh (*right*) acclimated to different salinities (6–60 psu). Data are the mean ± SEM for five crabs. *Asterisk* significantly different from the corresponding values in 35 psu salinity (one-way ANOVA, $p < 0.05$), *ND* not determined

and physiological adjustments (i.e., ion-transport systems or enzymes in gills) in relation to the degree of osmotic challenge. In posterior gills 6–8, a complex pattern of transcriptional regulation of Na^+/K^+-ATPase α-subunit mRNA and $Na^+/K^+/2Cl^-$ cotransporter mRNA dependent upon the direction of salinity change occurs in *N. granulata* from other geographical area (Luquet et al. 2005). Work in our laboratory showed differential adjustments in Na^+/K^+ATPase and alkaline phosphatase activity in chela muscle upon acclimation to 6 and 10 psu of crabs from the mudflat (Pinoni 2009).

The various molecular and biochemical changes such as increased gill Na^+/K^+-ATPase activity and/or hemolymph amino acids required by osmoregulatory adaptation to salinity need energy (Bianchini et al. 2008; Freire et al. 2008; McNamara and Faria 2012; Romano and Zeng 2012). A metabolic reorganization may occur leading to a modification of lipids, carbohydrate and/or protein metabolism, resulting in mobilization of energy reserves from distinct storage organs (Bianchini et al. 2008; Romano and Zeng 2012). The modulation of digestive enzymes as being a link between digestion and absorption could lead to differential availability of energy substrates for salinity acclimation (Li et al. 2008; Romano and Zeng 2012; Michiels et al. 2013). We have shown that male adult crabs of *N. granulata* from the mudflat and the saltmarsh without

acclimation (conditions in the natural environment) captured together with individuals acclimated to low and high salinity (this work) exhibited differential digestive and metabolic adjustments (Pinoni et al. 2011). We, then, determined and compared the effect of low (6 and 10 psu) and high (45 and 60 psu) salinity on the activity of key digestive enzymes in the hepatopancreas and energy reserves content to evaluate the possible occurrence of differential digestive and metabolic adjustments at the biochemical level in relation to habitat underlying osmoregulatory acclimation. We have previously shown that biochemical salinity adaptation in males of *N. granulata* from the mudflat involves the integrative modulation of several components such as Na^+/K^+-ATPase activity in gills, and Na^+/K^+-ATPase, the coexistent ouabain-insensitive Na^+ATPase (so called the second sodium pump) and alkaline phosphatase activities in chela muscle (Schleich et al. 2001; Pinoni et al. 2005; Pinoni 2009; Pinoni and López Mañanes 2009). The lower lipase activity in the hepatopancreas in low (6 and 10 psu) and high (45 and 60 psu) salinity (Fig. 3a) suggests that modulation of this activity is part of the biochemical changes occurring upon salinity adaptation of individuals from the mudflat. We have shown that modulation of key digestive enzymes activities constitutes one adjustment at the biochemical level upon acclimation to low and high salinity in *C. angulatus* (Michiels et al. 2013). Since total proteolytic, maltase and sucrase activities in the hepatopancreas of individuals from the mudflat were not affected (Figs. 3b and 4) and amylase activity was affected only at 10 psu as shown by previous work in our laboratory (Asaro et al. 2011), differential and specific mechanisms of modulation of digestive enzymes activities appear to occur in relation to salinity. The mechanisms of regulation (i.e., primary chemical messengers) or factors involved in the modulation of the activity and/or secretion of digestive enzymes in the hepatopancreas of crustaceans have been scarcely investigated and therefore are very far from having been elucidated. Previous work in our laboratory shows that in *C. angulatus*, lipase and proteolytic activities in the hepatopancreas are differentially affected by low and high salinity and dopamine injection, suggesting the occurrence of distinct digestive adjustments and mechanisms of regulation upon hyper- and hypo-osmoregulatory responses (Michiels et al. 2013). In the American crayfish *Orconectes limosus*, a differential release of digestive enzyme activity stimulated by vertebrate gastrointestinal hormones occurred in vitro (Resch-Sedlmeier and Sedlmeier 1999). In the lobster *Panulirus argus*, trypsin enzymes are regulated at the transcription and secretion level by distinct prandial signals (Perera et al. 2012). The digestive and absorptive processes at the biochemical level of *N. granulata* are still unknown. Dilucidating whether the modified lipase activity in the hepatopancreas upon acclimation to low and high salinity is related to a differential digestive (i.e., enhanced secretion of lipases) and/or absorptive capacity of lipids requires further experimental approach.

In crustaceans, lipids digested are mainly stored as triglycerides (over 80–90 %) which are a major source of energy in various species (Wright and Ahearn 1997; Sánchez-Paz et al. 2006; Dima et al. 2009). In *N. granulata* from Rio Grande city (Southern Brazil), mobilization of lipids would be related to the participation as energy sources during salinity acclimation (Luvizotto-Santos et al. 2003). The lower triglycerides content in chela muscle of individuals from the mudflat in low and high salinities compared to that in 35 psu (osmoconformation) (Fig. 5) suggests that a mobilization of these reserves occurs upon hyper- and hypo-regulation. The concomitant responses of lipase activity in the hepatopancreas and triglycerides content in chela muscle (Figs. 3a and 5) suggest that lipid metabolism would be involved in salinity acclimation of individuals of the mudflat. Since, as we pointed out above, the digestion–absorption routes are unknown in *N. granulata*, further experimental approach is needed to establish the possible interplay between activity of digestive enzymes in the hepatopancreas and metabolic pathways in energy storage tissues. The fact that triglyceride content was affected by salinity only in the chela muscle and not in the hepatopancreas (the major site of triglycerides storage in *N. granulata*, Fig. 5) suggests the occurrence of tissue-specific differential pathways and/or of differential mechanisms of regulation of these reserves. In *Carcinus maenas*, crustacean hyper-glycemic hormone regulates triglycerides content (Santos et al. 1997). Preliminary work in our laboratory shows that dopamine injection affects triglyceride content in storage tissues of *C. angulatus* (unpublished results). Contrary to individuals from the mudflat, the fact that low and high salinity had no effect on lipase activity in the hepatopancreas or on the content of triglycerides in storage tissues (Figs. 3a and 5) suggests that lipid metabolism would not have a role in biochemical adaptation to salinity in crabs from the saltmarsh. This appears to be in accordance with that we have found in individuals from the saltmarsh in the natural environment, in which the utilization of lipids as energy source appears to be diminished during autumn compared to mudflat (Pinoni et al. 2011), which could in part explain the differences found in the modulation of lipase and triglycerides storages in response to salinity between crabs from both sites (this work). Furthermore, these results support the idea of the occurrence of differential adjustments for the utilization of this energy reserve and then of distinct regulatory pathways operating in crabs from both habitats as we suggested to occur in the field (Pinoni et al. 2011). These differential metabolic adjustments could be further operating in biochemical

acclimation to low and high salinity. The influence of habitat in responses of various parameters to salinity occurs under laboratory conditions in individuals of the euryhaline crab *Carcinus maenas* from different geographical areas (Pinto et al. 2012).

We have shown that the anterior gills have an important role in biochemical adaptation to salinity and in the metabolism of carbohydrates in *N. granulata* (López Mañanes et al. 2000; Schleich et al. 2001; Artillo et al. 2008; Pinoni 2009; Pinoni et al. 2011; González et al. 2012, unpublished results). The lower glycogen content in anterior gills in individuals from the saltmarsh in 45 and 60 psu suggests a utilization of carbohydrate storages of these gills upon hypo-regulation. Since no changes occurred in low salinity (Fig. 6a), the variations in glycogen content in anterior gills would be dependent on the osmoregulatory response. Hyper- and hypo-osmoregulations in crabs appear to require different mechanisms (McNamara and Faria 2012; Romano and Zeng 2012; Michiels et al. 2013). Hypo- and hyper-osmotic stresses in vitro on muscle and hepatopancreas sections of *N. granulata* from other geographical areas induce different adjustments in metabolic pathways (i.e., changes in the carbon amino acid flux between gluconeogenesis and lipid synthesis) (Martins et al. 2011). Work in our laboratory shows that a mobilization of glycogen from anterior gills occurs only at high salinity in *C. angulatus* (Asaro et al. 2012). Since little is known about chemical messengers involved in the regulation of carbohydrate metabolism in gills of *N. granulata*, whether the glycogen content in anterior gills of crabs of the saltmarsh is under hormonal control requires further investigation. In vitro, insulin stimulated glucose uptake and glycogen synthesis in gills of individuals from other regions (Kucharski et al. 2002). Recently, we found that dopamine injection affects glycogen content in anterior gills of *C. angulatus* (del Valle et al. 2012). The change in glycogen content in anterior gills upon acclimation to high salinity only in individuals of *N. granulata* from the saltmarsh (Fig. 6a) further supports the idea of differential adjustments in metabolic pathways and mechanisms of regulation operating in response to salinity in relation to habitat. We showed that a differential modulation of glycogen concentration in the anterior gills in crabs from the mudflat and the saltmarsh occurred in the natural environment (Pinoni et al. 2011). The role of anterior gills and their distinct participation in biochemical adaptation to low and high salinity is further supported by the fact that free glucose content was also differentially affected (Fig. 6b). Whether the distinct free glucose content in anterior gills (while glycogen content in not affected) is related to differential modulation of gluconeogenic pathways which could lead to the formation of glucose from other sources requires further experiments. The fact that free glucose content in anterior gills of crabs from the mudflat was only affected in 10 psu supports the occurrence of differential responses also depending on the degree of osmotic challenge (Fig. 6b). This is in accordance with that we pointed out above about the occurrence of differential biochemical and physiological adjustments (i.e., ion-transport systems or enzymes in gills and in other tissues) in relation to the degree of hypo-osmotic challenge in this crab. In decapod crustaceans, the maintenance of suitable levels of glucose in the hemolymph is essential for supporting the regular functions of various organs, such as brain and muscle, and in response to various environmental stresses such emersion, temperature, salinity and pollutants (Verri et al. 2001; Lorenzon et al. 2005). The similar values of glycemia in crabs from both sites suggest that availability of glucose from the hemolymph would not be a constraint upon acclimation to low and high salinity. One of the main sources of hemolymphatic glucose comes from the hepatopancreas where it is stored as glycogen or synthesized by gluconeogenic pathways (Verri et al. 2001; Obi et al. 2011). We have shown that the hepatopancreas of *N. granulata* from the mudflat and the saltmarsh exhibited a low glycogen content under differential laboratory conditions and in the field (Artillo et al. 2008; Pinoni 2009; Pinoni et al. 2011; Fig. 6a, this work) but high levels of free glucose (Pinoni et al. 2011, this work, Fig. 6b), suggesting a role in the metabolism of carbohydrates probably in the maintenance of an adequate and sustained glucose supply. The chela muscle constitutes an important site of glycogen storage in *N. granulata* from Mar Chiquita coastal lagoon (Artillo et al. 2008; Pinoni et al. 2011; this work). However, since glycogen content in this tissue was not affected by exposure to low or high salinity (Fig. 6a), the mobilization of this reserve would not be involved in biochemical acclimation to salinity in individuals from both sites.

In various crustaceans, protein metabolism plays a key role in biochemical adaptation to salinity by supporting and adequate provision of amino acids necessary for the maintenance of osmoregulation (Shinji et al. 2012; Romano and Zeng 2012). In high salinities, some species increase free amino acids in the hemolymph (i.e., via breakdown of proteins in the hemolymph and/or storage organs), which enhances the osmotic pressure, although this is not a universal response (Romano and Zeng 2012). Since low or high salinity did not affect proteolytic activity in the hepatopancreas or protein content in storage organs of *N. granulata* from both sites (Figs. 3 and 6c), adjustments in protein metabolism (i.e., digestive and/or synthesis/degradation pathways) appear not to be a response involved in biochemical adaptation to salinity. In *N. granulata* from Brazilian populations, salinity affects protein metabolism in vitro, but variations are dependent of the

degree and type of osmotic challenge (Bianchini et al. 2008). This could be the case for *N. granulata* from Mar Chiquita coastal lagoon since preliminary work of our laboratory shows the occurrence of variations in protein content in the hepatopancreas upon an abrupt change in salinity (unpublished results).

In conclusion, the results of this work show that lipase activity in the hepatopancreas, triglycerides content in the chela muscle, and glycogen and free glucose concentrations in the anterior gills are differentially affected upon acclimation to low and high salinity in individuals of *N. granulata* from the mudflat and the saltmarsh, suggesting the occurrence of differential metabolic adjustments at the biochemical level upon hyper- and hypo-regulation and in relation to habitat. As we mentioned above, we have found that in the field differential adjustments in lipids, protein and carbohydrate metabolism (at the level of key digestive enzymes activities and utilization of energy reserves) occur in crabs from the mudflat and the saltmarsh, suggesting that distinct intrinsic mechanisms of regulation such as distinct signaling pathways could be operating likely triggered by the particular environmental characteristics in each site (i.e., salinity regime/food resources) (Pinoni et al. 2011). The fact that digestive enzyme activities (i.e., lipase activity) and energy reserves (i.e., triglycerides/carbohydrates), which are modulated in relation to habitat in the natural environment, are also differentially affected in crabs from both sites upon acclimation to low and high salinity (this work) suggests that these distinct intrinsic mechanisms could be operating upon osmotic adaptation to salinity leading to a differential use of digestive and metabolic pathways in relation to habitat. On the other hand, although they have a similar osmoregulatory pattern and capacity (Figs. 1 and 2), works in our laboratory show that individuals from the mudflat and the saltmarsh exhibit distinct responses of branchial ion-transporting systems upon salinity acclimation, suggesting that different osmoregulatory mechanisms would be operating in relation to habitat (Schleich et al. 2001; Pinoni et al. 2005; González et al. 2012; unpublished results). The distinct branchial osmoregulatory mechanisms could imply differential energy needs, which in turn could lead to the differences found between individuals from both sites in lipase activity in the hepatopancreas and use of energy reserves. A further experimental approach determining bioenergetics parameters such as oxygen consumption rate, a tool commonly used to evaluate energy demands in crustaceans (McGaw 2006; Normant et al. 2012), and/or metabolic routes involved is needed to test this hypothesis. Future studies should be focused on establishing the possible links and physiological role of the modulation of digestive enzymes and energy reserves to provide a better understanding of the complex integrative responses and mechanisms of regulation underlying biochemical adaptation to salinity and habitat occupancy in *N. granulata* in particular and in hyper-/hypo-regulating crabs in general.

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