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Digestive flexibility in a euryhaline crab from a SW Atlantic coastal lagoon: alkaline phosphatase activity sensitive to salinity in the hepatopancreas

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We studied biochemical characteristics and the response to low salinity at short and long-term after feeding of alkaline phosphatase (AP) activity in hepatopancreas of the osmoregulator crab Neohelice granulata *from Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina) (37°32′–37°45′S 57°19′–57°26′W). The hepatopancreas exhibited a levamisole-insensitive and a levamisole-sensitive AP activity with distinct characteristics. Levamisole-insensitive activity was similar within the range of pH 7.4–9.0 and exhibited a Michaelis–Menten kinetics. Levamisole-sensitive AP activity appeared to be maximal at pH 8.5 and appeared to exhibit an allosteric kinetics. In crabs acclimated to 10 psu (hyper-regulation conditions) levamisole-insensitive and levamisole-sensitive AP activity increased (about 16-fold) over time from short term (2–4 h) to long term (120 h) after feeding while no changes occurred in crabs acclimated to 35 psu (osmoconforming conditions). The changes of AP activity along with the higher values at 120 h after feeding in 10 psu compared with those in 35 psu, and the concomitant changes in proteolytic activity, suggest a role of AP in digestive and metabolic adjustments at the biochemical level upon hyper-regulatory conditions.*

Keywords: digestive flexibility, alkaline phosphatase, hepatopancreas, crabs

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INTRODUCTION

Estuaries and coastal lagoons constitute extremely challenging environments in which abiotic factors, particularly salinity, vary abruptly both spatial and temporally. Euryhaline crabs successfully occupying the intertidal area of coastal lagoons have to cope with abrupt and wide changes in environmental salinity (McNamara & Faria, 2012; Romano & Zeng, 2012). Biochemical adaptation to environmental salinity in such species is a complex process involving the participation of different enzymes and transport systems in branchial and extrabranchial tissues such as hepatopancreas and muscle (Pinoni & López Mañanes, 2004, 2008, 2009; Jahn *et al.*, 2006; Martins *et al.*, 2011; Michiels *et al.*, 2013; Pinoni *et al.*, 2013). However, some mechanisms of biochemical adaptation to low salinity, particularly from a metabolic perspective are still not fully understood (McNamara & Faria, 2012; Romano & Zeng, 2012). The hepatopancreas of decapod crustaceans is a multifunctional organ playing a key role in digestion and absorption (Ceccaldi, 1989; Verri *et al.*, 2001; Muhlia-Almazán & Garcia-Carreño, 2003; Zeng *et al.*, 2010). In some euryhaline species, the modulation of different digestive enzymes activities in the hepatopancreas in response to low salinity suggests the occurrence of digestive and metabolic adjustments at the biochemical level which could lead to a greater availability of substrates (i.e. aminoacids) for salinity acclimation (Li *et al.*, 2008; Asaro *et al.*, 2011; Romano & Zeng, 2012; Michiels *et al.*, 2013, 2015a, b; Pinoni *et al.*, 2013; Wang *et al.*, 2014). Alkaline phosphatases (AP) (EC 3.1.3.1) are membrane-bound glycoproteins that are widely found in animals which hydrolyse phosphate from a variety of molecules at a range of optimal pH above 7.0 (Ali *et al.*, 2013; Buchet *et al.*, 2013; Linder *et al.*, 2013). In the gastrointestinal tract of mammals, AP has a role in digestive and absorptive processes (Geddes & Philpott, 2008; Buchet *et al.*, 2013; Lallès, 2014). Although the physiological function of AP activity in the hepatopancreas of crustaceans has not been yet elucidated, it has been suggested to be involved in digestive enzyme synthesis, accumulation and secretion, as well as in the absorption and storage of digestive products (Momin & Rangneker, 1974; Barker & Gibson, 1977; Gibson & Barker, 1979; Wang *et al.*, 2014). Thus, level of AP activity in the hepatopancreas and its potential modulation by low salinity would play a central role in digestion, absorption and utilization of digestive products. However, nothing is known about the possible modulation by low salinity of AP in the hepatopancreas of osmoregulating crabs and the possible link with modulation of digestive enzymes. Moreover, information about the occurrence and biochemical characteristics of AP activity in the hepatopancreas of euryhaline crabs is still scarce and fragmentary. To our knowledge, *Scylla serrata* is

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the only euryhaline crab species in which studies of localization, characterization and modulation after feeding of AP of the digestive tract have been done (Momin & Rangneker, 1974; Barker & Gibson, 1977; Chen *et al.*, 2000).

Neohelice (*Chasmagnathus*) *granulata* (Decapoda: Varunidae; Dana, 1852) 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 South-western Atlantic from southern Brazil to the northern Argentinean Patagonia (Spivak, 1997; Iribarne *et al.*, 2003; Luppi *et al.*, 2013). In Mar Chiquita coastal lagoon (Argentina), *N. granulata* is one of the dominant crabs inhabiting areas with abrupt, frequent and highly variable changes in salinity (Spivak *et al.*, 1994; Iribarne *et al.*, 1997; Bortolus and Iribarne 1999; Fanjul *et al.*, 2008; Luppi *et al.*, 2013). Previous work from our lab shows that complex and integrative responses occur upon acclimation to low salinity (López Mañanes *et al.*, 2000; Schleich *et al.*, 2001; Pinoni *et al.*, 2005, 2013; Pinoni & López Mañanes, 2009; Asaro *et al.*, 2011; Michiels *et al.*, 2015a). In chela muscle of *N. granulata* levamisole-insensitive AP activity is differentially modulated by low salinity suggesting its role in adjustments underlying hyper-regulation (Pinoni *et al.*, 2005). The differential modulation of various digestive enzyme activities in the hepatopancreas and energy reserves in response to low salinity suggests the occurrence of complex digestive and metabolic adjustments at the biochemical level in relation to hyper-regulation in this crab (Pinoni, 2009; Asaro *et al.*, 2011; Pinoni *et al.*, 2013; Michiels *et al.*, 2015a). To increase knowledge of different aspects of the biology of *N. granulata* and as part of our studies on the identification of enzyme activities involved in biochemical adaptations to salinity in osmoregulating crabs, the aim of this work was to study biochemical characteristics and the responses to low salinity of AP activity in the short and long term after feeding in hepatopancreas of *N. granulata*.

MATERIALS AND METHODS

Chemicals

pNPP (p-nitrophenylphosphate), Tris-(hydroxymethylaminomethane) (Tris), bovine serum albumin, azocasein and levamisole (L [_]-2, 3, 5, 6-Tetrahydro-6-phenylimidazol [2,1-b] thiazole) were from Sigma (St. Louis, MO, USA), and sucrose was obtained from Merck (Darmstadt, Germany); magnesium sulphate and Coomassie blue G250 were from Fluka (Germany). All these chemicals were used as solutions prepared in glass-distilled water and buffered to correspond to Gomori (1955).

Animal collection and maintenance

The crabs were live trapped by hand from burrows of the mudflat area from Mar Chiquita coastal lagoon (Buenos Aires province, Argentina; 37°32′–37°45′S 57°19′–57°26′W), as we described (Pinoni *et al.*, 2011, 2013). For all the experiments, salinity was measured in practical salinity units (psu). Only adult male crabs with a carapace width greater than 2.5 cm were collected. Individuals were identified by unique and conspicuous morphological characteristics. Animals were transported to the laboratory in lagoon water under oxygenation on the day of collection. The time duration between catching ground and the laboratory was not longer than 45 min. The crabs were maintained in natural seawater (35 psu) or dilute seawater (10 psu) for 14 days (Pinoni *et al.*, 2005; Pinoni & López Mañanes, 2009). Dilute seawater was obtained by dilution of natural seawater with distilled water. The aquaria contained 36 L of water, continuously aerated and filtered. A regime of 12 h light/12 h dark was applied and the temperature was kept at 20 ± 2°C. The water was continuously filtered by means of an Atman filter (HF-0400). Crabs were fed three times a week with commercial food (Cichlind T.E.N., Wardley, USA) (about 0.07 g per individual). To study the effect of low salinity on AP activities in the hepatopancreas, crabs acclimated to 35 psu (control) and 10 psu, and unfed for 120 h, were individually fed. The 0 time was taken when the total amount of offered food was eaten which took up to 5 min. Crabs which did not eat or partially ate the offered food were discarded. AP activities were determined at the short term (2, 4 h) and long term (120 h) after feeding. These times were chosen based on studies in our lab which show that in male individuals of *N. granulata* from Mar Chiquita coastal lagoon digestive and metabolic adjustments relating to ingesta occur up to 120 h after feeding (Asaro *et al.*, 2009; Méndez *et al.*, 2011, 2012; Michiels *et al.*, 2015, unpublished results). Total proteolytic activity and protein concentration in the hepatopancreas were determined simultaneously with AP activities as an index of responses in protein metabolism.

Sampling procedures

The crabs were weighed and cold-anaesthetized by putting them on ice for about 25 min. A sample of haemolymph was withdrawn for assaying of osmolality as described below. Wet mass was measured to the nearest 0.01 g. After weighing, 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). 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) was added to supernatant samples before freezing (Ljungström *et al.*, 1984).

Biochemical assays

The assay for determining AP activity was performed as we previously described (Pinoni *et al.*, 2005; Pinoni & López Mañanes, 2008) with some modifications. In the standard assay, levamisole-insensitive AP activity 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 AP 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. 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. The AP activities were expressed as µmol pNP min^{-1} mg $protein^{-1}$. To study the effect of pH

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on AP activity, the procedure was the same as described above except that the activity was determined in the presence of varying pH of the reaction mixture. To study the effect of pNPP concentration on AP activity, the procedure was the same as described above except that the activity was determined in the presence of varying pNPP concentrations in the reaction mixture. Individuals acclimated to 35 psu and unfed for 48 h (Pinoni *et al.*, 2005) were used in these experiments.

Total proteolytic activity in the hepatopancreas was assayed as we previously described (Pinoni *et al.*, 2013). 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). 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). The unit of 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 $protein^{-1}$.

Protein was assayed according to Bradford (1976). Bovine serum albumin was used as standard.

Haemolymph osmolality

Haemolymph (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 2000 g for 3 min (IEC-Centra 7R, refrigerated). Osmolality (mOsm kg^{-1}) was measured in an aliquot of 50 µL of haemolymph and medium with a cryoscopic osmometer (Osmomat 030, Gonotec).

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 (one-way ANOVA or repeated measures ANOVA), non-parametric (Kruskal–Wallis) analysis of variance, or *t*-test was used to estimate the statistical significance of the differences and $P < 0.05$ was considered significant. A posteriori test to ANOVA (Holm-Sidak or Dunn) was used to identify differences. Results of effect of varying concentrations of pNPP on AP activity were analysed by means of non-linear regression analysis (GraphPad Prism 5.01 software). The mathematical models used were one-site binding, two-site binding and sigmoidal dose-response. The corresponding curves shown are those which best fit the experimental data.

RESULTS

AP activity in hepatopancreas of *N. granulata*: effect of pH and pNPP

In preliminary experiments, total AP activity in hepatopancreas was determined within the range of pH 7.0–9.0. Total AP activity was higher at pH 8.5 (data not shown). Then, AP activity at pH 8.5 was determined in the presence and in the absence of 16 mM levamisole (Pinoni *et al.*, 2005). The inhibition of total AP activity by levamisole revealed the presence in hepatopancreas of two AP activities: a levamisole-insensitive and a levamisole-sensitive activity (data not shown). Since, in the presence of 16 mM levamisole, inhibition of total AP activity was maximal, this concentration of inhibitor was used for further experiments described below.

No significant differences were found in levamisole-insensitive AP activity within the range of pH 7.4–9.0 (Figure 1A). Levamisole-sensitive AP activity was maximal at pH 8.5, decreasing about 25% at pH 9.0. Within the range of pH 7.4–8.0 levamisole-sensitive AP activity was about 67% lower than maximal activity (Figure 1B).

The effect of pNPP concentrations on AP activity in hepatopancreas of *N. granulata* is shown in Figure 2. Levamisole-insensitive AP activity exhibited Michaelis–Menten kinetics ($K_m = 0.22$ mM) (Figure 2A). The plot of levamisole-sensitive AP activity *vs* pNPP concentration gave a sigmoidal curve suggesting an allosteric kinetics (Figure 2B).

Effect of low salinity on AP activity of hepatopancreas of *N. granulata* at different times after feeding

The haemolymph osmolality of the crabs was significantly higher from the external medium at 10 psu while no differences were detected at 35 psu (Table 1).

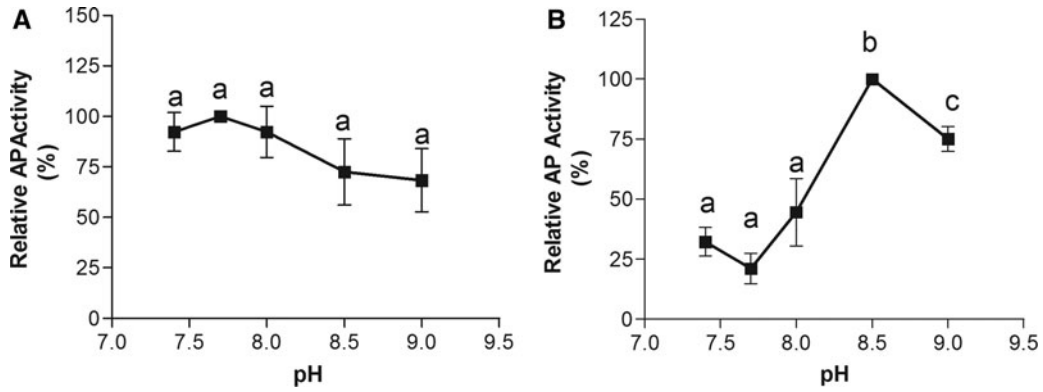

Fig. 1. Effect of pH (7.4–9.0) on levamisole-insensitive (A) and levamisole-sensitive (B) AP activity in hepatopancreas of *N. granulata* acclimated to 35 psu. The values of AP activity are expressed as relation to the activity at pH 7.7 (levamisole-insensitive) and pH 8.5 (levamisole-sensitive) (100%). Data are the mean ± SE for five individuals. Different letters indicate significant differences (RM-ANOVA, $P < 0.05$).

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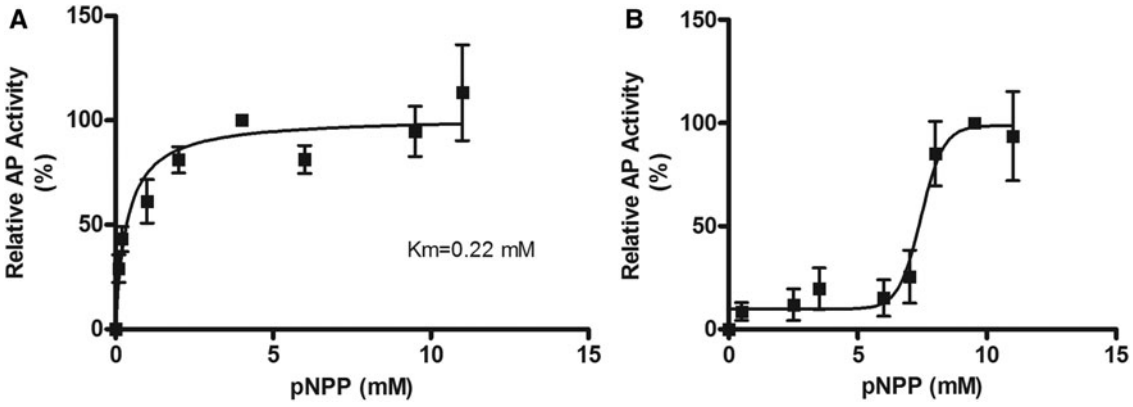

Fig. 2. Effect of pNPP on levamisole-insensitive (A) and levamisole-sensitive (B) AP activity at pH 7.7 and 8.5, respectively, in hepatopancreas of *N. granulata* acclimated to 35 psu. The curves are the ones which best fit the experimental data (GraphPad Prism 4). The values of AP activity are expressed as a relation to the activity at 9.5 mM pNPP (100%). In some cases, deviation bars were smaller than the symbols used. Data are the mean ± SE for five individuals. When corresponding, K_m value (Michaelis–Menten constant) was estimated by analysis of data using a Lineweaver–Burk plot.

Table 1. Osmolality (mOsm kg^{-1}) in external medium and in *N. granulata* haemolymph

	35 psu [a]		10 psu [a]	
	Medium	**Haemolymph**	**Medium**	**Haemolymph**
Osmolality	899.0 ± 20.3	853.1 ± 41.6	291.0 ± 22.5	674.0 ± 41.6*

[a]Haemolymph of crabs maintained to either 35 or 10 psu.
*Significantly different from the corresponding concentration of the external medium (*t*-test, $P < 0.05$). Data are the mean ± SE, N = 10–15.

In individuals acclimated to 35 psu, levamisole-insensitive and levamisole-sensitive AP activities in hepatopancreas were similar at 2, 4 and 120 h after feeding (Figure 3A).

In 10 psu, levamisole-insensitive and levamisole-sensitive AP activities were higher (848 ± 394 and 260 ± 134 µmol pNP min^{-1} mg $protein^{-1}$, respectively) at 120 h after feeding than the corresponding values at 2 and 4 h after feeding (39 ± 20 and 19 ± 13 µmol pNP min^{-1} mg $protein^{-1}$, respectively) (Figure 3B).

In 10 psu, both levamisole-insensitive and levamisole-sensitive AP activities at 120 h after feeding were higher than the corresponding values in 35 psu (31 ± 18 and 4 ± 2 µmol pNPP min^{-1} mg $protein^{-1}$, respectively).

Effect of low salinity on proteolytic activity and protein concentration in hepatopancreas of *N. granulata* at different times after feeding

In 35 psu, total proteolytic activity in hepatopancreas was similar at 2, 4 and 120 h after feeding (Figure 4A).

In individuals acclimated to 10 psu, total proteolytic activity was higher at 120 h after feeding (4.8 ± 0.6 U min^{-1} mg $protein^{-1}$) than the activity at 2 and 4 h (1.9 ± 0.4 and 2.5 ± 0.4 U min^{-1} mg $protein^{-1}$, respectively) (Figure 4B).

In 35 psu, protein concentration in the hepatopancreas was similar at 2, 4 and 120 h after feeding (Figure 4C). In 10 psu, protein concentration in the hepatopancreas was higher at 4 h (28 ± 3.6 mg protein g $tissue^{-1}$) and decreased by 120 h after feeding (12.7 ± 1.8 mg protein g $tissue^{-1}$) (Figure 4D).

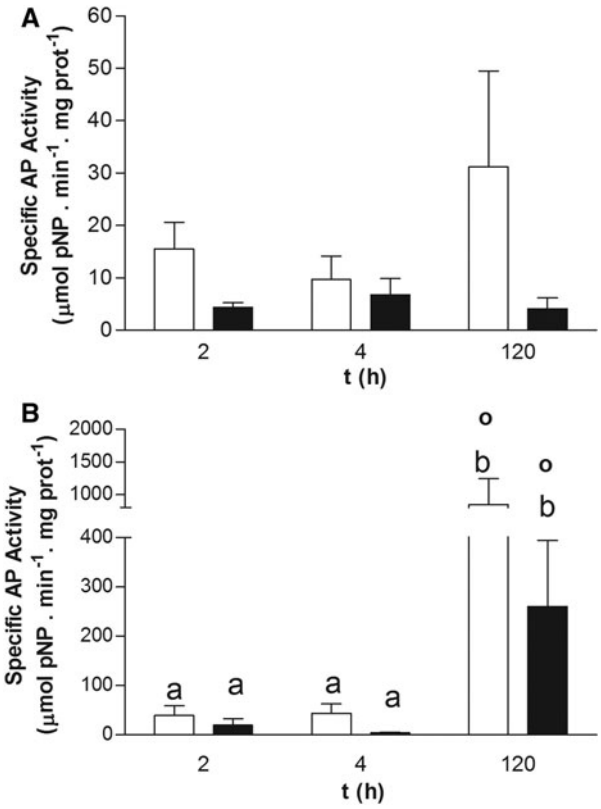

Fig. 3. AP activity in hepatopancreas of *N. granulata* maintained to 35 (A) and 10 psu (B) 2, 4 and 120 h after feeding. Open bars: levamisole-insensitive AP activity. Black bars: levamisole-sensitive AP activity. Different letters indicate significant differences between corresponding values at the same salinity (ANOVA, $P < 0.05$). °Indicate significantly different from the corresponding activity at 35 psu (*t*-test, $P < 0.05$). Data are the mean ± SE for five individuals.

DISCUSSION

Our results show the occurrence of levamisole-insensitive and levamisole-sensitive AP activities in hepatopancreas of the euryhaline crab *N. granulata* from Mar Chiquita coastal lagoon and their modulation after feeding in low salinity. Levamisole is an inhibitor of AP commonly used to discriminate between different mammals' AP isoforms (Chan & Stinson, 1986; Mota *et al.*, 2008; Diez-Zaera *et al.*, 2011).

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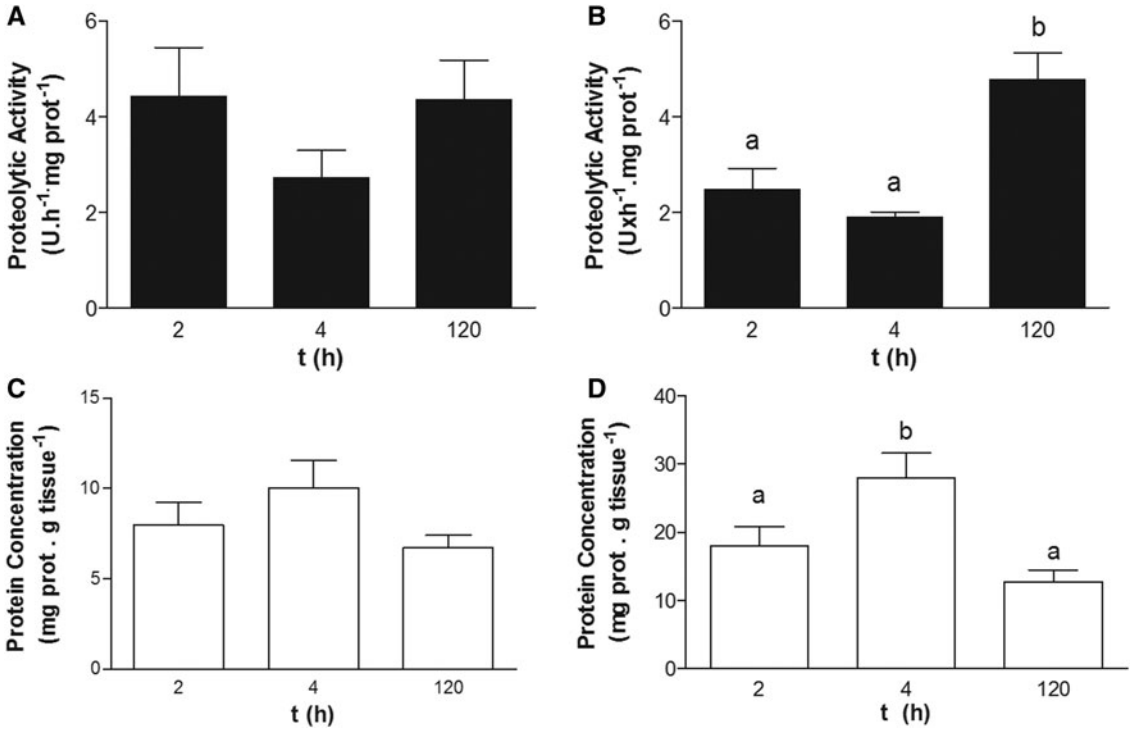

Fig. 4. Total proteolytic activity and protein concentration in hepatopancreas of *N. granulata* maintained to 35 (A, C) and 10 psu (B, D) 2, 4 and 120 h after feeding. Different letters indicate significant differences between corresponding values at the same salinity (ANOVA, $P < 0.05$). Data are the mean ± SE for 5–10 individuals.

The inhibition of total AP activity by levamisole in hepatopancreas of *N. granulata* revealed the presence of two AP activities: a levamisole-insensitive and a levamisole-sensitive AP. This is similar to that we previously found in the chela muscle of this crab and of the euryhaline crab *Cyrtograpsus angulatus* (Pinoni *et al.*, 2005; Pinoni & López Mañanes, 2008). AP activity in mammals is characterized by exhibiting a high pH optimum (Ohkubo *et al.*, 1974; Chan & Stinson, 1986; Simao *et al.*, 2007). In invertebrates, AP activities show a range of optimum pH values between 7.1 and 10.5 (Lovett *et al.*, 1994; Mazorra *et al.*, 2002; Linton *et al.*, 2014). Levamisole-insensitive and levamisole-sensitive AP activities in hepatopancreas of *N. granulata* exhibited a quite different pH profile (Figure 1). This agrees to that found for these activities in chela muscle of this crab (Pinoni *et al.*, 2005) and in gills of *C. sapidus* (Lovett *et al.*, 1994). The maximal levamisole-sensitive AP activity at pH 8.5 in hepatopancreas of *N. granulata* (Figure 1B) was distinct to the activity in chela muscle (Pinoni *et al.*, 2005), and in gills of *C. sapidus* (Lovett *et al.*, 1994). The response to pH of AP activity of the hepatopancreas of *N. granulata* (Figure 1) was also different to that found for AP activity in hepatopancreas of the shrimp *Pandalus borealis* (Olsen *et al.*, 1991) and the viscera of the crab *S. serrata* (Chen & Zhou, 1998). The Michaelis–Menten kinetics in response to varying pNPP concentrations of levamisole-insensitive AP activity of hepatopancreas of *N. granulata* (Figure 2A) are in agreement with that found in chela muscle of this crab (Pinoni *et al.*, 2005), in gills of *C. sapidus* (Lovett *et al.*, 1994) and for the AP purified from the viscera of *S. serrata* (Chen *et al.*, 2000). The allosteric kinetics exhibited by levamisole-sensitive AP activity in hepatopancreas of *N. granulata* in response to pNPP concentrations (Figure 2B) is quite different to that found for this activity in chela muscle of this crab (Pinoni *et al.*, 2005). Some mammalian AP isoenzymes have been described to exhibit allosteric kinetics which suggests that these activities could be more sensitive and affected by the binding of substrate molecules (Millán, 2006; Simao *et al.*, 2007). Whether the different biochemical characteristics of levamisole-insensitive and levamisole-sensitive AP activities in the hepatopancreas of *N. granulata* (this work) to those of the activities in chela muscle (Pinoni *et al.*, 2005) are due to the occurrence of different tissue-specific forms, with distinct functions, requires further investigation.

Biochemical adaptation to salinity implies various molecular and biochemical changes such as those in haemolymph amino acid (Freire *et al.*, 2008; McNamara & Faria, 2012; Romano & Zeng, 2012; Larsen *et al.*, 2014). *Neohelice granulata* from the mudflat of Mar Chiquita coastal lagoon behaves as a hyper-regulator at low salinity since it exhibits haemolymph osmolality values higher from those of the corresponding external medium upon acclimation to 10 psu, while it osmoconforms in 35 psu (López Mañanes *et al.*, 2000; Schleich *et al.*, 2001; Pinoni *et al.*, 2005, 2013; Pinoni & López Mañanes, 2009; Asaro *et al.*, 2011; Michiels *et al.*, 2015a; Table 1, this work). We have shown that biochemical acclimation to low salinity involves the integrative modulation of several components in gills and extrabranchial tissues (López Mañanes *et al.*, 2000; Schleich *et al.*, 2001; Pinoni, 2009; Pinoni & López Mañanes, 2009; Asaro *et al.*, 2011; Pinoni *et al.*, 2013; Michiels *et al.*, 2015a), among them, the levamisole-insensitive AP activity in chela muscle (Pinoni *et al.*, 2005). The concomitant modulation of various digestive enzyme activities in the hepatopancreas and energy reserves content suggests the occurrence of digestive and metabolic adjustments at the biochemical level in this crab upon

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hyper-regulation conditions (Pinoni, 2009; Asaro *et al.*, 2011; Pinoni *et al.*, 2013). The increase in both levamisole-insensitive and levamisole-sensitive AP activities by 120 h after feeding in 10 psu (hyper-regulation conditions), while no changes occurred in 35 psu (osmoconforming conditions) (Figure 3), suggests that modulation of these activities is another component involved in response to low salinity. This is further supported by the fact that in crabs acclimated to 10 psu, AP activities in the hepatopancreas by 120 h after feeding were higher than the corresponding values in 35 psu (Figure 3). In various crustaceans, an adequate protein intake is essential to support amino acid provision necessary for the maintenance of osmoregulation (Sánchez-Paz *et al.*, 2006; Romano & Zeng, 2012; Larsen *et al.*, 2014). Digestive enzymes, being a link between digestion and absorption, could be modulated leading to a differential availability of energy substrates for salinity acclimation (Li *et al.*, 2008; Asaro *et al.*, 2011; Romano & Zeng, 2012; Michiels *et al.*, 2013; Pinoni *et al.*, 2013). Although not yet elucidated, it has been suggested that one role of AP in the hepatopancreas involved its participation in the process of synthesis and accumulation of digestive enzymes (Barker & Gibson, 1977; Wang *et al.*, 2014). In the hepatopancreas of *S. serrata*, AP activity in the brush border of R-cells has been associated with the luminal absorption of nutrients (Monin & Rangneker, 1974). The hepatopancreas of decapod crustaceans appears to have a role in the initial steps of macromolecules synthesis (i.e. proteins) after feeding (McGaw & Curtis, 2013; Carter & Mente, 2014). The higher protein concentration in the hepatopancreas of *N. granulata* at 4 h after feeding in low salinity (Figure 4) suggests that this could be the case for *N. granulata* under hyper-regulating conditions. The lower AP activities by 2–4 h suggest that these activities would not be involved in this initial adjustment in protein metabolism in the hepatopancreas after feeding in low salinity. However, over time further increase in AP activity along with that in total proteolytic activity (Figures 3 and 4) suggests a role for AP activity in posterior metabolic adjustments (i.e. decrease in protein concentration). This similar profile and response to low salinity of AP and total proteolytic activities in the hepatopancreas of *N. granulata* (Figures 3 and 4) suggests the occurrence of concomitant mechanisms of modulation. It has been proposed that when the internal reserves must be mobilized from the hepatopancreatic cells of crustaceans, digestive enzymes that are secreted during the digestive cycle could be activated intracellularly and finely regulated (Sánchez-Paz *et al.*, 2006). Studies *in vitro* in our lab show the occurrence of a modulation of intracellular lipase and proteolytic activity in the hepatopancreas of *N. granulata* and the euryhaline crab *C. angulatus* by primary chemical and intracellular messengers (i.e. cyclic AMP) (Pinoni & López Mañanes, 2014; Michiels *et al.*, 2015b; unpublished results). Whether the increase in both AP activities in the hepatopancreas is related to a role as components in signalling pathways leading to the modulation (i.e. increase) of proteolytic activities in the hepatopancreas under low salinity conditions remains to be investigated. Modulation of AP activity could be related to an increase for dephosphorylation of components involved in the regulation of proteolytic activities. Further experimental approaches are needed to test this hypothesis. Many physiological processes in animals are under regulation via the formation (phosphorylation) or cleavage (dephosphorylation) of phosphate esters in which the dephosphorylation process is catalysed by different APs (Linder *et al.*, 2013). The fact that both levamisole-insensitive and levamisole-sensitive AP were affected by low salinity (Figure 4) is in agreement with that shown by Lovett *et al.* (1994) in the gills of *C. sapidus*. These authors suggested that modulation of levamisole-sensitive and levamisole-insensitive AP activities in the gills of *C. sapidus* upon acclimation to low salinity could be involved in the regulation of the synthesis or delivery of polyamines which in turn affect the activity of branchial key enzymes such as Na^+K^+ATPase. The modulation of AP activity by primary chemical messengers in crustaceans has not yet been elucidated. We have shown that AP activity in chela muscle of *C. angulatus* is regulated by dopamine (Pinoni & López Mañanes, 2004, 2008). We have shown that dopamine increases *in vivo* lipase activity in hepatopancreas of *C. angulatus*, and *in vitro* lipase and N-aminopeptidase activities in hepatopancreas of *N. granulata*, suggesting that this biogenic amine would be one primary chemical messenger involved in the regulation of digestive enzyme activities in euryhaline crabs (Michiels *et al.*, 2013, 2015a, b). Whether the responses of AP activity in hepatopancreas of *N. granulata* in low salinity are also related to a regulation by dopamine remains to be investigated.

In summary, our results show the existence of levamisole-insensitive and a levamisole-sensitive AP activities in hepatopancreas of *N. granulata* which are modulated after feeding in low salinity suggesting their participation as components of the complex process of biochemical adaptation to environmental salinity. The exact physiological role of AP activity (i.e. in modulation of digestive and metabolic pathways) remains to be investigated to provide a better understanding of the integrative responses and mechanisms of regulation underlying biochemical adaptation to low salinity in osmoregulating crabs.

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