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ORIGINAL PAPER

Biochemical characteristics and modulation by external and internal factors of aminopeptidase-N activity in the hepatopancreas of a euryhaline burrowing crab

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Abstract Strikingly, in spite of its physiological importance, information about occurrence, biochemical characteristics and mechanisms of regulation of aminopeptidase-N (APN) in the hepatopancreas of intertidal euryhaline crabs is still lacking. In this work, we determined the occurrence, biochemical characteristics, response to environmental salinity and dopamine of APN in the hepatopancreas of the euryhaline crab *Neohelice granulata* (Dana 1851) from the open mudflat of Mar Chiquita coastal lagoon (Buenos Aires province, Argentina). APN activity was maximal at pH and temperature range of 7.6–9.0 and 37–45 °C, respectively. APN activity exhibited Michaelis–Menten kinetics (apparent Km = 0.19 ± 0.10 mM) (pH 7.6, 37 °C) and appeared to be sensitive to bestatin (I_{50} = 15 mM) and EDTA (I_{50} = 9 mM). In crabs acclimated to 10 psu (hyper-regulation conditions) and 37 psu (hypo-regulation conditions), APN activity was about 45 and 160 % higher, respectively, than in 35 psu (osmoconformation). APN activity in the hepatopancreas was stimulated in vitro (about 137 %) by 10^{-4} M dopamine. Higher dopamine concentrations produced a similar extent of increase. The responses of APN activity to salinity and dopamine in vitro suggest the role of APN in digestive adjustments upon hyper and hypo-regulatory conditions and its modulation via direct mechanisms on hepatopancreas by dopamine.

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Introduction

Phenotypic flexibility implies within individual reversible changes in phenotypic traits (from molecular to organisms) which can increase the chances of survival for animals facing changes in external conditions allowing them to adapt favorably to the prevailing environmental regime (Piersma and Drent 2003; Pfenning et al. 2010; Kelly et al. 2012). Several organisms inhabiting heterogeneous habitats exhibit digestive flexibility at the biochemical level (i.e. modulation of digestive enzymes activities) upon changes in environmental conditions (Li et al. 2008; Karasov et al. 2011; del Valle et al. 2004, 2006). However, in spite of their ecological importance studies in euryhaline estuarine crabs are still scarce and fragmentary (Asaro et al. 2011; Michiels et al. 2013; Pinoni et al. 2013). Euryhaline crabs successfully occupying the intertidal area of coastal lagoons have to cope with abrupt and wide changes in environmental salinity. Biochemical adaptation to low and high salinity in such species appears to be a complex process involving the participation of enzymes and transport systems in branchial and 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; Pinoni et al. 2013). However, some mechanisms of biochemical adaptation to low and high salinity, particularly from a metabolic perspective are still not fully understood. Modulation of digestive enzymes in the hepatopancreas (the main site of digestive enzymes synthesis and with an important role in the absorption and storage of nutrients) as being a link between digestion and

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absorption could lead to a greater availability of substrates (i.e. amino acid) for salinity acclimation (Li et al. 2008; Romano and Zeng 2012; Michiels et al. 2013). However, and strikingly, information about occurrence, biochemical characteristics or mechanisms of regulation of key proteolytic enzymes such as aminopeptidase-N in the hepatopancreas of euryhaline crabs is still lacking.

L-alanine aminopeptidase-N or Aminopeptidase-N (APN) (E.C. 3.4.11.2), a membrane-bound exopeptidase that catalyzes the sequential release of N-terminal amino acid of peptides (Hooper 1994; Sanderink et al. 1988; Luciani et al. 1998; Mentlein 2004; Wong et al. 2012; Chen et al. 2013) plays a main role in the final steps of digestion of dietary proteins by producing di-/tri-peptides and single amino acid which are absorbed by amino acid and peptide transporters (Alpers 1987; Mentlein 2004; Goodman 2010; Fairweather et al. 2012). In this way, APN is commonly used as an indicator of capacity to digest proteins (Ramirez-Otarola et al. 2011). The occurrence and level of APN activity in the hepatopancreas, would then determine the ability for total digestion and/or utilization of dietary proteins. Furthermore, its modulation could represent a digestive flexible response to face environmental and/or physiological challenges (del Valle and López Mañanes 2011).

Little is known about primary chemical messengers involved in the regulation of digestive enzyme activities in the hepatopancreas of decapod crustaceans. Dopamine (DA) an important neurotransmitter and neurohormone in crustaceans (Fingerman et al. 1994; Clark et al. 2008; Christie 2011) is involved in the regulation of various processes, metabolic pathways, and enzyme activities (Mo et al. 1998; Morris 2001; Lucu and Towle 2003; Halperin et al. 2004; Pinoni and López Mañanes 2004; Cheng et al. 2005; Lorenzon et al. 2005; Chiu et al. 2006; Genovese et al. 2006; Hsieh et al. 2006; Yeh et al. 2006; Chang et al. 2007; Liu et al. 2008; Avramov et al. 2013; Swetha et al. 2014; Pan et al. 2014). Various chemical messengers, such as biogenic amines including DA have been detected in the hepatopancreas, which appears also to be an important endocrine organ (Huang et al. 2005). Recently, we have shown that DA injections produced an enhancement of lipase activity in the hepatopancreas of *C. angulatus* suggesting also its role as one primary chemical messenger involved in the regulation of digestive functions at the biochemical level (Michiels et al. 2013).

Neohelice granulata 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 (Méndez-Casariego et al. 2011; Luppi et al. 2013). Interpopulation differences exist in biochemical and physiological adaptations to salinity (Pinoni 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 ocurr upon acclimation to low and high salinity (López Mañanes et al. 2000; Schleich et al. 2001; Pinoni and López Mañanes 2009; Asaro et al. 2011; Pinoni et al. 2005, 2013). The differential modulation of amylase and lipase activity in the hepatopancreas in response to low and high salinity suggests the occurrence of complex and specific digestive adjustments at the biochemical level in relation to hyper- and hyporegulation (Asaro et al. 2011; Pinoni et al. 2013). 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 and on mechanisms of modulation of digestive enzymes in euryhaline crabs, the aims of this work were to determine the occurrence and biochemical characteristics of APN activity in the hepatopancreas and its response to low and high environmental salinity and to DA in vitro.

Materials and methods

Chemicals

L-Alanine-*p*-nitroanilide (L-Ala *p*NA), Tris-(hydroxymethylamino-methane), (Tris), ethylene glycol *N*,*N*′,*N*′-tetraacetic acid (EGTA), bovine serum albumin and dopamine (DA) (3-hydroxytyramine) were from Sigma (St. Louis, MO, USA); Coomasie blue G250 was from Fluka (Germany). All chemicals were of analytical grade. All solutions were prepared in glass-distilled water.

Animal collection and maintenance

The crabs were caught from the mudflat area of Mar Chiquita coastal lagoon (Buenos Aires, Province Argentina) (37°32′–37°45′S; 57°19′–57°26′W). 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. Animals were transported to the laboratory in lagoon water on the day of collection. The crabs were maintained in natural seawater (35 psu), dilute sea-water (10 psu) or concentrated seawater (37 psu) for at least 10 days prior to use (López Mañanes et al. 2000; Schleich et al. 2001; Pinoni et al. 2005; Pinoni and López Mañanes 2009). Dilute 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 (Michiels et al. 2013; Pinoni et al. 2013). 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 22 ± 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 (Tetrapond Koi Vibrance, USA: 53 % carbohydrates, 31 % proteins, 5 % lipids, 2 % fiber) (about 0.07 g individual^{-1}) but they were starved 24–48 h prior to the experiments (Asaro et al. 2011; Pinoni et al. 2013). No differences in the feeding behavior occurred in the experimental conditions used. This research project was done following the norms and statements of Ethics Commite CICUAL (OCA 1499/12) FCEyN Universidad Nacional de Mar del Plata.

Preparation of enzyme hepatopancreas extract

The crabs were cryoanesthetized by putting them on ice for about 20 min. The hepatopancreas was immediately excised, mixed with homogenizing medium (0.5 M Tris/HCl pH 7.4; 4 ml g^{-1} of hepatopancreas tissue) and homogenized (CAT homogenizer × 120, tool T10) on ice. The homogenate was centrifuged at 10000×*g* for 15 min (Sorval, rotor SS34, refrigerated). The hepatopancreas from one individual was used for each preparation of enzyme extract. The supernatant was fractionated into 500 ul aliquots and stored at −20 °C until use.

Assay of APN activity

The APN activity was determined using L-alanine-*p*-nitroanilide (L-Ala *p*NA) as substrate (Roncari and Zuber 1969) as described (del Valle and López Mañanes 2008, 2011; Naya et al. 2011) with some modifications. In the standard assay, the reaction was initiated by adding the substrate (final concentration 0.41 mM) to a reaction mixture containing an adequate aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) in 0.08 mM Tris buffer pH 7.6. After incubation for 15 min, the reaction was stopped by the addition of 0.5 mL of cold acetic acid 2 M and absorbance was determined at 384 nm. To study the effect of pH and temperature on APN activity, the procedure was the same as described above except that the activity was determined in the presence of varying pH (range 6.6–10) (50 mM Tris–HCl buffer pH 6.6–9.0; 50 mM Glycine buffer pH 10.0) and temperature (4–50 °C) of the reaction mixture. To study the effect of L-Ala-*p*NA concentration on APN activity, the procedure was the same as described above except that the activity was determined in the presence of varying L-Ala-*p*NA concentrations (0.083–0.5 mM) in the reaction mixture. To study the effect of bestatin and EDTA on APN activity, the activity was measured as described before but in the absence and/or the presence of increasing bestatin and EDTA concentrations. Bestatin, an antibiotic of microbial origin, is an inhibitor commonly used as a tool to characterize APN activity (Bauvois and Dauzonne 2006; Chen et al. 2013). Individuals acclimated to 35 psu were used in these experiments.

The determination of enzyme activity was always performed with samples that had been stored at −20 °C, without any previous thawing.

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

Hemolymph osmolality

Hemolymph (about 500 µl) was sampled from the infrabranchial sinus of 5–10 individuals by means of a syringe at the base of the cheliped and transferred to an iced centrifuge tube. Serum was separated by centrifugation at 10000×*g* (Beckman, Microfuge, B) for 30 s as we described before (Michiels et al. 2013). Osmolality was measured with a micro-osmometer (Osmomat 030 D, GONOTEC).

Effect of dopamine (DA) on APN activity in hepatopancreas

Sections of hepatopancreas (100 mg) from crabs maintained in 35 psu were incubated in the absence and or the presence of varying concentrations of DA (10^{-6}–10^{-2} M) in 2 ml of a medium containing (mM): 400 NaCl, 13 KCl 10 $MgCl_2$ 8.8 H_3BO_3, pH 7.6 at 30 °C. After 30 min of incubation, tissue was homogenized in buffer Tris–HCl 50 mM pH 7.4 (4 ml × g tissue^{-1}) and APN activity was assayed as described above.

Statistical analysis

The results of the effect of different substrate concentrations on the enzymatic activities were analyzed by a nonlinear regression analysis (GraphPad Prism 4.0 software). The curve that appears is the one which best fit to the experimental data according to stimation by GraphPad Prism 4.0 software, showing adjustment to the Michaelis–Menten model. Km values (Michaelis–Menten constant) were estimated from this curve (GraphPad Prism 4.0 software). I_{50} (bestatin concentration at which APN activity was 50 % inhibited) was calculated from inhibition curves (GraphPad Prism software). The statistical analysis of the data was realized using the Sigma 3.0 program for Windows, which automatically performs previous test of equality of variances and normality. Analysis of variance (one-way ANOVA or repeated measures ANOVA) or *t* tests were used to estimate the statistical significance of the

differences and $P < 0.05$ was considered significant. A posteriori test to ANOVA or repeated measures ANOVA (Student–Newman–Keuls, Holm-SidaK, respectively) was used to identify differences.

Results

APN activity of hepatopancreas of *Neohelice granulata*: effect of pH, temperature, L-Ala-*p*NA and bestatin

APN activity was maximal within the range of 7.6–9.0. At pH 6.6 APN activity was about 75 % of the activity at pH 7.6. At pH 10.0 the activity decreased, but it was about 60 % of the activity at pH 7.6 (Fig. 1a). Figure 1b shows the effect of temperature (4–50 °C) on APN activity. The activity increased between 4 and 37 °C being maximal between 37 and 45 °C. At 50 °C the activity decreased being about 75 % of the activity at 37 °C. The effect of L-Ala *p*NA concentration (0.08–0.5 mM) on APN activity is shown in Fig. 1c. APN activity in hepatopancreas of *N. granulata* exhibited Michaelis–Menten kinetics (apparent Km = 0.19 ± 0.10 mM). The effect of bestatin concentrations on APN activity in hepatopancreas is shown in Fig. 1d. APN activity was dose-dependently inhibited by bestatin (I_{50} = 15 mM). Maximal inhibition (about 80 %) was reached with 28 mM bestatin. Higher bestatin concentrations (up to 56 mM) produced a similar percentage of inhibition (Fig. 1d). APN activity was inhibited by 8.5–10 mM EDTA (about 30 and 60 %, respectively) (I_{50} = 9 mM). Lower concentrations of EDTA (1.5–6.5 mM) did not affect APN activity (Fig. 1e).

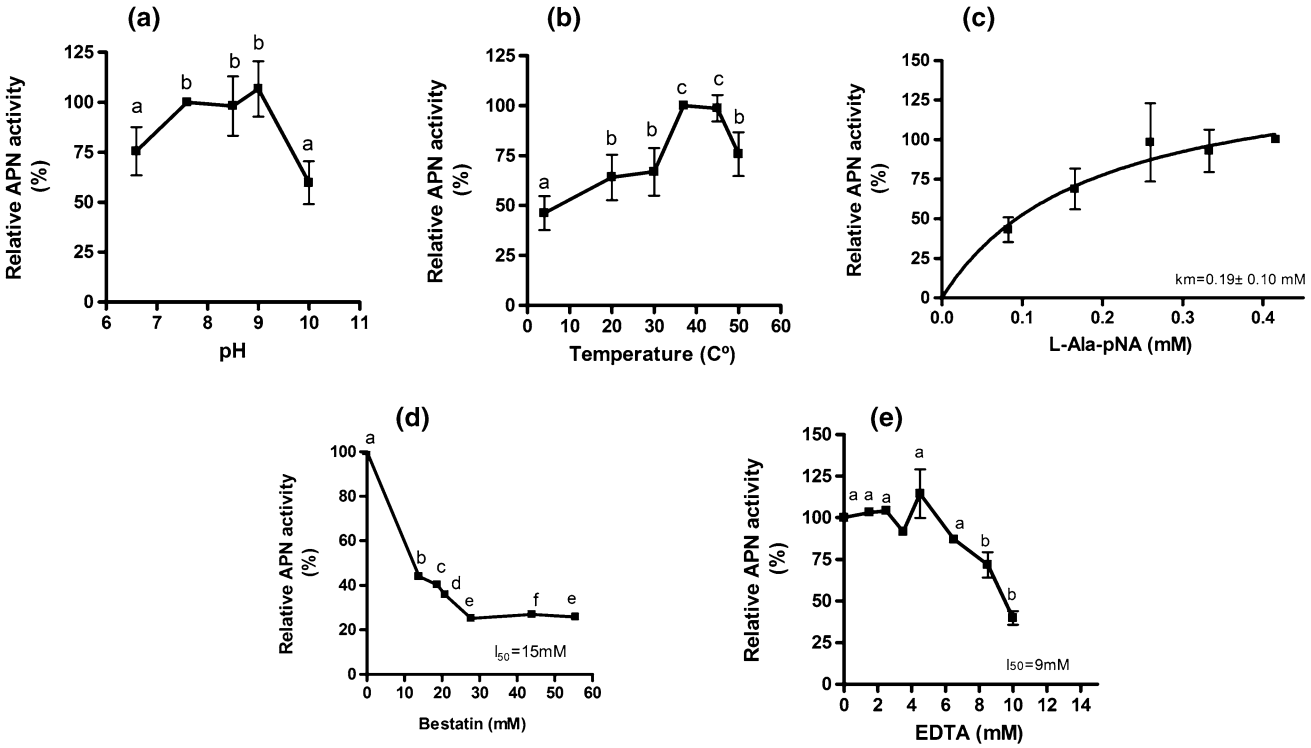

Fig. 1 **a** Effect of pH (6.6–10.0) on APN activity in hepatopancreas of *N. granulata.* The activity was measured at 37 °C and in the presence of 0.41 mM L-Ala *p*NA. The APN activity values are expressed as a relation to the specific activity at pH 7.6 (100 %, 3.9 ± 0.5 μmol min^{-1} mg $prot^{-1}$). Data are the mean ± SE for five individuals. **b** Effect of temperature (4–50 °C) on the APN activity in hepatopancreas of individuals of *N. granulata*. The activity was measured at pH 7.6 and in the presence of 0.41 mM L-Ala *p*NA. The activity is expressed in relation to the activity at 37 °C (100 %, 3.4 ± 0.6 μmol min^{-1} mg $prot^{-1}$). Data are mean ± SE for five individuals. **c** Effect of L-Ala *p*NA concentration (0.08–0.5 mM) on APN activity in hepatopancreas of *N. granulata*. The activity was measured at 37 °C and at pH 7.6. The activity values are expressed as a relation to the corresponding activity in the presence of 0.41 mM L-Ala *p*NA (100 %, 3.5 ± 0.7 μmol min^{-1} mg $prot^{-1}$). Data are the mean ± SE for five individuals. **d** Effect of bestatin on APN activity in hepatopancreas of *N. granulata* acclimated to 35 psu salinity. APN activity in the absence of bestatin was taken as 100 % (3.9 ± 0.25 μmol min^{-1} mg $prot^{-1}$). I_{50} Bestatin concentration that produced 50 % of inhibition, were calculated by GraphPad Prism 2.01. Data are the mean ± SE for 3–5 individuals. *Different letters* indicate significant differences ($p < 0.05$). *Different letters* indicate significant differences ($p < 0.05$). In some cases, *deviation bars were smaller than symbols* used. **e** Effect of EDTA on APN activity in hepatopancreas of *N. granulata* acclimated to 35 psu salinity. APN activity in the absence of EDTA was taken as 100 % (4.3 ± 0.08 μmol min^{-1} mg $prot^{-1}$). I_{50}: EDTA concentration that produced 50 % of inhibition, were calculated by GraphPad Prism 2.01. Data are the mean ± SE for 3–5 individuals. *Different letters* indicate significant differences ($p < 0.05$)

Effect of environmental salinity on APN activity in hepatopancreas of *Neohelice granulata*

The hemolymph osmolality of the crabs was significantly higher and lower from the external medium at 10 and 37 psu, respectively, while no differences were detected at 35 psu (Table 1).

In individuals acclimated to low (10 psu) and high (37 psu) salinity, APN activity was about 45 and 160 %, respectively, higher than at 35 psu (2.2 µmol pNP min^{-1} mg $prot^{-1}$) (Fig. 2).

Effect in vitro of dopamine on APN activity in hepatopancreas of *Neohelice granulata*

APN activity in the hepatopancreas was not affected by 10^{-6} M or 10^{-5} M DA. 10^{-4} M DA significantly increased in vitro (about 137 %) APN activity in the hepatopancreas (Fig. 3). Higher DA concentrations (10^{-3} M–10^{-2} M) produced similar increases of APN activity (Fig. 3).

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

10 psu		35 psu		37 psu	
Medium	Hemolymph	Medium	Hemolymph	Medium	Hemolymph
256 ± 45	575 ± 38*	913 ± 32	810 ± 57	1007 ± 96	832 ± 94*

Data are the mean ± S.E. $n = 4$–9

* Significantly different from the corresponding concentration of the external medium ($p < 0.05$)

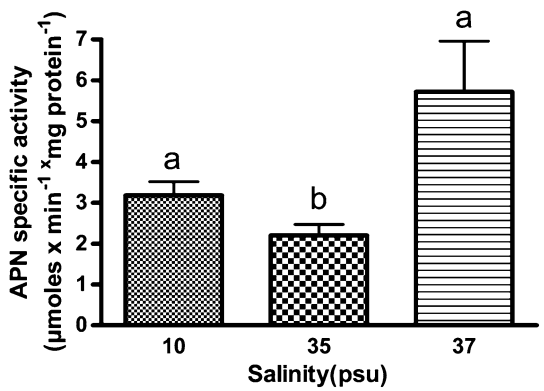

Fig. 2 Effect of acclimation to low (10 psu) and high salinity (37 psu) on APN activity in hepatopancreas of *N. granulata*. The activity was measured as described in the "Materials and methods" section in the presence of 0.41 mM L-Ala *p*NA at pH 7.6 and at 37 °C. *Different letters* indicate significant differences ($P < 0.05$). Data are mean ± SE for 5–6 individuals

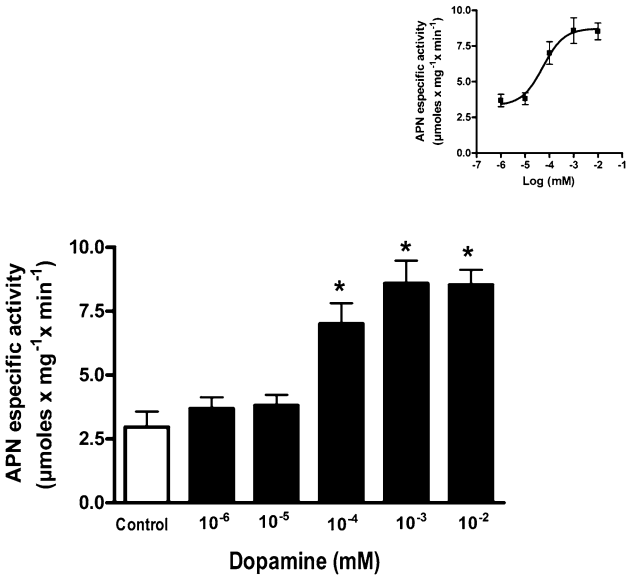

Fig. 3 In vitro effect of various DA concentrations on APN activity in hepatopancreas of *N. granulata* acclimated at 35 psu salinity. Slices of hepatopancreas were incubated as described in the "Materials and methods" section in the absence (control) and in the presence of varying DA concentrations. After incubation, APN activity was measured as described in the "Materials and methods". *Inset* semi-log plot of DA concentrations versus APN activity. Data are mean ± SE for 4–5 individuals. *Asterisk* significantly different from the activity in the absence of DA ($P < 0.05$)

Discussion

Our results show the occurrence of APN activity in hepatopancreas of *N. granulata* from Mar Chiquita coastal lagoon and the response to low and high environmental salinity and dopamine. The occurrence of APN activity in the hepatopancreas of *N. granulata* would be in accordance with the omnivorous–detritivorous dietary habit of this crab in the mudflat of Mar Chiquita lagoon (Botto et al. 2005; Bas et al. 2014) and suggests the ability to perform extracellular total degradation of dietary proteins (Alpers 1987; Mentlein 2004; Ramirez-Otarola et al. 2011; Fairweather et al. 2012). In various crustaceans, the presence and levels of specific digestive enzyme activities in the hepatopancreas is related to the nature of the dietary components being potentially used for metabolic processes (Pavasovic et al. 2007; Figueiredo and Anderson, 2009). Distinct aminopeptidase activities have been detected in the hepatopancreas of several crustaceans (Galgani et al. 1984; Dendinger 1987; Ceccaldi 1989; Galgani and Nagayama 1987; De la Ruelle et al. 1992; Figueiredo et al. 2001), APN activity has been found in the hepatopancreas of crustaceans Munida (Rossano et al. 2011). However, to our knowledge, no report is available about biochemical characteristics of APN activity in hepatopancreas of euryhaline crabs and, in

fact, of decapod crustaceans in general. The pH value for maximal APN activity in hepatopancreas of *N. granulata* (7.6–9.0) (Fig. 1a) is in agreement with that found for this enzyme activity in human intestine (Sanderink et al. 1988) and intestinal homogenates of birds (Sabat et al. 1998). APN activity in hepatopancreas of *N. granulata* appeared to be maintained at high levels at low temperature (4 °C) (50 % of activity at 37 °C) and high temperature (50 °C) (about 75 % of activity at 37 °C) (Fig. 1b). Since in Mar Chiquita coastal lagoon *N. granulata* is exposed to a wide range of temperatures (Spivak et al. 1994; Luppi et al. 2013; personal observations), an extreme temperature tolerant APN activity in the hepatopancreas could be related with a role in thermal acclimation (i.e. a higher digestion of proteins) as suggested for other proteases in eucarid crustaceans (Dittrich 1992). However, further experimental approaches are needed to test this hypothesis. In the striped hamster, cold acclimation induced an increase in intestinal APN (Zhao et al. 2014). Aminopeptidase activity found in the hepatopancreas of the crayfish *Procambrus clarkii* exhibited optimal temperature of 55 °C (De La Ruelle et al. 1992). The Michaelis–Menten kinetics of APN activity of hepatopancreas of *N. granulata* (Fig. 1c) is in accordance with that described for this activity in intestinal homogenates of birds (Sabat et al. 1998) and for APN purified from human and chicken intestine (Sanderink et al. 1988; Mane et al. 2010). Bestatin, an antibiotic of microbial origin, is commonly used as a tool to characterize APN activity (Bauvois and Dauzonne 2006; Chen et al. 2013). In mammalian intestine, APN is the major bestatin-sensitive enzyme involved in the degradation of oligopeptides on the surface of intestine brush borders (Scornik and Botbol 2001). APN in the hepatopancreas of *N. granulata* appeared to be sensitive to bestatin ($I_{50} = 15$ mM) (Fig. 1d) which further support the idea of the occurrence of a APN activity with similar biochemical characteristics to mammalian intestine APN (Scornik and Botbol 2001). Whether the fact that 100 % inhibition was not reached even at high concentrations (13–55 mM) of bestatin (Fig. 1d) is due to a lower sensitivity to bestatin of APN activity in the hepatopancreas and/or to the presence of bestatin-sensitive and bestatin-insensitive APN activities require further investigation. As expected, since APN has been described to be a metalloenzyme (Chen et al. 2013), APN activity in the hepatopancreas of *N. granulata* was inhibited by the metal chelator EDTA as shown for distinct vertebrate aminopeptidases (Garner and Behal 1974; Vanderheyden et al. 2006).

Biochemical adaptations to salinity imply various molecular and biochemical changes such as those in hemolymph amino acid (Freire et al. 2008; McNamara and Faria 2012; Romano and Zeng 2012; Larsen et al. 2014). 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 and Zeng 2012). Digestive enzymes as being a link between digestion and absorption could be modulated leading to a differential availability of energy substrates and/or metabolites (i.e. amino acid) for salinity acclimation (Li et al. 2008; Romano and Zeng 2012; Michiels et al. 2013; Pinoni et al. 2013). *Neohelice granulata* behaves as hyper/hypo-regulator since it exhibits hemolymph osmolalities values higher and below from those of the corresponding external medium upon acclimation to 6–10 psu and 37–60 psu, respectively, while osmoconforms in 35 psu (López Mañanes et al. 2000; Schleich et al. 2001; Pinoni and López Mañanes 2009; Asaro et al. 2011; Pinoni et al. 2005, 2013; Table 1 this work). We have previously shown that biochemical salinity acclimation in males of *N. granulata* from the mudflat of Mar Chiquita coastal lagoon involves the integrative modulation of several components in gills, muscle and hepatopancreas (López Mañanes et al. 2000; Schleich et al. 2001; Pinoni et al. 2005; Pinoni and López Mañanes 2009; Asaro et al. 2011; Pinoni et al. 2013). The higher APN activity in hepatopancreas of crabs acclimated to 10 and 37 psu (Fig. 2) suggests that modulation of this activity is another component at the biochemical level involved in response to low and high salinity in this crab. In this context, since APN is involved in intermediate and final steps of degradation of digested proteins, the increased APN activity under low and high salinity conditions (Fig. 2) implying hyper and hypo-regulatory responses (Table 1), respectively, could lead to a potential enhanced protein digestive capacity and therefore to a higher supply of amino acid to be used in biochemical adjustments in relation to differential salinity. In vitro experiments on hepatopancreas sections of *N. granulata* from other geographical areas suggests that gluconeogenesis from amino acid is one pathway involved in the adjustment of the intracellular concentration of nitrogenated compounds during hypo-osmotic stress and that, hyper-osmotic stress induces an enhancement in the amino acid uptake (Schein et al. 2005; Martins et al. 2011). Some amino acid as being a major sources of organic osmolytes play important functions in osmotic responses in crabs and other crustaceans facing, for instance, hyperosmotic exposure (Romano and Zeng 2012; Larsen et al. 2014). In mammalian intestine, APN and neutral amino acid transporter B0AT1 form complexes which alters the kinetic parameters of the transporter (Fairweather et al. 2012). Further experimental approach is needed to establish the possible interplay between enhanced APN activity in low and high salinity and amino acid flux and transporters in hepatopancreas of *N. granulata*. Crustacean hepatopancreas has been shown to contain amino acid transporters (Ahearn 1982; Ahearn and Clay 1988; Simmons et al. 2012; Duka and Ahearn 2013). To our knowledge no work occurs

about amino acid transporters in the hepatopancreas of *N. granulata.* In the shrimp *Litopenaeus setiferus* hepatopancreatic brush border membrane vesicles appears to exhibit a cation-dependent, amino acid transporter stimulated by transmembrane gradients of either sodium or potassium (Duka and Ahearn 2013). Work in our lab shows that APN activity in the hepatopancreas of *N. granulata* is also modulated upon an abrupt change from high to low salinity (unpublished results) which further supports the idea of an important physiological role of this activity in biochemical responses to salinity. In the euryhaline teleost, *Dicentrarchus labrax* expression of intestinal APN is influenced by salinity (Boutet et al. 2006). In mammals, kidney APN is linked to renal Na^+ handling by reducing basolateral Na^+-K^+-ATPase levels upon high salt conditions (Kotlo et al. 2007).

The primary chemical messengers involved in the modulation of the activity of digestive enzymes in the hepatopancreas of decapod crustaceans are far from having been elucidated (Resch-Sedlmeier and Sedlmeier 1999), particularly, in euryhaline crabs. DA is a pleiotropic compound that acts as a neurotransmitter and a hormone. The hepatopancreas of decapod crustaceans appears to have an important role as endocrine organ being the source of several primary chemical messengers such as DA (Fingerman et al. 1994; Huang et al. 2005). Recently, we have shown the effect of 10^{-4} M DA injections on lipase activity in hepatopancreas of *C. angulatus*, for the first time in a euryhaline crab (Michiels et al. 2013) and the effect in vitro of 10^{-4} M DA on lipase activity in the hepatopancreas of *N. granulata* (Michiels et al. 2015). These findings suggest that DA is one primary chemical messenger involved in the regulation of digestive enzyme activities in the hepatopancreas. The effect in vitro of DA on APN activity in hepatopancreas of *N. granulata* (Fig. 3) supports this idea and suggests a direct effect on the hepatopancreas. The physiological actions of dopamine are known to be mediated by membrane-bound G protein-coupled receptors (Beaulieu and Gainetdinov 2011). *N. granulata* has been suggested to exhibit D_1- and D_2-like DA receptors in posterior gills (Genovese et al. 2006). Since nothing is known, to our knowledge, about the occurrence of dopaminergic receptors in the hepatopancreas, further studies are required to establish whether the effect of DA on APN activity involves its binding to specific receptors on this tissue. On the other hand, since APN is an exopeptidase described to have an extracellular active site and an intracellular domain (Chen et al. 2013) further experimental approach is needed to establish the mechanisms of action of dopamine (i.e. by direct activation of APN, activation/inhibition of endogenous activators or inhibitors, respectively, retranslocation of APN). The structural features of mammal APN enable it to have multifunctional roles as those in metabolism of various peptides and interaction with other proteins (Chen et al. 2013).

In conclusion, the results of this study show the occurrence of APN activity in the hepatopancreas of *N. granulata* which is affected upon acclimation to low and high salinity suggesting its participation in the biochemical adaptation process to environmental salinity. The fact that APN activity in hepatopancreas of *N. granulata* was affected in vitro by DA supports the idea of this biogenic amine having a role in mechanisms of regulation of digestive enzymes and furthermore suggests that APN is under control of differential modulation pathways (i.e. by internal and external factors).

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