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Two sodium pumps in the hepatopancreas of the intertidal euryhaline crab *Neohelice granulata*: biochemical characteristics and differential modulation after feeding

E. Méndez[1], C. Caruso Neves[2], A. A. López Mañanes[1*]

[1]Instituto de Investigaciones Marinas y Costeras (IIMyC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Universidad Nacional de Mar del Plata, Funes 3250, Mar del Plata, Argentina.

[2]Instituto de Biofísica Carlos Chagas Filho UNFRJ, Brasil

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*Corresponding author:

Dra. Alejandra López Mañanes

Instituto de Investigaciones Marinas y Costeras (IIMyC) Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Universidad Nacional de Mar del Plata, Argentina.

Funes 3250

7600 Mar del Plata, Argentina

Tel. +54-223-4753554

mananes@mdp.edu.ar

Two sodium pumps in the hepatopancreas of the intertidal euryhaline crab Neohelice granulata: biochemical characteristics and differential modulation after feeding

E. Méndez, C. Caruso Neves, A. A. López Mañanes

Abstract

No study occurs about the existence, biochemical characteristics and modulation of K^+-independent ouabain-insensitive Na^+ ATPase activity (the second sodium pump) in the digestive tract of intertidal euryhaline crabs and moreover on the co-existence and modulation under distinct physiological and/or environmental conditions of different sodium pumps. We determined the occurrence, characteristics and responses at different times (0, 1, 24, 48 and 120 h) after feeding upon distinct salinities of Na^+ ATPase activity and Na^+-K^+ ATPase in hepatopancreas of *Neohelice granulata* (Dana,1852), a model species. The stimulation by Na^+ under total inhibition of Na^+-K^+ ATPase activity revealed the occurrence of Na^+ ATPase activity which was totally inhibited by 2 mM furosemide, exhibits Michaelis–Menten kinetics for ATP (apparent Km= 0.52 ± 0.16 mM) and highest activity at around pH 7.4. In crabs acclimated to 35 psu (osmoconforming conditions), Na^+ ATPase activity was highly increased (about 15 fold) (532±58 nmol Pi $xmin^{-1}x$ $mgprotein^{-1}$) at 48 h after feeding in hepatopancreas. In 10 psu (hyper-regulating conditions) Na^+ ATPase activity decreased at 24 h (7±9 nmol Pi $xmin^{-1}x$ $mgprotein^{-1}$) after feeding and recovered initial values by 48 h (24±35 nmol Pi $xmin^{-1}x$ $mgprotein^{-1}$). Unlike Na^+ATPase, Na^+-K^+ ATPase activity did not change after feeding at any salinity, suggesting the specific modulation of the second sodium pump and its role in postprandial adjustments in hepatopancreas.

Keywords: K^+-independent ouabain-insensitive Na^+ ATPase activity, hepatopancreas, sodium pumps, feeding, *Neohelice granulata*, crab, Southwestern Atlantic

Introduction

The hepatopancreas of decapod crustaceans is a multifunctional organ which plays a central role in digestion and nutrient absorption (Zeng et al. 2010; Wang et al. 2014; Saborowski 2015; Ribeiro et al. 2016). In the small intestine of mammals, Na^+ gradients are required for supporting the function of facilitated transports involved in nutrient absorption. Basolateral Na^+-K^+ ATPase (the classical sodium pump) is pointed out as the main responsible for the primary active Na^+ transport to support Na^+ gradients (Chang and Leung 2014; Zhang et al. 2016). However, this process appears not to be exclusively mediated by the Na^+-K^+ ATPase. A link between K^+-independent, ouabain-insensitive, furosemide-sensitive Na^+ ATPase activity (Na^+-ATPase) (usually named as the second sodium pump) and Na^+ transport also occurs (del Castillo and Robinson 1985*a,b*; Rocafull et al. 2012; Thomas et al. 2013). In the hepatopancreas of various decapod crustaceans, some Na^+-dependent transporters appear to work during nutrient assimilation (Simmons et al. 2012; Duka and Ahearn 2013, 2014; Lignot and Charmantier 2015; Saborowski 2015). This supports the idea of the importance of maintaining suitable Na^+ gradients and the participation of sodium pumps in this process in the hepatopancreas. In this context, Na^+-K^+ ATPase and Na^+ ATPase activities in the hepatopancreas and its potential differential modulation could be important for digestive and absorptive process under different physiological and/or environmental conditions. However, studies on biochemical characteristics and modulation of these sodium ATPases in the hepatopancreas are lacking. Moreover, nothing is known yet about the occurrence of Na^+ ATPase activity in hepatopancreas of groups of ecological importance such as intertidal euryhaline crabs.

The Na^+ ATPase is a member of the P-type ATPase family which participates in extrusion of Na^+ in various non-polarized and polarized cells and thus, in the maintenance and/or adjustments of Na^+ gradients (Caruso-Neves et al. 1997, 2001, 2002; Rocafull et al. 2011, 2012; Thomas et al. 2013; Vieira-Filho et al. 2014; Vieyra et al. 2016). This enzyme has been purified and cloned from guinea-pig enterocytes (Rocafull et al. 2011). Na^+ ATPase has been identified in various tissues of several invertebrates (Caruso-Neves and Lopes, 2000; Pagliarani et al. 2006, 2008; Pinoni and López Mañanes 2009; Vieyra et al. 2016) but, to our knowledge, the work of Zilli et al. (2003) in the shrimp, *Marsupenaeus japonicas* is the only one describing the detection of Na^+ ATPase activity in the digestive tract of a decapod crustacean.

The intertidal euryhaline burrowing crab *Neohelice granulata* is an animal model for biochemical, physiological and ecological research (Spivak 2010). We previously determined Na^+ ATPase activity in chela muscle showing, for the first time, the occurrence of this enzyme in an intertidal euryhaline crab (Pinoni and

López Mañanes, 2009). In various species of euryhaline crabs including *N. granulata*, the differential modulation of digestive enzymes activities in the hepatopancreas suggests that distinct digestive and metabolic adjustments at the biochemical level occur in response to environmental salinity (Asaro et al.2011; Romano and Zeng 2012; Michiels et al. 2013, 2015; Wang et al. 2014; Pinoni et al. 2013. 2015). In this context, previous work of our lab showed a differential dynamics (i.e. temporal) of the modulation of different digestive enzymes after feeding in the hepatopancreas of *N. granulata* which also suggested the occurrence of concomitant adjustments in postprandial absorptive processes (Pinoni 2009; Pinoni et al. 2015, 2016; Michiels 2015). The aims of this work were to determine the co-existence and biochemical characteristics of Na^+-K^+ ATPase and Na^+ ATPase activities and their response at different times after feeding in the hepatopancreas of *N. granulata* in different environmental salinities.

Materials and methods

Animal collection and maintenance

Neohelice granulata is one of the dominant euryhaline burrowing crabs in intertidal areas of the southwestern Atlantic (Spivak et al.1994; Spivak 1997; Luppi et al. 2013). Male adults (carapace width greater than 2.5 cm) (n=60) were caught in the mudflat area of Mar Chiquita coastal lagoon (Buenos Aires, Province Argentina) (37°32′–37°45′S; 57°19′–57°26′W) and taken to the laboratory in lagoon water the same day. Salinity was determined in practical units (psu). The crabs were maintained for at least 10 days at two different salinities: 35 psu (906± 26 mOsm $kg^{-1;}$, osmoconforming condition) or 10 psu (274±34 mOsm kg^{-1}, hyper-regulation condition) (Pinoni and López Mañanes 2009; Michiels et al.2015; Pinoni et al. 2013, 2015; this work) Water of aquaria (36 L) was under continuous aeration and filtration and the photoperiod was 12 h light and temperature of water 22 ± 2 °C). Food (Tetrapond Koi Vibrance, USA: 53 % carbohydrates, 31 % proteins, 5 % lipids, 2 % fiber) (about 0.07 g $crab^{-1}$) was given three times a week. To determine enzyme activities in hepatopancreas at different times after feeding, crabs acclimated to 35 psu or 10 psu, unfed for 120 h, were individually fed. The 0 time referred at the time when all given food was eaten (this took up to 5 min). Crabs which did not eat or partially ate were not used in experiments. Na^+ ATPase and Na^+-K^+ ATPase activities were determined at the short (1 h) and long term (24, 48 and 120 h) after feeding. These times were selected according to experimental work in our lab showing that in males of this crab postprandial digestive and

metabolic changes at the biochemical level occur up to 120 h (Asaro et al. 2009; Méndez et al. 2011, 2012; Michiels 2015; Michiels et al. 2015; Pinoni et al. 2015; Asaro 2016; unpublished results of our lab). The feeding behavior was not changed by 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.

Sampling procedures

The crabs were weighed and cold anaesthetized for about 25 min. After weighing, the hepatopancreas was homogenized in 0.25 M sucrose/0.5 mM EGTA-Tris, pH 7.4; 4 ml g^{-1} of tissue (CAT homogenizer×120, tool T10) on ice. The homogenate was immediately used for enzyme activities assays. The hepatopancreas from one individual was utilized for each preparation.

Assay of Na^+–K^+ ATPase activity

Na^+-K^+ ATPase activity was assayed by quantifying ATP hydrolysis as we previously described (Pinoni and López Mañanes 2009). Briefly, Na^+-K^+ ATPase activity was determined as the difference between two assays containing 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EGTA in 20 mM imidazole buffer (pH 7.4) and the same medium in the absence of KCl but with 1 mM ouabain. After pre-incubation of the sample in the assay mixture for 5 min at 30 °C, ATP (final concentration 5 mM) was added and incubation proceed for 15 min. To stop the reaction 2 ml of cooled Bonting's reagent (560 mM sulphuric acid, 8.1 mM ammonium molybdate and 176 mM ferrous sulphate) was added and left at room temperature for 20 min. The amount of released Pi was quantified measuring the absorbance at 700 nm of the reduced phosphomolybdate complex (Bonting, 1970). To determine adequate relation of concentrations of sodium/potassium for Na^+-K^+ ATPase activity, this activity was assayed in the presence of varying concentrations of sodium and potassium but always keeping constant the total sodium plus potassium concentration at 130 mM. To study the effect of ouabain, the activity was measured as described before but in the absence and/or the presence of different ouabain concentrations (0.1 to 1.0 mM). To study the effect of ATP concentration and pH, the activity was assayed in the presence of varying ATP concentrations (0.1 to 10 mM) or at pHs (6.3-9.0) of the assay mixture. Crabs maintained at 35 psu were used in these experiments.

Assay of K^+ independent, ouabain-insensitive Na^+ ATPase activity

K^+ independent, ouabain insensitive Na^+ ATPase activity was determined as we previously described (Pinoni and López Mañanes, 2009). Briefly, Na^+ ATPase activity was determined in an assay mixture containing 100 mM NaCl, 10 mM MgCl2, 0.5 mM EGTA, 1 mM ouabain and 1 mM sodium azide (to inhibit F-ATPases) in 20 mM imidazole buffer (pH 7.4). Mg^{2+}–ATPase activity (determined in the absence of NaCl) was subtracted in all cases. The sample was pre-incubated for 5 min at 30 °C and then ATP (final concentration 3 mM) was added and incubation was carried out for 15 min. As described above, 2 ml of cooled Bonting's reagent was used to stop the reaction and to determine PI released. To study the effect of ATP concentration and pH on ouabain-insensitive Na^+ ATPase activity, the activity was assayed in the presence of varying ATP concentrations (0.1 to 12.0 mM) or at different pH (6.3–9.0) of the assay mixture. To study the effect of furosemide, the activity was assayed in the absence and/or the presence of different furosemide concentrations (0.1 to 3mM). Crabs maintained at 35 psu were used in these experiments.

Protein analysis

Protein concentration was determined as we described (Michiels et al. 2015). According to Bradford (1976) which monitors the increase in absorption at 595 nm resulting from the binding to protein of the dye Coomasie Brilliant Blue G-50. Bovine serum albumin was used as standard.

Hemolymph osmolality

Hemolymph osmolality was determined as we previously described (Pinoni et al. 2013, 2015; Michiels et al. 2015). Briefly, hemolymph (about 500 µl) was sampled from the infrabranchial sinus with a syringe, transferred to an iced centrifuge tube and centrifuge at 10000×g (Beckman, Microfuge, B) for 30 s to separate serum. Osmolality was measured with a micro-osmometer (Osmomat 030 D, 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), was used to estimate the statistical significance of the differences and $P<0.05$ was considered significant. A posteriori test to ANOVA (Bonferroni) was used to identify differences (Zar 1999). Results of

effect of varying concentrations of Na^+ and ATP on Na^+ ATPase and Na^+-K^+ ATPase activities were analyzed 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 according to estimation by GraphPad Prism 5.01 software. Km values (Michaelis–Menten constant) were estimated from these curves (GraphPad Prism 5.01 software). I_{50} (the concentration of ouabain or furosemide at which the corresponding ATPase activity was 50% inhibited) was calculated from the inhibition curve (GraphPad Prism 5.01 software).

Results

Hepatopancreas of *N. granulata* presents both ouabain-sensitive and -insensitive sodium pumps

Initially, we determined Na^+-K^+ ATPase activity in hepatopancreas by measuring ATPase activity in the presence of different concentrations of sodium plus potassium (keeping constant the total sodium plus potassium concentration at 130 mM). Na^+-K^+ ATPase activity was increased as sodium concentration enhanced up to 100 mM sodium and exhibited Michaelis-Menten kinetics (apparent K_m of 36.07± 5.16 mM sodium) (Fig. 1A). Ouabain (0.1 to 1 mM) (a well-known specific inhibitor of Na^+-K^+ ATPase activity) induced the dose-dependent inhibition of Na^+-K^+ ATPase activity in hepatopancreas (Fig. 1B). The maximal inhibition was obtained at 1 mM (F=11.666 p=0.003). The I_{50} (the concentration of ouabain that produced half-maximal inhibition) was 0.07 mM. We also determined the effect of ATP concentration (0.1-10 mM) on Na^+-K^+ ATPase activity. The kinetic parameters obtained were: apparent Km = 0.71 ± 0.31 mM and Vmax (maximal rate) = 286.7 ± 44.78 nmol Pi x mg^{-1} x min^{-1} (Fig. 1C). In addition, we studied the effect of pH on Na^+/K^+-ATPase activity (Fig. 1D). Na^+-K^+ ATPase activity increased when the pH was enhanced from 6.3 to 8.0 but it decreased by further enhancement in pH.

In a second step, the presence of an ouabain-insensitive Na^+ ATPase activity in hepatopancreas was investigated. Sodium dependence (0.25-150 mM) of the ATPase activity was determined in the presence of ouabain 1 mM and in the absence of potassium added. Under these conditions, Na^+-K^+ ATPase activity is completely inhibited (Pinoni and Lopez Mañanes, 2009; Fig. 1B this work). The kinetic parameters obtained were: apparent Km = 11.96 ± 2.87 mM and the maximal rate (Vmax) = 507.3 ± 64.58 nmol Pi x mg^{-1} x min^{-1} (Fig. 2A). It has been described that ouabain-insensitive Na^+-ATPase activity is inhibited by furosemide

(Rocafull et al. 2012; Vieyra et al. 2016). Figure 2B shows the effect of furosemide (0.1-3.0 mM) on the ouabain-insensitive Na^+ ATPase activity in hepatopancreas. Ouabain-insensitive Na^+ ATPase activity was completely inhibited by furosemide at 3 mM (F=3.518 p=0.035). I_{50} (the concentration of furosemide that produced half-maximal inhibition of ouabain-insensitive ATPase activity) was about 0.6 mM. The effect of ATP concentrations on ouabain-insensitive Na^+ ATPase activity in hepatopancreas is shown in Figure 2C. Na^+-ATPase activity exhibited Michaelian profile for ATP (apparent Km = 0.52 ± 0.16 mM and Vmax = 442.7 ± 48.37 nmol Pi x mg^{-1} x min^{-1}). Ouabain-insensitive Na^+-ATPase activity increased from pH 6.3 to 7.4 (Fig 2D). Further increase in pH decreased the enzyme activity being at pH 9.0 about 20% of the activity at pH 7.4.

So far our results show that hepatopancreas of *N. granulata* express two sodium pumps: the classical ouabain-sensitive Na^+-K^+ ATPase activity and the ouabain-insensitive Na^+ ATPase activity.

Sodium pumps at different times after feeding in hepatopancreas of *N. granulata* acclimated to different salinities

The values of osmolality of the hemolymph were 831±78 and 625±40 in 35 and 10 psu, respectively (mean+SE, 10–15 crabs for each condition).

In individuals acclimated to 35 psu, Na^+ ATPase activity in hepatopancreas was not changed by 1 and 24 h after feeding. At 48 after feeding, Na^+ ATPase activity was markedly increased (about 15 fold compared to the activity at 0 time). At 120 h after feeding, Na^+ ATPase activity returned to values similar to the initial time (F=90.474 P <0.001) (Fig. 3A, Table 1).

In individuals acclimated to 10 psu, Na^+ ATPase activity was decreased by 1 h up to 24 h after feeding (Fig. 3B). At 48 h after feeding, Na^+ ATPase activity recovered values similar to that at 0 time maintaining constant at 120 h (F=4.036 P=0.019) (Fig. 3B, Table 1).

On the other hand, Na^+-K^+ ATPase activity in hepatopancreas did not change after feeding in individuals acclimated to either 35 or 10 psu (ANOVA P <0.05) (Figs. 4 A and B, Table 1).

Discussion

Our results show the co-existence of both a K^+-independent ouabain-insensitive Na^+ ATPase activity (Na^+ ATPase) and the classic sodium pump Na^+-K^+ ATPase activity in hepatopancreas of the intertidal euryhaline crab *N. granulata* and the specific post-feeding modulation of Na^+ ATPase activity.

To the best of our knowledge, there are not available reports about biochemical characteristics of Na^+-K^+ ATPase activity in the hepatopancreas of decapod crustaceans. The pattern of response to sodium, potassium and ATP concentrations, and the strong pH dependency of Na^+-K^+ ATPase activity in hepatopancreas of *N. granulata* is in agreement with that found in gills of various decapod crustaceans (Neufeld et al. 1980; D'Orazio and Holliday 1985; Corotto and Holliday 1996**;** Lucu and Towle 2003; Leone et al. 2014, 2017). In gills homogenates of *N. granulata* the optimum pH of Na^+-K^+ ATPase activity is about 7.4-7.6 and markedly decreases at lower and higher pHs (Castilho et al. 2001). Na^+-K^+ ATPase activity in chela muscle of the euryhaline crab *Cyrtograpsus angulatus* also shows a strong pH dependency (Pinoni and López Mañanes 2008).

In several vertebrate and invertebrate tissues, the insensitivity to ouabain (a well-established specific inhibitor of Na^+-K^+ ATPase; Kaplan 2002; Nesher et al. 2007; Miles et al. 2013; Leone et al. 2017) and the inhibition by furosemide (unlike Na^+-K^+ ATPase) allowed to identify the occurrence of a K^+- independent Na^+ ATPase activity **(**Proverbio et al. 1986, 1991; Caruso-Neves et al.1999; Beltowski et al.2004; Rocafull et al. 2012; Vieyra et al. 2016). By using this approach, in this work we showed, for the first time in a euryhaline crab, the occurrence of K^+ independent, ouabain-insensitive Na^+ ATPase activity in hepatopancreas of *N. granulata*. The co-existence of Na^+ ATPase and Na^+-K^+ ATPase in the hepatopancreas is similar to that described for small intestine of mammals (Rocafull et al. 2012) and to that we found in chela muscle of this crab (Pinoni and López Mañanes 2009). Similarly to chela muscle (Pinoni and López Mañanes 2009), maximal inhibition by furosemide of Na^+-ATPase activity in hepatopancreas was reached at 2 mM, a concentration that fully inhibits this activity in insects and mammals (del Castillo and Robinson 1985*b*; Moretti et al.1991; Caruso-Neves and Lopes 2000; Caruso-Neves et al. 2002; Beltowski et al. 2004). The Michaelis–Menten kinetics of Na^+ ATPase activity in hepatopancreas of *N. granulata* is in agreement with this activity in chela muscle (Pinoni and López Mañanes, 2009) and in various vertebrate and invertebrate tissues (Ventrella et al. 1992; Caruso-Neves et al. 2002; de Almeida-Amaral et al. 2008). Compared to chela muscle (Pinoni and López Mañanes 2009) Na^+ ATPase activity in the hepatopancreas was more sensitive to pH being lower at acidic (6.3) and at alkaline (9.0) pH. Whether this different sensitivity to pH is due to the occurrence of tissue-specific forms requires further investigation.

The hepatopancreas is the main site of nutrients absorption in decapod crustaceans and of initial steps of macromolecules synthesis after feeding (McGaw and Curtis 2013; Carter and Mente 2014; Saborowski 2015). Previous works of our lab showed that feeding triggers distinct responses of key digestive enzymes in the

hepatopancreas of *N. granulata* in 35 psu (osmoconforming conditions) and in low salinity (10 psu, hyper-regulation condition) suggesting that different postpandrial digestive and metabolic adjustments occur depending on salinity (Pinoni 2009; Michiels 2015; Pinoni et al. 2015). To determine the possible differential modulation after feeding of sodium pumps activities in the hepatopancreas, we determined Na^+ ATPase activity and Na^+-K^+ ATPase at different times after feeding in crabs acclimated to 35 or 10 psu. The acute and marked enhancement of Na^+ ATPase activity in the hepatopancreas of *N. granulata* 48 h after feeding in 35 psu, would suggest its role in postprandial digestive and absorptive adjustments (i.e. by supporting Na^+ gradients). In small intestine of mammals, single amino acids resulting from the activity of membrane-bound aminopeptidase-N (APN) are absorbed by different mostly Na^+ -dependent transporters (Fairweather et al. 2012; Chang and Leung 2014; Vuille-dit-Bille et al. 2015). We previously showed that APN activity increased in the hepatopancreas of *N. granulata* 24 h after feeding in 35 psu, suggesting a postprandial enhancement of final digestion of dietary proteins (Michiels 2015; Michiels et al. 2015; unpublished results of our lab). The increase after feeding in Na^+-ATPase activity in hepatopancreas of *N. granulata* in 35 psu could then be necessary for supporting possible enhancement in Na^+-dependent absorption of digestion products. In the hepatopancreas of various decapod crustaceans, Na^+-dependent transporters work during nutrient assimilation (Lignot and Charmantier 2015; Saborowski 2015). In mammalian intestinal and kidney cells, modulation of Na^+ ATPase activity results in modifications in Na^+ absorption (Caruso-Neves et al. 2000, 2001; Gomes et al. 2008; Líbano-Soares et al. 2011; Wengert et al. 2005, 2007; Rocafull et al. 2012; Dias et al. 2014; Thimm et al. 2015; Vieyra et al. 2016). In enterocytes of the fish *Sparus aurata*, Na^+ ATPase activity supports the functioning of secondary active transport processes (Dópido et al. 2004). We previously found that acclimation to low salinity (10 psu) affects short-term responses after feeding of digestive enzymes activities in hepatopancreas of *N. granulata* suggesting a distinct dynamics of postprandial digestive and absorptive adjustments depending on environmental salinity (Michiels 2015: Pinoni et al. 2015, unpublished results of our lab). In this context, the differential adjustments of APN and total proteolytic activities after feeding in 10 psu could be related to concomitant changes in Na^+-dependent mechanisms (i.e. lower amino acids absorption) and consequently to a lower demand of Na^+ flux. The short-term decrease of Na^+ ATPase activity in the hepatopancreas of *N. granulata* after feeding in 10 psu found in this work would support this idea. Furthermore, the differential responses of Na^+ ATPase activity points out this enzyme as one of the components of the distinct postprandial responses to low salinity in this crab.

In cells that transport Na^+ at very high rates such as in small intestine, Na^+ ATPase would be necessary for the absorption of sodium, nutrients, and water without a significant increase in intracellular K^+ thus, preventing poisoning by this ion (Rocafull et al. 2012). Since Na^+-K^+ ATPase activity did not change after feeding in neither 35 or 10 psu, it appears to occur a specific postprandial modulation of Na^+ ATPase activity in hepatopancreas of *N. granulata*. This specific modulation of Na^+ ATPase activity after feeding could allow a fine-tuning regulation of Na^+ flux as suggested for mammalian intestine (Rocafull et al. 2012; Vieyra et al. 2016). In mammals, insects and bivalve tissues and in chela muscle of *N. granulata*, the coexistent Na^+ ATPase and Na^+-K^+ ATPase activities differentially response to intrinsic and/or environmental conditions and/or exogenous chemicals (Caruso-Neves and Lopes 2000; Ventrella et al. 2001; Caruso-Neves et al. 2004; Beltowski et al. 2004; Wengert et al. 2005; Pagliarani et al. 2006, 2008; Pinoni 2009; Pinoni and López Mañanes 2009). The results of this work supports that this is also the case for these activities in the hepatopancreas of *N. granulata*.

In summary, our results show for the first time the occurrence of Na^+ ATPase activity, co-existent to the classical sodium pump, in hepatopancreas of an intertidal euryhaline crab. The specific modulation of Na^+ ATPase activity in the hepatopancreas of *N. granulata* after feeding suggests its role in postprandial digestive and absorptive adjustments and to be one component of post-feeding responses at the biochemical level to environmental salinity.

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Table 1 One-way Analysis of Variance. Analysis of variation of enzymatic activities in the hepatopancreas after feeding in different salinities (factor: time).

	Source of Variation	DF	SS	MS	*F*	*P*
Na+ ATPase activity	t (hs)	4	1126979,64	281744,91	90,474	<0,001*
35 ‰	Residual	21	65396,062	3114,098		
	Total	25	1192375,7			
Na+ ATPase activity	t (hs)	4	13395,148	3348,787	4,036	0,019*
10 ‰	Residual	16	13276,269	829,767		
	Total	20	26671,418			
Na+/K+ ATPase Activity	t (hs)	4	13053,871	3263,468	0,719	0,592
35 ‰	Residual	15	68107,022	4540,468		
	Total	19	81160,894			
Na+/K+ ATPase Activity	t (hs)	4	9085,963	2271,491	1,177	0,36
10 ‰	Residual	15	28938,436	1929,229		
	Total	19	38024,4			

One–way ANOVA was used to estimate the statistical significance of the differences and $P < 0.05$ was considered significant. * Significantly different.

Figure captions

Fig. 1: (A) Na^+/K^+ ATPase activity in the presence of varying concentrations of sodium/potassium at a constant total and Na^+ plus K^+ concentration of 130 mM in hepatopancreas of *N. granulata.*. Na^+/K^+ ATPase activity was assayed as described in Materials and Methods in the presence of 10 mM $MgCl_2$ and 0.5 mM EGTA at pH 7.4 and 30 °C. in the presence of 5mM Na_2ATP The values of Na^+/K^+ ATPase activity are expressed as relation to the activity at 100 mM Na^+/ 30 mM K^+ (100%; 292.82 ± 51.36 nmol Pi x min^{-1} x mg $prot^{-1}$). The curve is the one which best fits the experimental data. Data are the mean ± S.E for five individuals. (B) Effect of different concentrations of ouabain (0-1 mM) on Na^+/K^+ ATPase activity in hepatopancreas of individuals of *N. granulata*. Na^+/K^+ ATPase activity was assayed as described in Materials and Methods in the presence of 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$, 0.5 mM EGTA and 5mM Na_2ATP at pH 7.4 and 30 °C in the absence and/or the presence of different ouabain concentrations (0.1 to 1.0 mM). The values of activity are expressed as relation to the activity in the absence of ouabain (100%, 241.73 ± 57.44 nmol Pi x min^{-1} x mg $prot^{-1}$). Data are the mean ± S.E for four individuals. *indicate different from the activity without ouabain ($P < 0.05$) (C) Effect of different concentrations of ATP on Na^+/K^+ ATPase activity in hepatopancreas of *N. granulata*. Na^+/K^+ ATPase activity was assayed as described in Materials and Methods in the presence of 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EGTA at pH 7.4 and 30 °C in the presence of varying Na_2ATP concentrations (0.1 to 10 mM). The values of activity are expressed as relation to the corresponding activity in the presence of 5 mM ATP (100%, 245.83 ± 9241 nmol Pi x min^{-1} x mg $prot^{-1}$). The curve is the one which best fits the experimental data (GraphPad Prism 5.01). Data are the mean ± S.E. for four to five individuals. (D) Effect of pH (6.3–8.0) on Na^+/K^+ ATPase activity in hepatopancreas of *N. granulata*. Na^+/K^+ ATPase activity was assayed as described in Materials and Methods in the presence of 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EGTA at different pHs (6.3-9.0) and 30 °C in the presence of 5 mM Na_2ATP. The values of Na^+/K^+ activity are expressed as relation to the corresponding activity at pH 7.4 (100%, 259.59 ± 24.76 nmol Pi x min^{-1} x mg $prot^{-1}$). Data are the mean ± S.E. for four to five individuals.

Fig. 2: (A) Effect of Na^+ on ouabain-insensitive ATPase activity in hepatopancreas of *N. granulata*. Na^+ ATPase activity was determined as described in Materials and Methods in the presence of 10 mM MgCl2, 0.5 mM EGTA, 1 mM ouabain, 1 mM sodium azide, 3 mM Na_2 ATP at pH 7.4 and 30 °C and in the presence of varying

sodium concentrations. The values of Na^+ ATPase activity are expressed as relation to the activity at 100 mM Na^+ (100%, 460.24 ± 113.07 nmol Pi x min^{-1} x mg $prot^{-1}$). The curve is the one which best fits the experimental data. Data are the mean ± S.E for five individuals. (B) Dose-dependent inhibition of ouabain-insensitive Na^+ ATPase activity by furosemide in hepatopancreas of *N. granulata*. Na^+ ATPase activity was determined as described in Materials and Methods in the presence of 100 mM NaCl, 10 mM $MgCl^2$, 0.5 mM EGTA, 1 mM ouabain, 1mM sodium azide, 3 mM Na_2 ATP at pH 7.4 and 30 °C and in the absence and/or the presence of different furosemide concentrations (0.1 to 3mM). The values of ouabain-insensitive Na^+ATPase are expressed as relation to the activity in the absence of inhibitor (100%, 17.21 ± 2.07 nmol Pi x min^{-1} x mg $prot^{-1}$). I_{50}: furosemide concentration that produced 50% of inhibition, and was calculated by GraphPad Prism 5.01. Data are the mean±S.E for four to five individuals. *indicate different from the activity without furosemide ($P < 0.05$) (C) Effect of different concentrations of ATP on ouabain-insensitive Na^+ ATPase activity in hepatopancreas of *N. granulata*. Na^+ ATPase activity was determined as described in Materials and Methods in the presence of 100 mM NaCl, 10 mM $MgCl^2$, 0.5 mM EGTA, 1 mM ouabain, 1mM sodium azide at pH 7.4 and 30 °C and in the absence and/or the presence of varying ATP concentrations (0.1 to 12.0 mM). The values of activity are expressed as relation to the corresponding activity in the presence of 3 mM ATP (100%, 423.99 ± 83.8 nmol Pi x min^{-1} x mg $prot^{-1}$). The curve is the one which best fits the experimental data. (GraphPad Prism 5.01). Data are the mean±S.E. for four to five individuals. (D) Effect of pH (6.3–9.0) on ouabain-insensitive Na_+ ATPase activity. Na^+ ATPase activity was determined as described in Materials and Methods in the presence of 100 mM NaCl, 10 mM MgCl2, 0.5 mM EGTA, 1 mM ouabain, 1mM sodium azide, 3 mM Na_2 ATP at 30 °C and different pHs (6.3–9.0). The values of Na^+ ATPase activity are expressed as relation to the corresponding activity at pH 7.4 (100%, 91.76 ± 42.79 nmol Pi x min^{-1} x mg $prot^{-1}$). Data are the mean ± S.E. for four to five individuals.

Fig. 3: Na^+ ATPase at different times after feeding in hepatopancreas of *N. granulata* acclimated at 35 psu (A) and 10 psu (B). Na^+ ATPase activity was determined as described in Materials and Methods in the presence of 100 mM NaCl, 10 mM $MgCl^2$, 0.5 mM EGTA, 1 mM ouabain, 1mM sodium azide, 3 mM **Na_2 ATP** at pH 7.4 and 30 °C. Data are the mean±S.E for four or six individuals.*indicate different from the corresponding activity at t_0 ($P < 0.05$).

Fig. 4: Na^+/K^+ ATPase at different times after feeding in hepatopancreas of *N. granulata* acclimated at 35 psu (A) and 10 psu (B). Na^+/K^+ ATPase activity was assayed as described in Materials and Methods in the presence of 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$, 0.5 mM EGTA and 5mM **Na_2ATP** at pH 7.4 and 30 °C. Data are the mean±S.E for four or five individuals.

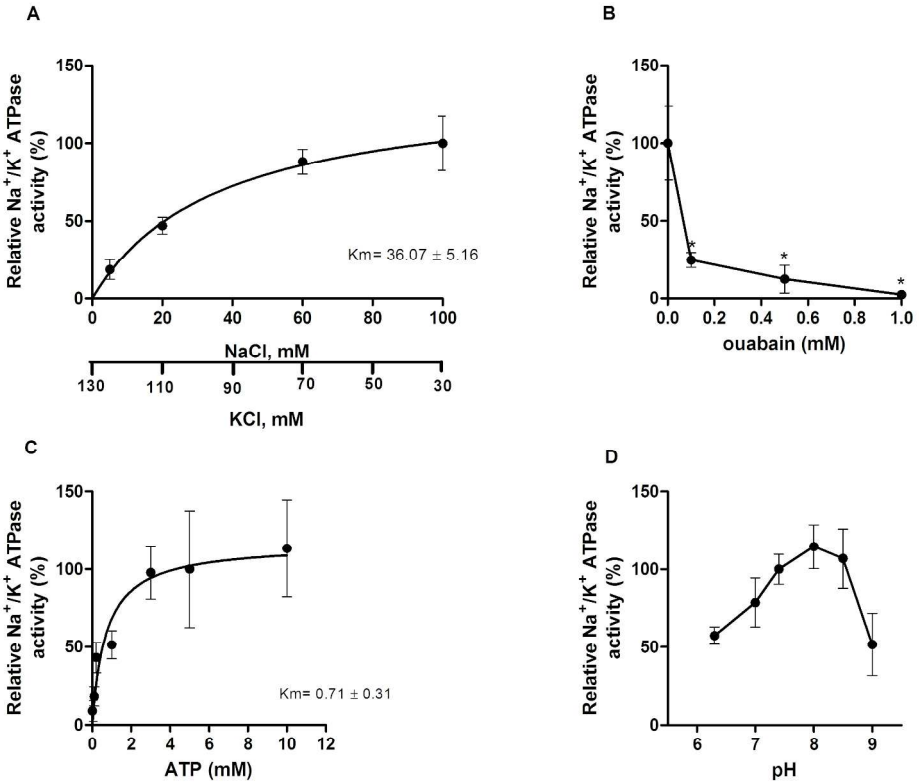

Figure 1

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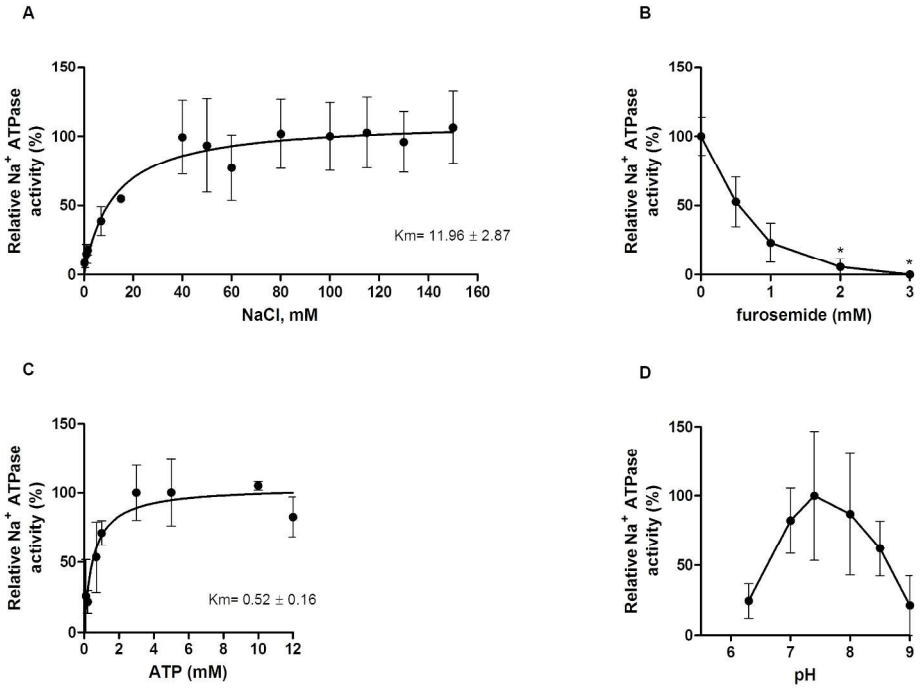

Figure 2

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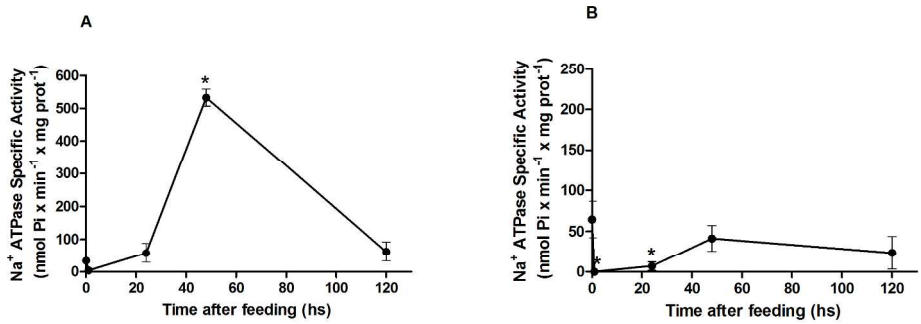

Figure 3

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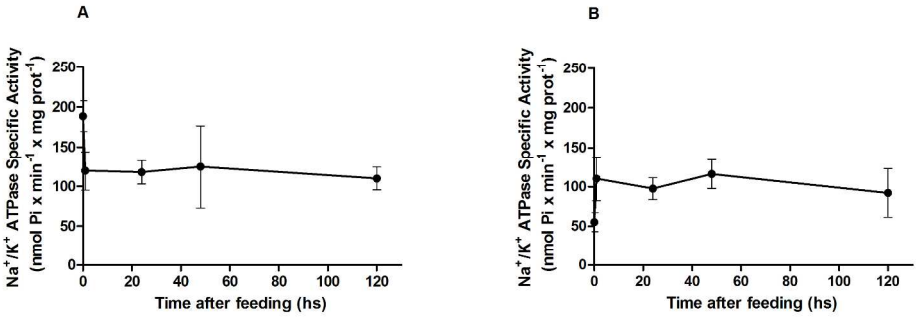

Figure 4

260x95mm (300 x 300 DPI)