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Digestive flexibility in the euryhaline crab *Cyrtograpsus angulatus* (Decapoda, Brachyura, Varunidae) from Mar Chiquita coastal lagoon (Buenos Aires, Argentina): responses to salinity of key enzymes in hepatopancreas

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Abstract: In Mar Chiquita coastal lagoon (Argentina) *Cyrtograpsus angulatus* is one of the dominant intertidal euryhaline crabs having an important ecological role. However, studies on digestive physiology and flexibility (i.e. occurrence and modulation of key digestive enzymes) are lacking. We determined the occurrence and characteristics of alkaline phosphatase (AP) activity in hepatopancreas and studied the responses to low salinity at short and long-term after feeding of AP and proteolytic activities in hepatopancreas. The hepatopancreas exhibited a levamisole-insensitive and a levamisole-sensitive AP activity with distinct biochemical characteristics. In crabs acclimated to 10 psu (hyper-regulation conditions), levamisole-insensitive AP activity decreased at long term (48 h) after feeding, while no changes occurred in crabs acclimated to 35 psu (osmoconforming conditions). No changes occurred in levamisole-sensitive AP activity at any salinity tested. In 35 psu, proteolytic activity decreased at short term after feeding (2- 4 h) while no changes were found in 10 psu. The different responses after feeding of levamisole-insensitive AP and proteolytic activities of hepatopancreas at 10 and 35 psu, suggest a role of these activities in digestive and metabolic adjustments underlying biochemical adaptation to environmental salinity.

Keywords: intertidal crabs, environmental salinity, digestive enzymes, post-feeding adjustments

Resumen: Flexibilidad digestiva en el cangrejo eurihalino *Cyrtograptus angulatus* (Decapoda, Brachyura, Varunidae) de la laguna costera de Mar Chiquita (Buenos Aires, Argentina): respuestas a la salinidad de enzimas clave en hepatopáncreas. En la laguna costera de Mar Chiquita (Argentina) *Cyrtograptus angulatus* es uno de los cangrejos dominantes con un importante papel ecológico. Sin embargo, faltan estudios sobre su fisiología y flexibilidad digestiva (ej. existencia y modulación de enzimas digestivas clave). En este estudio caracterizamos la actividad de fosfatasa alcalina (AP) en hepatopáncreas y estudiamos las respuestas post-ingesta a corto y largo plazo a baja salinidad ambiental (10 psu) de actividades de AP y proteolítica en hepatopáncreas. El hepatopáncreas exhibió actividades de AP insensible y sensible a levamisol con características bioquímicas diferentes. En cangrejos aclimatados a 10 psu (hiper-regulación), la actividad de AP levamisol-insensible disminuyó a largo plazo (48 h) post-ingesta, mientras que no se produjeron cambios en 35 psu (osmoconformación). No hubo cambios en la actividad de AP levamisol-sensible en ninguna de las salinidades estudiadas. En 35 psu, la actividad proteolítica se redujo a corto plazo post-ingesta (2- 4 h), mientras que no se encontraron cambios en 10 psu. Las diferentes respuestas post-ingesta de las actividades de AP levamisol-insensible y proteolítica en hepatopáncreas en 10 y 35 psu sugieren un papel de estas actividades en ajustes digestivos y metabólicos

subyacentes a la adaptación bioquímica a la salinidad ambiental.

Palabras claves: cangrejos intermareales, salinidad ambiental, enzimas digestivas, ajustes postingesta

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 then to cope with abrupt and wide changes in environmental salinity (McNamara & Faria 2012, Romano & Zeng 2012, Larsen *et al.* 2014). In animals inhabiting heterogeneous habitats, the ability to balance the acquisition, storage and use of energy under challenging environmental conditions is critical for survival (Karasov & Martinez del Rio 2007). In this context, studies on characteristics and mechanisms of regulation of digestive enzymes are crucial due to their main physiological role as a link between digestion, absorption and storage of nutrients (del Valle & López Mañanes 2011, 2012, Karasov & Douglas 2013). A differential modulation of key digestive enzymes activity in the digestive tract appears to be an important strategy in various animals allowing digestive flexibility upon distinct environmental conditions (del Valle & López Mañanes 2011, 2012, Romano & Zeng 2012; Karasov & Douglas 2013). However, studies on digestive physiology and flexibility (i.e. occurrence and modulation of digestive enzymes) in key species of intertidal euryhaline crabs of ecological importance such as *Cyrtograpsus angulatus* are still scarce and fragmentary.

C. angulatus is found from Rio de Janeiro (Brazil) to Patagonia (Argentina) on the Atlantic coast and in Peru and Chile on the Pacific coast (Spivak 1997). In Mar Chiquita coastal lagoon (37°32′–37°45′S; 57°19′–57°26′W, Argentina), *C. angulatus* inhabits areas with abrupt, frequent, and wide changes in environmental salinity (Spivak *et al.* 1994, Spivak 1997). Previous work from our lab show that in *C. angulatus* complex and integrative responses in branchial and extrabranchial tissues occur upon acclimation to low salinity. The acclimation to low salinity upon hyperegulatory conditions distinctly affect Na^+K^+ATPase activity in individual gills, alkaline phosphatase and Na^+K^+ATPase in the chela muscle and lipase and proteolytic activity in the hepatopancreas (López Mañanes *et al*. 2002, Pinoni & López Mañanes 2004, 2008, Pinoni 2009; Michiels *et al*., 2013). However, nothing is known about the effect of low salinity on postpandrial responses. Recently, we have shown that in individuals of the intertidal euryhaline crab *Neohelice granulata* from the mudflat of Mar Chiquita coastal lagoon, differential post-feeding adjustments in key enzymes of the hepatopancreas occurred in relation to environmental salinity (i.e. variations in alkaline phosphatase and proteolytic activities after feeding in crabs acclimated at low salinty) (Pinoni 2009, Pinoni *et al.* 2015, Michiels *et al*. 2015a).

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 & García-Carreño 2003, Zeng *et al.* 2010). Several studies suggest that the modulation of different enzyme activities in the hepatopancreas, in response to salinity, may lead to a differential availability of substrates (e.g. 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, 2015,Wang *et al.* 2014). Alkaline phosphatases (AP) (EC 3.1.3.1) are membrane-bound glycoproteins that are widely found in animals which hydrolyze 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 mammals, AP in the gastrointestinal tract is involved in digestive and absorptive processes (Geddes & Philpott 2008, Buchet *et al.* 2013, Lallès 2014). In hepatopancreas of crustaceans, AP activity has been related to digestive enzyme synthesis, accumulation, secretion, as well as absorption and storage of digestive products (Momin & Rangneker 1974, Barker & Gibson 1977, Gibson & Barker 1979, Wang *et al.* 2014). However, little is known about the occurrence and biochemical characteristics of AP activity in the hepatopancreas of euryhaline crabs as well as the possible modulation by low salinity of this activity. The level of proteolytic activity in the hepatopancreas will determine the ability for digestion and/or utilization of proteins. In previous studies we showed that levels of AP and proteolytic activity in the hepatopancreas of *N. granulata* in the natural ambient differ between habitats (mudflat and saltmarsh) in Mar Chiquita coastal lagoon (Buenos Aires, Province, Argentina), suggesting a role in adaptive, digestive and metabolic strategies to face the differential

environmental conditions (i.e. salinity/food availability) (Pinoni *et al*. 2011).

Previous works of our lab have shown the occurrence of different responses (activation/inhibition; different timing) to environmental salinity of enzymes activities in gills, muscle and hepatopancreas of *C. angulatus* to those in the other crab, *N. granulata*, of Mar Chiquita coastal lagoon (Schleich *et al.* 2001, López Mañanes *et al*. 2002, Pinoni *et al.* 2005, Pinoni & López Mañanes 2004, 2009, Michiels *et al.* 2015a) suggesting the occurrence of species-specific mechanisms of adjustments and moreover, pointing out, the need to evaluate responses to key environmental cues such as salinity in particular species. Furthermore, in spite of ecological importance of *C. angulatus* little is known about its digestive physiology and flexibility at the biochemical level (presence of key enzymes in digestion and absorption process in the hepatopancreas). Therefore, we were interested to establish the occurrence of AP activity in the hepatopancreas and the possible linked postpandrial adjustments of AP activity and proteolytic activity in low salinity in this crab. In this context, the aim of this work was to study the occurrence, biochemical characteristics of AP activities in the hepatopancreas, and the responses to low salinity of AP and total proteolytic activities at short and long term after feeding in *C. angulatus* from the mudflat of Mar Chiquita coastal lagoon.

Materials and Methods

Chemicals: p-nitrophenylphosphate (pNPP), 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 caught from the mudflat area from Mar Chiquita coastal lagoon (Buenos Aires province, Argentina; 37º32'–37º45'S, 57º19'–57º26'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. 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 crabs were maintained in aquaria (25 individuals per aquarium) with natural seawater (35 psu) or dilute seawater (10 psu) for at least 10 days prior to use (Pinoni & López Mañanes 2004, 2008, Michiels *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 20 ± 2ºC. Aquaria were shielded by black plastic to reduce disturbance. Crabs were fed three times a week with commercial food (Cichlind T.E.N., Wardley, USA) (about 0.07 g per individual). Dilute seawater was obtained by dilution of natural seawater with distilled water. The water conditions were checked daily and it was renovated when necessary.

To study the effect of low salinity at different times after feeding crabs acclimated to 35 psu and 10 psu were individually separated, unfed for 120 h (pre-feeding condition=PF) and fed posteriorly. The zero 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 and total proteolytic activities were determined at the short term (2, 4 h) and long term (48 h) after feeding. These times were chosen based on our previous works (Pinoni & López Mañanes 2004, 2008, Pinoni 2009; Michiels *et al.* 2013).

Sampling procedures: The crabs were weighed and cold-anaesthetized by putting them on ice for about 15 min. Haemolymph (about 500 mL) 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. Serum was separated by centrifugation at 10,000 g (Beckman, Microfuge, B) for 30 s. A sample of haemolymph was withdrawn for assaying of osmolality and ions concentration as described below. Wet mass was measured to the nearest 0.01 g. After weighing, the hepatopancreas was homogenized in 0.1 MTris/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 mL aliquots and stored at -20°C until to be used for enzymatic assays. Glycerol (1.3% v v^{-1}) was added to supernatant samples before freezing (Ljungström *et al.* 1984).

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 1mM $MgSO_4$ in 0.1 M Tris-HCl buffer (pH 8.0) 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 1mM $MgSO_4$ in 100 mM Tris–HCl buffer (pH 8.0) in the absence (total AP activity) and in the presence of 16 mM levamisole. Levamisole is an inhibitor of AP commonly used to discriminate between different AP isoforms (Chan & Stinson 1986, Mota *et al.* 2008, Diez-Zaera *et al.* 2011, Pinoni *et al.* 2005). 16 mM levamisole totally inhibited AP activity in chela muscle of this crab in both salinities studied (Pinoni 2009, Pinoni & López Mañanes 2008). 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 30 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 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. Individuals acclimated to 35 psu and unfed for 48 h (Pinoni & López Mañanes 2008, Pinoni *et al.* 2015) were used in these experiments.

Total proteolytic activity in the hepatopancreas was assayed as we previously described (Pinoni 2009, Pinoni *et al.* 2013). An aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) was added to a reaction mixture containing 1% w v^{-1} azocasein in 0.1 M Tris–HCl buffer (pH 7.5). After incubation at 45°C for 30 min, the reaction was arrested by adding 0.75 mL of cold trichloroacetic acid (TCA) (10% w v^{-1}). Overnight absorbance was measured at 440 nm (A_{440}) in the supernatant resulting after centrifuging at 1,800 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.

Measurement of hemolymph osmolality and ionic concentration: Osmolality was measured with a micro-osmometer (Osmomat 030 D, GONOTEC). Na^+ and K^+ were determined by flame photometry (Radiometer Copenhagen, FLM3). Cl^- was determined by a colorimetric method (Randox Commercial Kit) based on the formation of a blue Fe-2,4,6-tri-(2-pyridyl)-1.3.5-triazine-ferrous sulphate complex.

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 (repeated measures ANOVA, *t*-test or two-way ANOVA) analysis of variance 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 Bonferroni) was used to identify differences. Repeated measures ANOVA was used to estimate the significant differences between values of AP activities the same sample of hepatopancreas at different pHs of the reaction mixture and $P < 0.05$ was considered significant (Zar 1999). Two–way ANOVA was used to estimate the statistical significance of the differences and $P < 0.05$ was considered significant when analyzing enzymatic activities and protein content in the hepatopancreas after feeding in different salinities (factors: time and salinity).

Results

AP activity in hepatopancreas of C. angulatus: effect of levamisole and pH: Initially, total AP activity in hepatopancreas was determined in individuals maintained in osmoionoconformation conditions (35 psu, Table I) at pH 8 (Fig. 1). Then, AP activity at pH 8 was determined in the presence and in the absence of 16 mM levamisole (Pinoni *et al.* 2005, 2015; Pinoni & López Mañanes 2008). The inhibition of total AP activity by levamisole revealed the presence of two AP in hepatopancreas of *C. angulatus*: a levamisole-insensitive and a levamisole-sensitive activity (Fig. 1). 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 8.0–9.5, decreasing about 48 % at pH 7.4 and 10.0 (Fig. 2A). Levamisole-sensitive AP activity was similar within the range of pH 7.4–9.0. This activity was not detected at pH 9.5 (Fig. 2B).

Table I. Concentration of ions (mEq L^{-1}) and osmolality (mOsm kg^{-1}) in external medium and in *C. angulatus* haemolymph

	35 psu		10 psu	
	Medium	Haemolymph	Medium	Haemolymph
Na^+	420.8±18.8	406.7±18.8	179.0±3.2	332.8±6.6 *
K^+	10.2±2.7	7.8±1.9	3.8±0.3	8.6±0.4 *
Cl^-	497.6±34.2	440.0±12.1	152.0±3.3	363.3±16.1 *
Osmolality	954.8±4.6	894.6±38.3	273.8±1.3	644,5±27.4 *

* Significantly different from the corresponding value of the external medium (t-test, $p<0.05$). Data are the mean ± S.E. for 4-9 individuals.

Similar results concerning values of pH for maximal AP activities, detection of two AP (levamisole-insensitive and a levamisole-sensitive activity) and total inhibition by 16 mM levamisole were found when crabs acclimated to low salinity (10 psu) were used (not shown).

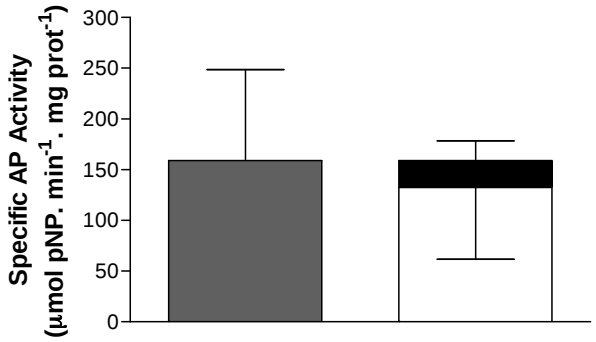

Figure 1. Total (grey bar), levamisole-insensitive (open bar) and levamisole-sensitive (back bar) AP activities in hepatopancreas of *C. angulatus*. Data are the mean ± S.E. for 3 individuals.

Effect of feeding on proteolytic activity and protein concentration of hepatopancreas at different salinities:At 35 psu, total proteolytic activity in hepatopancreas of C. angulatus was lower at 2 and 4 h after feeding (4.8±1.3 and 8.5±1.2 U min^{-1} mg $protein^{-1}$, respectively) than pre-feeding condition (15.5±0.9 U min^{-1} mg $protein^{-1}$).

Effect of feeding on AP activities of hepatopancreas at different salinities: The effect of feeding on AP activities was study in specimens acclimated to 35 and 10 psu, salinities at which this crab osmoionoconform and hyper-regulate, respectively (Table I). In individuals acclimated to 35 psu, levamisole-insensitive AP activity in hepatopancreas was similar at 2, 4 and 48 h after feeding than pre-feeding (30.5±12.9 µmol pNP min^{-1} mg $protein^{-1}$). At 10 psu, levamisole-insensitive AP activity was lower 48 h after feeding (8.6±2.8 µmol pNP min^{-1} mg $protein^{-1}$) than this activity at pre-feeding condition (55.5±22.3 µmol pNP min^{-1} mg $protein^{-1}$) (Fig. 3 A; Table II). Levamisole-sensitive AP activity was similar at pre-feeding and after feeding in crabs acclimated to both salinities (Fig. 3B; Table II).

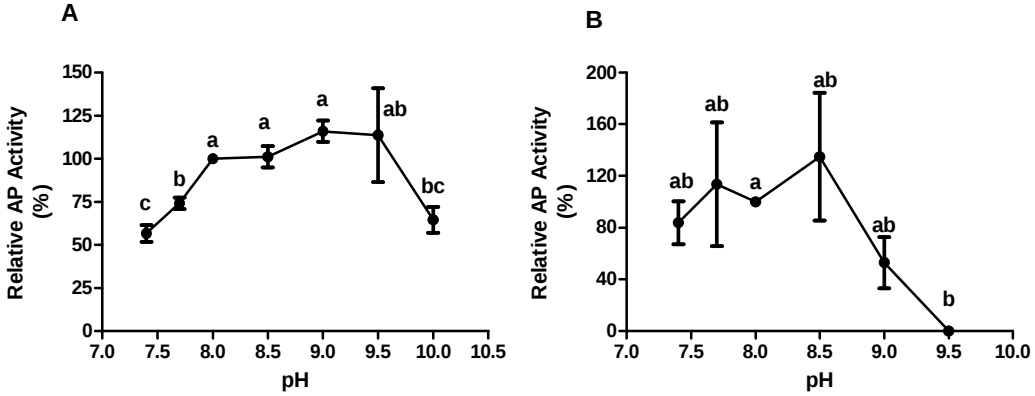

Figure 2. Effect of pH (7.4-10.0) on levamisole-insensitive (A) and levamisole-sensitive (B) AP activities in hepatopancreas of *C. angulatus* maintained at 35 psu. Data are the mean ±S.E. for 3-5 individuals. Different letters indicate significantly differences (Repeated Measure-ANOVA, $p<0.05$). The values of AP activity are expressed as relation to the activity at pH 8 (100%).

Table II. Two-way Analysis of Variance. Analysis of variation of enzymatic activities and protein content in the hepatopancreas after feeding in different salinities (factors: time and salinity). Two–way ANOVA was used to estimate the statistical significance of the differences and $P < 0.05$ was considered significant. * Significantly different

	Source of Variation	DF	SS	MS	F	P
Levamisole-Insensitive AP Activity						
	t	3	7047.07	2349.02	0.83	0.49
	Salinity	1	1378.64	1378.64	0.49	0.49
	t x Salinity	3	18206.52	6068.84	2.15	0.12
	Residual	29	81979.34	2826.87		
	Total	36	109511.97	3041.99		
Levamisole-Sensitive AP Activity						
	t	3	430.27	143.42	1.51	0.23
	Salinity	1	62.61	62.61	0.66	0.42
	t x Salinity	3	965.75	321.92	3.39	0.03*
	Residual	30	2848.99	94.97		
	Total	37	4332.49	117.09		
Proteolytic Activity						
	t	3	172.67	57.55	4.24	0.013*
	Salinity	1	134.36	134.36	9.91	0.004*
	t x Salinity	3	113.52	37.84	2.79	0.058
	Residual	30	406.91	13.56		
	Total	37	856.49	23.15		
Protein Concentration						
	t	3	633.25		3.67	0.022*
	Salinity	1	1041.88	1041.88	18.09	<0.001*
	t x Salinity	3	616.07	205.36	3.57	0.025*
	Residual	32	1843.17	57.60		
	Total	39	4331.40	111.06		

In individuals acclimated to 10 psu, no changes occur in proteolytic activity after feeding (Fig. 4A; Table II). At 35 psu, protein concentration in the hepatopancreas was similar at 2, 4 and 48 h after feeding than pre-feeding conditions. At 10 psu, protein concentration in the hepatopancreas was not significantly different after feeding at 2 and 4 h but was higher by 48 h than prefeeding (PF: 9.9±3.2, 48h: 29±6.6 mg protein g tissue^{-1}) (Fig. 4B; Table II).

Effect of feeding on proteolytic activity and protein concentration of hepatopancreas at different salinities: At 35 psu, total proteolytic activity in hepatopancreas of *C. angulatus* was lower at 2 and 4 h after feeding (4.8±1.3 and 8.5±1.2 U min^{-1} mg protein^{-1}, respectively) than pre-feeding condition (15.5±0.9 U min^{-1} mg protein^{-1}). In individuals acclimated to 10 psu, no changes occur in proteolytic activity after feeding (Fig. 4A; Table II). At 35 psu, protein concentration in the hepatopancreas was similar at 2, 4 and 48 h after feeding than pre-feeding conditions. At 10 psu, protein concentration in the hepatopancreas was not significantly different after feeding at 2 and 4 h but was higher by 48 h than prefeeding (PF: 9.9±3.2, 48h: 29±6.6 mg protein g tissue^{-1}) (Fig. 4B; Table II).

Discussion

In Mar Chiquita coastal lagoon (Argentina), adult males of the euryhaline crab *C. angulatus* are exposed to a wide range of environmental salinity (Spivak *et al.* 1994, Spivak 1997). Results of this work suggest that differential modulation after feeding of levamisole-insensitive AP, levamisole-sensitive AP and proteolytic activities in hepatopancreas is a component underlying biochemical adaptation to environmental salinity.

In the hepatopancreas of crustaceans AP is suggested to be related to digestive enzyme synthesis, accumulation, secretion, absorption and storage of digestive products (Momin & Rangneker 1974, Barker & Gibson 1977, Gibson & Barker 1979, Wang *et al*. 2014).

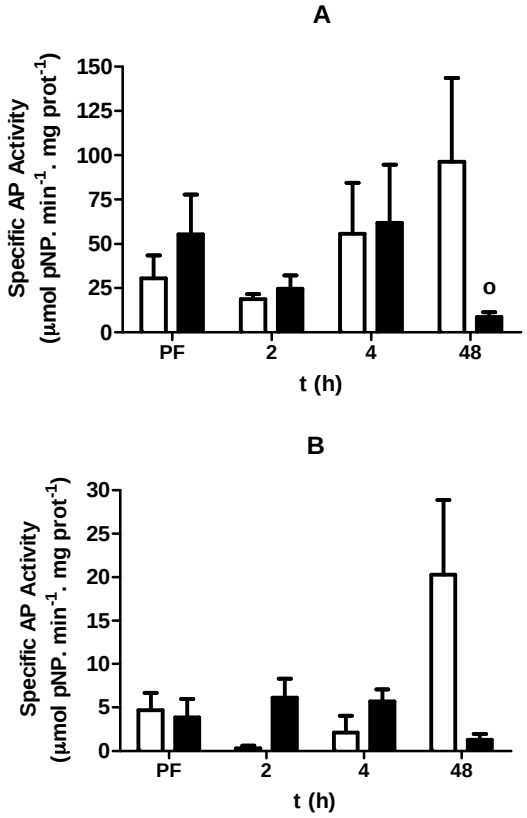

Figure 3. AP activities in hepatopancreas of *C. angulatus* maintained to 35 (open bars) and 10 (black bars) psu at different times after feeding. A: levamisole-insensitive AP activity. B: levamisole-sensitive AP activity. Data are the mean ±SE for 5 individuals. º Indicate significantly different from the corresponding activity PF=pre-feeding (Two-Way ANOVA, $p< 0.05$).

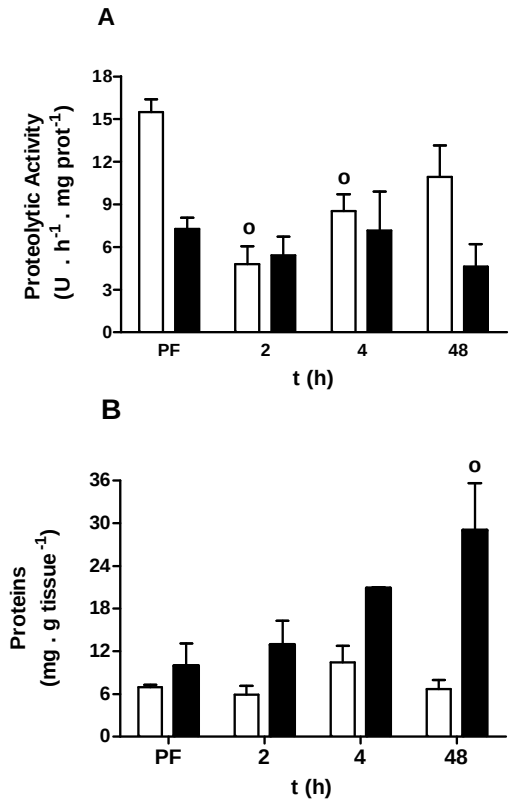

Figure 4. Total proteolytic activity (A) and protein concentration (B) in hepatopancreas of *C. angulatus* maintained to 35 (open bars) and 10 (black bars) psu at different times after feeding. Data are the mean ± S.E. for 5-8 individuals. º Indicate significantly different from the corresponding activity or concentration in PF=pre-feeding (Two-Way ANOVA, $p< 0.05$).

We have previously showed that differential modulation of levamisole-insensitive and levamisole-sensitive AP activities in chela muscle of *C. angulatus* are part of the responses underlying acclimation to low salinity in *C. angulatus* (Pinoni & López Mañanes 2004, 2008). To study the possible occurrence of modulation of these activities after feeding in the hepatopancreas in response to low salinity, we first determined the occurrence of both activities in the hepatopancreas and determined biochemical characteristics. The inhibition of total AP activity by levamisole in hepatopancreas of *C. angulatus* revealed the presence of levamisole-insensitive and levamisole-sensitive AP activities (Fig. 1). This is similar to that we previously found in the chela muscle of this crab, and in chela muscle and hepatopancreas of the euryhaline crab *N. granulata* (Pinoni *et al.* 2005, 2015, 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, Pinoni *et al.* 2015). Levamisole-insensitive and levamisole-sensitive AP activities in hepatopancreas of *C. angulatus* exhibited a different pH profile (Fig. 2). This agrees to that we found for these activities in chela muscle and hepatopancreas of *N. granulata* (Pinoni *et al.* 2005, 2015).

The maximal levamisole-insensitive AP activity in the range of pH 8.0-9.0 in hepatopancreas of *C. angulatus* (Fig. 2A) was distinct to the activity in chela muscle which presented maximal activity at pH 7.4-8.0 (Pinoni *et al.* 2005) but similar to hepatopancreas of *N. granulata* (Pinoni *et al.* 2015). The pH profile of the levamisole-sensitive AP activity (Fig. 2 B) was similar to this activity in muscle (Pinoni *et al.* 2005) but different to that

found in hepatopancreas of *N. granulata* (Pinoni *et al.* 2015). The response to pH of AP activities of the hepatopancreas of *C. angulatus* (Fig. 2) 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 *Scylla serrata* (Chen & Zhou 1998).

C. angulatus from the Mar Chiquita coastal lagoon behaves as a hyper-regulator at low salinity since it exhibits hemolymph osmolality and ions values higher from those of the corresponding external medium upon acclimation to 10 psu, while it osmoionoconforms in 35 psu (Table I). The decrease in levamisole-insensitive AP activity by 48 h after feeding in 10 psu (hyper-regulation conditions), while no changes occurred in 35 psu (osmoionoconforming conditions) (Figure 3 A; Table I), suggests that modulation of this activity is a component involved in response to low salinity. The fact that no changes of AP activities occurred at 2–4 h after feeding in any salinity suggests that these activities would not be involved in short-term adjustment in hepatopancreas in response to feeding. However, the overtime decrease in levamisole-insensitive AP activity (Fig. 3) suggests a role for this activity in posterior metabolic adjustments in response to environmental salinity. Future experimental approach would be focus in the exact physiological consequence (i.e. differential adjustments in digestive enzymes production and/or storage of nutrients) of the modulation of levamisole-insensitive AP activity in hepatopancreas by 48 h after feeding in 10 psu. 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). If this is the case for AP of *C. angulatus* remains to be investigated.

The decrease of total proteolytic activity in the hepatopancreas of *C. angulatus* 2-4 h after feeding in 35 psu but not in 10 psu (Fig. 4 A) shows the occurrence of differential digestive modulation and consequently adjustments in the protein metabolism in relation to osmoregulatory condition. This suggests that post-feeding modulation of total proteolytic activity in hepatopancreas is another component of biochemical adaptation to salinity in *C. angulatus*. Whether the maintenance of total proteolytic activity in the hepatopancreas after feeding in crabs acclimated to low salinity is related to changes (i.e. increases) in protein synthesis and storage in the hepatopancreas remains to be investigated. Our results concerning the protein concentration values in the hepatopancreas after feeding in 10 and 35 psu appear to support this idea (Fig. 4B). 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). It has been proposed that digestive enzymes that are secreted during the digestive cycle could be modulated intracellularly and finely regulated under certain physiological conditions (Sanchez-Paz *et al.* 2006). We have shown the modulation *in vitro* of intracellular lipase and proteolytic activity in the hepatopancreas of *N. granulata* and *C. angulatus* by primary chemical and intracellular messengers (i.e. cyclic AMP) which lead to concomitant changes in energy reserves concentrations (Pinoni & López Mañanes 2014, Michiels 2015, Michiels *et al.* 2015b, unpublished results). Furthermore, the fact that total proteolytic activity in hepatopancreas of *C. angulatus* was lower by 2-4 h after feeding in 35 psu while no changes occurred in 10 psu (Fig. 4 A) suggests that differential and specific mechanisms of modulation of proteolytic activity appear to occur in relation to salinity. The mechanisms of regulation of digestive enzyme in the hepatopancreas are far from having been elucidated. In the lobster *Panulirus argus*, trypsin enzymes are regulated at the transcriptional and secretion level by distinct prandial signals (Perera *et al.* 2012, 2015). In the American crayfish *Orconectes limosus*, a differential release of digestive enzyme activity stimulated by vertebrate gastrointestinal hormones occur *in vitro* (Resch-Sedlmeier & Sedlmeier 1999). As we pointed out above, we have shown the *in vitro* regulation of digestive enzymes in the hepatopancreas of *N. granualata* and *C. angulatus* suggesting the direct effect of biogenic amines and glucagon on the hepatopancreas (Pinoni & López Mañanes 2014, Michiels 2015, Michiels *et al.* 2015a,b; unpublished results).

In conclusion, the results of this study suggest that the modulation of levamisole-insensitive AP activity and total proteolytic activity in the hepatopancreas of *C. angulatus* at different times after feeding constitutes a part of the complex response underlying biochemical adaptation to environmental salinity.

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