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Effect of environmental salinity and dopamine injections on key digestive enzymes in hepatopancreas of the euryhaline crab *Cyrtograpsus angulatus* (Decapoda: Brachyura: Varunidae)

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SUMMARY: We studied the occurrence and characteristics of lipase activity and the response of lipase and proteolytic activity to salinity and dopamine injections in hepatopancreas of the euryhaline crab (*Cyrtograpsus angulatus*). Lipase activity was maximal at pH 8.5; it exhibited Michaelis-Menten kinetics (apparent K_m=0.019 mM), was higher at 37°C and appeared to be cold tolerant, being also high at 4°C. In 10 psu (hyper-regulation conditions), lipase and proteolytic activity were about 3 and 5 times higher, respectively, than in 35 psu (osmoconformation). In 40 psu (hypo-regulation), lipase activity was about three times higher than in 35 psu, while proteolytic activity was similar. Lipase activity was inhibited in vivo by 10^{-4} M dopamine in 35 psu but not in 10 or 40 psu. Proteolytic activity was not affected by 10^{-4} M dopamine. The differential responses of lipase and proteolytic activity to salinity and dopamine suggest the occurrence of distinct digestive adjustments and mechanisms of regulation upon osmoregulatory conditions. This study contributes to a better understanding of the complexity of the biochemical adaptations to salinity in euryhaline crabs. The fact that higher digestive enzyme activities could be associated with a differential digestive capacity potentially leading to enhanced availability of energy substrates is discussed.

Keywords: *Cyrtograpsus angulatus*, euryhaline crabs, salinity, hepatopancreas, digestive enzymes, dopamine, Mar Chiquita lagoon.

RESUMEN: EFECTO DE LA SALINIDAD AMBIENTAL Y DE LA INYECCIÓN DE DOPAMINA SOBRE ENZIMAS DIGESTIVAS CLAVE EN HEPATOPÁNCREAS DEL CANGREJO EURIHALINO *CYRTOGRAPSUS ANGULATUS* (DECAPODA: BRACHYURA: VARUNIDAE). – Se estudió la existencia y características bioquímicas de actividad de lipasa y la respuesta a salinidad y a dopamina de actividad de lipasa y proteolítica total en el hepatopáncreas del cangrejo eurihalino *Cyrtograpsus angulatus*. La actividad de lipasa fue máxima a pH 8,5; mostró una cinética michaeliana (K_m=0.019 mM); fue mayor a 37°C, y resistente a baja temperatura (4°C). En 10 psu (condición de hiperregulación), las actividades de lipasa y proteolítica fueron 3 y 5 veces mayores, respectivamente, que en 35 psu (condición de osmoconformación). En 40 psu (condición de hiporregulación), la actividad de lipasa fue de alrededor 3 veces mayor que en individuos aclimatados a 35 psu, mientras que la actividad proteolítica fue similar. La actividad de lipasa fue inhibida *in vivo* por dopamina 10^{-4} M en 35 psu pero no fue afectada en 10 o 40 psu. Dopamina 10^{-4} M no afectó la actividad proteolítica. Las respuestas de las actividades de lipasa y proteolítica a salinidad y a dopamina permiten sugerir la existencia de ajustes digestivos y mecanismos de regulación diferenciales en relación al estado osmorregulatorio. Los resultados constituyen un aporte relevante al conocimiento sobre las complejas adaptaciones a nivel bioquímico a la salinidad en cangrejos eurihalinos.

Palabras clave: *Cyrtograpsus angulatus*, cangrejos eurihalinos, salinidad, hepatopancreas, enzimas digestivas, dopamina, Laguna de Mar Chiquita.

INTRODUCTION

Euryhaline crabs have to cope with frequent changes in environmental salinity and therefore require strategies at different levels for controlling movements of water and ions between the individuals and their medium (Anger 2001, Kirschner 2004). Hypo and hyper-osmoregulation involves the absorption or secretion of ions, respectively, to maintain hemolymph ion homeostasis (McNamara and Faria 2012, Romano and Zeng 2012). Hyper-osmoregulation requires the involvement of various branchial ion transport mechanisms such as Na^+/K^+-ATPase activity and carbonic anhydrase, although the extent, timing and activity in individual gills can be species-specific; hypo-osmoregulation responses appear more variable and may require different mechanisms (López Mañanes *et al*. 2000, 2002, Schleich *et al*. 2001, Bianchini *et al*. 2008, Freire *et al.* 2008, McNamara and Faria 2012, Romano and Zeng 2012). Biochemical adaptation to environmental salinity appears to be a complex process involving also the participation of enzymes and transport systems in extrabranchial tissues (Jahn *et al.* 2006, Pinoni and López Mañanes, 2004, 2008, 2009, Martins *et al*. 2011). The biochemical changes occurring in salinity adaptation require a metabolic reorganization to support increasing energy demands (Freire *et al.* 2008, McNamara and Faria 2012, Romano and Zeng 2012). Since digestive enzymes constitute a link between digestion and absorption, modulation of their activity in the hepatopancreas (the major site of digestive enzyme production) could lead to a greater availability of essential energy substrates (i.e. lipids and proteins) for salinity acclimation. The level of lipolytic and proteolytic activity in the hepatopancreas will determine the ability for digestion and/or utilization of lipids and proteins, respectively. The information on the occurrence and biochemical characteristics of digestive enzyme activities in the hepatopancreas is still scarce and fragmentary. We have recently shown the effect of low salinity acclimation on amylase activity in the hepatopancreas of *Neohelice granulata* in what is, to our knowledge, the first work on the subject in a euryhaline crab (Asaro *et al.* 2011). In field studies we showed that levels of lipase and proteolytic activity in the hepatopancreas of this crab differ between habitats in Mar Chiquita coastal lagoon (Buenos Aires, Province, Argentina), suggesting their participation in adaptive digestive and metabolic strategies to face the differential environmental conditions (i.e. salinity) (Pinoni *et al*. 2011). In the hepatopancreas of the euryhaline pacific white shrimp *Litopenaeus vannamei*, the responses of digestive enzymes activity to extreme environmental salinity has been suggested to be related to the possibility of deriving extra energy upon osmoregulation (Li *et al*. 2008).

Dopamine (DA) is an important neurotransmisor and neurohormone in crustaceans (Fingerman *et al*. 1994, Clark *et al*. 2008, Christie 2011) and has been shown to participate in the regulation of metabolic responses to environmental stresses (Lorenzon, 2005, Hsieh *et al*. 2006). In euryhaline crabs, DA is involved in the regulation of osmoregulation process by modulating osmolyte concentration in the hemolymph and branchial transport of Na^+ and Na^+/K^+ ATPase activity (Mo *et al*. 1998, Schleich *et al*. 1999, Morris 2001, Halperin *et al*. 2004, Genovese *et al.* 2006). Nothing is known about the effect of this biogenic amine on digestive enzyme activity in the hepatopancreas.

C. angulatus is a euryhaline crab that 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). Field studies indicate that *C. angulatus* behaves as omnivorous-detritivorous (Spivak 1997, Botto *et al*. 2005) but the digestive battery at the biochemical level (i.e. occurrence and level of lipase activity) underlying this feeding behavior has not been yet elucidated. In Mar Chiquita coastal lagoon, *C. angulatus* inhabits areas with abrupt, frequent, and highly variable changes in salinity ranging from 4 to 40 psu (and sometimes lower and higher values), indicating an extremely high degree of osmoregulatory capacity (Anger *et al*. 1994, Spivak *et al*. 1994, López Mañanes *et al*. 2002, Pinoni and López Mañanes 2004, 2008, Pinoni 2009). We have shown that biochemical acclimation to salinity of *C. angulatus* involves complex and integrative responses (López Mañanes *et al*. 2002, Pinoni and López Mañanes 2004, 2008, Pinoni 2009). The modulation of alkaline phosphatase activities in the hepatopancreas suggests the occurrence of digestive and metabolic adjustments in relation to salinity (Pinoni 2009, unpublished results). However, studies on the modulation of digestive enzymes are lacking. To increase knowledge of different aspects of the biology of *C. angulatus* and as part of our studies on the identification of enzyme activities involved in biochemical adaptation to salinity in hyper/hypo-regulating crabs, the aim of this work was to determine the occurrence and characteristics of lipase activity in the hepatopancreas and the response of lipase and total proteolytic activity to low and high salinity and dopamine injections**.**

MATERIALS AND METHODS

Chemicals

Azocasein, *p*-nitrophenylpalmitate, Tris-(hydroxymethylamino-methane) (Tris), ethyleneglicol *N,N',N'*-tetraacetic acid (EGTA), bovine serum albumin and dopamine (3-hydroxytyramine) were from Sigma (St. Louis, MO, USA); sucrose and triclhoroacetic acid (TCA) were from Merck (Darmstadt, Germany); Coomasie blue G250 were from Fluka (Germany)**.** All chemicals were of analytical grade. All solutions were prepared in glass-distilled water.

Animal collection and maintenance

The crabs (n=60) 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 (20 individuals per aquarium) were maintained in natural seawater (35 psu), dilute sea-water (10 psu) or concentrated seawater (40 psu) for at least 10 days prior to use (Pinoni and López Mañanes 2004, 2008). 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 (López Mañanes *et al*. 2000, Pinoni and López Mañanes 2004, 2008, Pinoni 2009). 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). Aquaria were shielded by black plastic to reduce disturbance. Crabs were fed three times a week with Vita fish commercial food (30% carbohydrates, 44% protein, 12% fat; about 0.07 g individual^{-1}) but they were starved 24 h prior to the experiments. No differences in the feeding behavior occurred in the experimental conditions used.

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 enzyme activities

In the standard assay, lipase activity was determined by measuring *p*-nitrophenylpalmitate (pNPP) hydrolysis (Markweg *et al.* 1995) with some modifications. The reaction was initiated by the addition of pNPP (final concentration 0.7 mM) to a reaction mixture containing a suitable aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) in 50 mM Tris-HCl buffer (pH 8.5)/4 µl of Tween 80. Incubation was carried out at 37°C for 5 min. The reaction was stopped by addition of 0.5 ml of 0.2 % w/v of TCA. The amount of released p-nitrophenol (pNP) was determined by reading the absorbance at 410 nm (Metrolab 330). To study the effect of pH and temperature on lipase activity, the procedure was the same as described above except that the activity was determined in the presence of varying pH (range 5.4-10.0) (50 mM phosphate buffer pH 5.4-6.4; 50 mM Tris-HCl buffer pH 7.2-8.5; 50 mM Glycine buffer pH 10.0) and temperature (4-45°C) of the reaction mixture. To study the effect of pNPP concentration on lipase activity, the procedure was the same as described above except that the activity was determined in the presence of varying pNPP concentrations (0.09-0.9 mM) in the reaction mixture. Individuals acclimated to 35 psu were used in these experiments.

Total proteolytic activity was assayed by adding an aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) to a reaction mixture containing 1% w/v azocasein in 0.1 M Tris-HCl buffer (pH 7.5). The assay conditions used were optimal for this activity in the hepatopancreas of *C. angulatus* as described in previous work of our laboratory (Pinoni 2009). 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) (Pinoni 2009, Pinoni *et al*. 2011). Overnight absorbance was measured at 440 nm (A_{440}) (Metrolab 330) in the supernatant resulting after centrifuging at 1800 × g for 20 min (IEC-Centra 7R, refrigerated). One unit activity (U) was defined as the amount of enzyme extract that produced an increase of 1 in A_{440}. The proteolytic activity was expressed as U h^{-1} mg protein^{-1}.

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. Absorbance was measured with a Metrolab 330 spectrophotometer.

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. Osmolality was measured with a micro-osmometer (Osmomat 030 D, GONOTEC).

Effect of dopamine (DA) on digestive enzyme activity in hepatopancreas

The effect of DA in vivo was determined as we described previously (Pinoni and López Mañanes 2004). After being cryoanesthetized for 20 min, individuals (n=5-10) were injected by means of a syringe at the base of the cheliped into the infrabranchial sinus with 100 µl of saline solution (400 mM NaCl, 10 mM KCl) as control, or saline solution plus DA (final concentration 10^{-4} M). At 30 min after the injection, the corre-

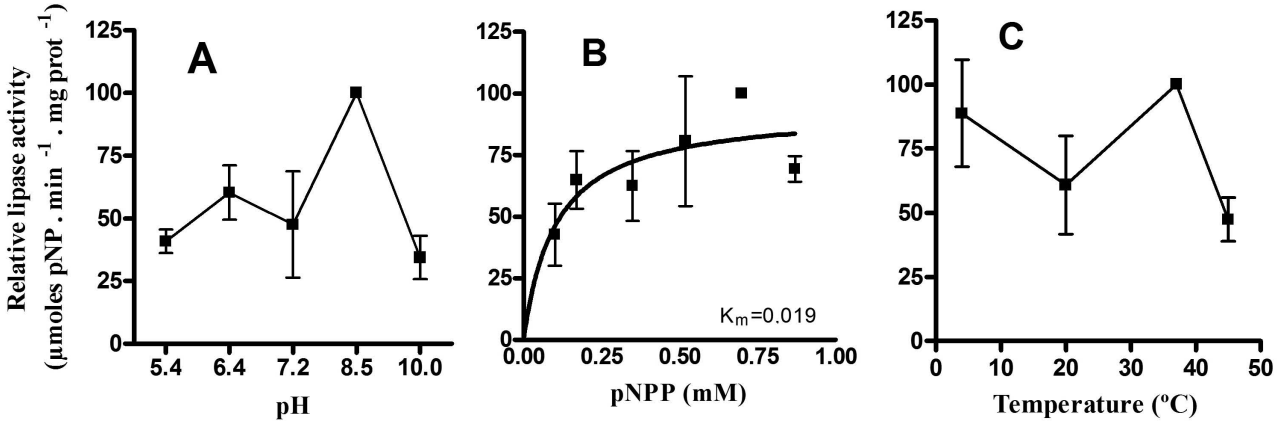

FIG. 1. – (A) Effect of pH (5.4-10.0) on lipase activity in hepatopancreas of *C. angulatus*. The activity was measured at 37°C and in the presence of 0.7 mM p-nitrophenylpalmitate (pNPP). The lipase activity values are expressed as a relation to the specific activity at pH 8.5 (100%, 59±20 µmoles pNP min^{-1} mg $prot^{-1}$). Data are the mean ±SE for 5 individuals. (B) Effect of pNPP concentration on lipase activity in hepatopancreas of *C. angulatus*. The activity was measured at 37°C and at pH 8.5. The activity values are expressed as a relation to the corresponding activity in the presence of 0.7 mM pNPP (100%, 50±18 µmoles pNP min^{-1} mg $prot^{-1}$). Data are the mean ±SE for 5 individuals. (C) Effect of temperature (4-45°C) on the lipase activity in hepatopancreas of individuals of *C. angulatus.* The activity was measured at pH 8.5 and in the presence of 0.7 mM pNPP. The activity is expressed in relation to the activity at 37°C (100%, 32±18 µmol pNP min^{-1} mg $prot^{-1}$). Data are mean ±SE for 3 individuals.

sponding activity was determined in hepatopancreas as described above (standard assay conditions). For each individual (control and treated) the corresponding activities were assayed at least in triplicate. No mortality occurred during the experiment.

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 that best adjusts to the experimental data. The values of K_m (constant of Michaelis-Menten) were estimated by the analysis of the data using the graph of Lineweaver-Burk (GraphPad Prism 4.0 software). The statistical analysis of the data was realised using the Sigma 3.0 program for Windows, which automatically performs a previous test of equality of variances and normality. A parametric analysis of variance was used (one-way ANOVA).

RESULTS

Lipase activity of hepatopancreas of *Cyrtograpsus angulatus*: effect of pH, p-nitrophenylpalmitate (pNPP) and temperature

Lipase activity in the hepatopancreas was determined within the range of pH 5.4-10.0. Maximal activity was found at pH 8.5. The activity was similar within the range 5.4-7.2 being about 40%-55% of the activity at pH 8.5. At pH 10.0 the activity decreased markedly, being about 35% of the activity at pH 8.5 (Fig. 1A). The effect of pNPP concentrations on lipase activity is shown in Figure 1B. Lipase activity in hepatopancreas of *C. angulatus* exhibited Michaelis-Menten kinetics

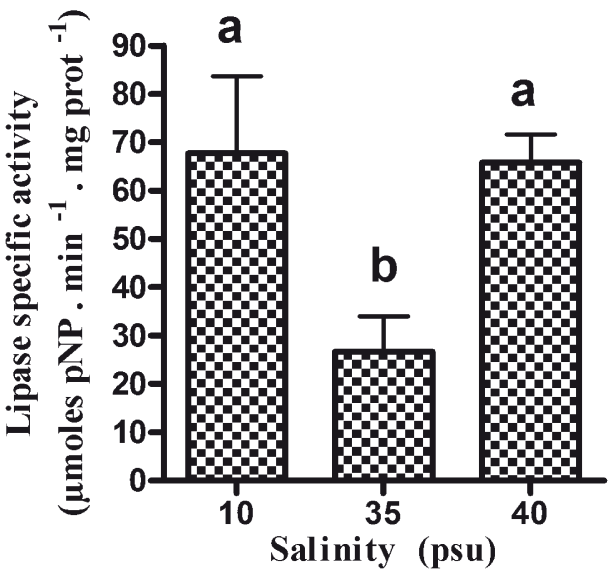

FIG 2. – Effect of acclimation to low and high salinity on lipase activity in hepatopancreas of *C. angulatus.* The activity was measured as described in the Materials and Methods section in the presence of 0.7 mM p-nitrophenylpalmitate (pNPP) at pH 8.5 and at 37°C. Different letters indicate significant differences ($P<0.05$). Data are mean ±SE for 5 individuals.

(apparent K_m=0.019 mM). Figure 1C shows the effect of temperature (4-45°C) on lipase activity. The activity decreased between 4°C and 20°C. At 37°C activity increased, reaching values similar to those at 4°C and at 45°C it decreased to about 47% of the activity at 37°C.

Effect of environmental salinity on digestive enzymes in hepatopancreas of *Cyrtograpsus angulatus*

To determine the effect of environmental salinity on lipase and total proteolytic activity in hepatopan-

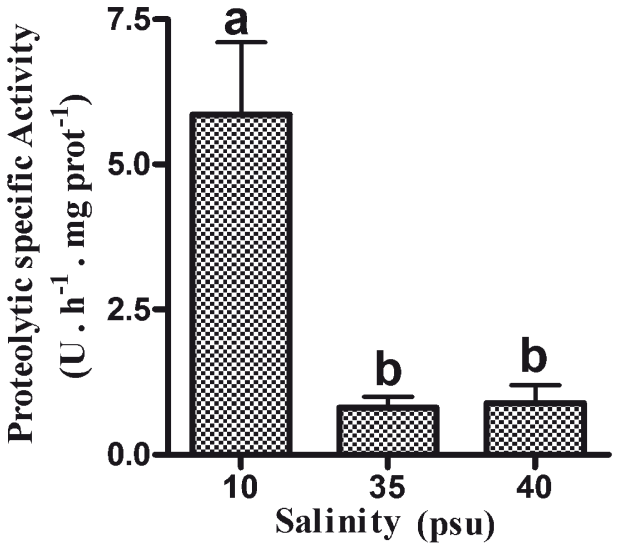

FIG 3. – Effect of acclimation to low and high salinity on proteolytic activity in hepatopancreas of *C. angulatus.* The activity was determined as described in the Materials and Methods section in the presence of 1% w/v azocasein at pH 7.5 and 45°C. Different letters indicate significant differences (P<0.05). Data are mean ±SE for 5 individuals.

creas, crabs were acclimated to 35, 10 and 40 psu. The hemolymph osmolality of the crabs was significantly higher and lower from the external medium at 10 (medium =269±15; hemolymph =653±14) and 40 psu (medium =1015±16; hemolymph =878±21) respectively, while no differences were detected at 35 psu (medium =882±6; hemolymph =872±21).

In individuals acclimated to low (10 psu) and high (40 psu) salinity, lipase activity was about three times higher than at 35 psu (27 μmoles pNP min^{-1} mg $prot^{-1}$) (Fig. 2). Lipase activity of crabs acclimated to 10 and 40 psu was not significantly different from each other (Fig. 2).

Crabs acclimated to 10 psu also exhibited a higher proteolytic activity in hepatopancreas (about five times) than crabs acclimated to 35 psu. On the other hand, crabs exposed to 40 psu exhibited a similar proteolytic activity to crabs acclimated to 35 psu (Fig. 3).

Effect of dopamine injections on digestive enzymes in hepatopancreas of *Cyrtograpsus angulatus*

DA 10^{-4} M significantly decreased lipase activity (about 45%) in hepatopancreas of crabs acclimated to 35 psu but had no effect on this activity in individuals exposed to 10 or 40 psu (Fig. 4A). DA 10^{-4} M did not affect proteolytic activity in hepatopancreas at any salinity (Fig. 4B).

DISCUSSION

Our results show the occurrence of lipase activity in hepatopancreas of *C. angulatus* from Mar Chiquita coastal lagoon and the differential response of lipase and total proteolytic activity to low and high environmental salinity and dopamine. In crustaceans, the occurrence in the hepatopancreas of specific digestive enzyme activities has been related to the nature of the dietary components that could be potentially used for metabolic processes (Pavasovic *et al*. 2007, Figueiredo and Anderson 2009). The high lipase activity in hepatopancreas of *C. angulatus* is in accordance with the dietary habit of this crab in Mar Chiquita lagoon (Spivak 1997, Botto *et al*. 2005) and suggests the ability to perform lipid degradation and its potential use

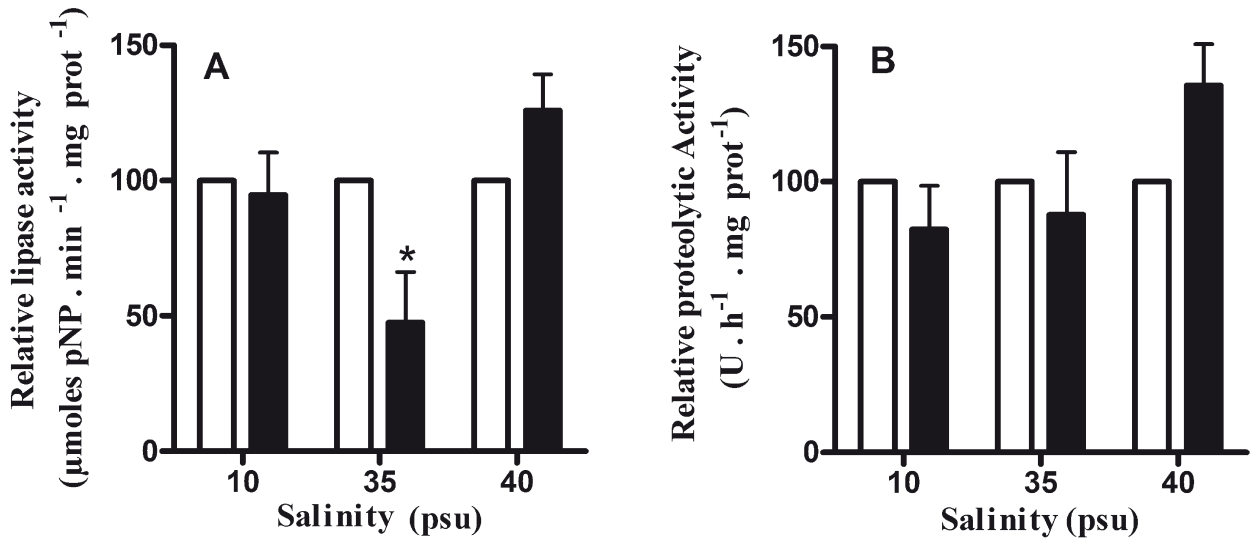

FIG 4. – Effect of DA on lipase activity and total protease activity in hepatopancreas of *C. angulatus* acclimated to different salinities. Lipase activity was measured as described in the Materials and Methods section in the presence of 0.7 mM p-nitrophenylpalmitate (pNPP) at pH 8.5 and at 37°C. Total protease activity was determined as described in the Materials and Methods section in the presence of 1% w/v azocasein at pH 7.5 and 45°C. Activity is expressed as a percentage (%) of the activity of the corresponding control (without DA) (100%). Data are mean ±SE for 5-10 individuals. Open bars, control; black bars, DA 10^{-4} M. *Significantly different from the corresponding control (P<0.05).

as an energy source. The pH value for maximal lipase activity and the high level of activity throughout a wide range of pH (5.4-8.5) (Fig. 1A) is in agreement with that found for this enzyme in hepatopancreas of the euryhaline crab *N. granulata* (Michiels *et al*. 2011) and of the marine green crab *Carcinus mediterranus* (Cherif *et al*. 2007, Cherif and Gargouri 2009, Smichi *et al*. 2012). The high lipase activity at 37°C in hepatopancreas of *C. angulatus* (Fig. 1C) is also similar to that found in *N. granulata* (Michiels *et al*. 2011) but quite different from that described in *C. mediterraneus* (Cherif and Gargouri 2009, Smichi *et al*. 2012). Lipase activity in hepatopancreas of *C. angulatus* appeared to be strikingly high at low temperature (4°C) (Fig. 1C). Since in Mar Chiquita coastal lagoon this crab is commonly exposed to low temperature (as low as 1°C) (Spivak *et al*. 1994, personal observations), a cold tolerant lipase activity in the hepatopancreas could be related to a role in thermal acclimation (i.e. a higher digestion of lipids), as suggested for the copepod *Calanus glacialis* (Freese *et al*. 2012). However, this hypothesis requires further investigation. The Michaelis–Menten kinetics of lipase activity of hepatopancreas of *C. angulatus* (Fig. 1B) is in agreement with our preliminary results in hepatopancreas of *N. granulata*, to our knowledge, the only data on crabs (Michiels *et al*. 2011) and with those reported in the crayfish *Procambrus clarki* (Hammer *et al*. 2003) and the prawn *Macrobrachium borellii* (Pasquevich *et al*. 2011).

Osmoregulatory adaptation in response to salinity requires various molecular and biochemical changes such as increased gill Na^+/K^+-ATPase activity and/or hemolymph amino acids, which need energy (Freire *et al*. 2008, McNamara and Faria 2012, Romano and Zeng 2012). While this phenomenon has been studied extensively, some mechanisms are not fully understood, particularly from a metabolic perspective. In cultured crustaceans species, it has been suggested that dietary manipulation has the potential to improve the osmoregulatory abilities of individuals subjected to osmotically stressful conditions because osmoregulation requires energy that is sourced from lipids and or protein (Romano and Zeng 2012). Modulation of key digestive enzymes (i.e lipase and proteolytic activity) in the hepatopancreas could then lead to a greater availability of essential energy substrates (i.e lipids and protein metabolites) upon osmoregulation. *C. angulatus* behaves as a hyper/hypo-regulator because it exhibits hemolymph osmolality values higher and below lower than those of the corresponding external medium upon acclimation to 10 psu and 40 psu, respectively, whereas it osmoconforms at 35 psu (López Mañanes *et al*. 2002, Pinoni and López Mañanes 2008, this study). These osmoregulatory responses reached after 10 days' acclimation are maintained stable for longer periods (up to three months) at all salinities tested (López Mañanes *et al*. 2002, Pinoni and López Mañanes 2008, Pinoni 2009, del Valle *et al*. 2012, unpublished results). We have shown that biochemical salinity adaptation in *C. angulatus* involves the differential and integrative modulation of Na^+/K^+-ATPase activity in individual anterior and posterior gills and Na^+/K^+-ATPase and alkaline phosphatase activity in chela muscle (López Mañanes *et al*. 2002, Pinoni and López Mañanes 2004, 2008, Pinoni 2009). The higher lipase activity in hepatopancreas of crabs acclimated to 10 psu and 40 psu (Fig. 2) suggests that modulation of this activity is another component of the biochemical adaptation to differential salinity. The enhanced lipase activity under salinity conditions implying hyper and hypo-regulatory responses (Fig. 2) could be related to a potential enhanced digestive capacity for lipids, leading in turn to a higher availability of energy metabolites upon osmoregulation. Since nothing is known about metabolic pathways and interrelation between organs and tissues in *C. angulatus*, a further experimental approach is required to test this hypothesis. In *N. granulata*, the changes in lipid metabolism (i.e mobilization from storage sites) must be related to the participation of these compounds as energy sources during salinity acclimation (Luvizotto-Santos *et al*. 2003, Pinoni and López Mañanes 2011). An adequate protein intake is essential to support the amino acid provision necessary to maintain key essential functions such as osmoregulation (Sánchez-Paz *et al*. 2006, Romano and Zeng 2012). Similarly to what we pointed out above concerning lipase activity, the higher proteolytic activity in the hepatopancreas of *C. angulatus* in low salinity (10 psu) than at 35 psu (osmoconformation) (Fig. 3) suggests a potential higher capability for the digestion of proteins upon hyper-regulation. Hyper and hypo-osmoregulation appear to require different mechanisms (McNamara and Faria 2012, Romano and Zeng 2012). The fact that lipase activity in hepatopancreas of *C. angulatus* was affected at both low and high salinity while proteolytic activity was only affected at low salinity (Figs 2 and 3) suggests the occurrence of differential digestive adjustments and consequently adjustments in the lipid and protein metabolism in relation to different osmoregulatory (hyper or hypo-regulation) responses. Parallel work in our laboratory shows that amylase activity in the hepatopancreas of *C. angulatus* would not be affected by salinity under the same conditions (unpublished results) and that a mobilization of glycogen from gills occurs only at high salinity (Asaro *et al*. 2012), further supporting the hypothesis of different specific metabolic adjustments for differential osmoregulatory responses. We have recently shown the different responses to low salinity of amylase and disaccharidase activity in the hepatopancreas of *N. granulata* (Asaro *et al*. 2011). In vitro experiments on muscle and hepatopancreas of *N. granulata* showed the occurrence of different adjustments in metabolic pathways (i.e. changes in the carbon amino acid flux between gluconeogenesis and lipid synthesis) in response to hypo and hyper-osmotic stress (Martins *et al*. 2011).

We have shown that injection of 10^{-4} M DA increased hemolymph glucose levels and inhibited

alkaline phosphatase activity in chela muscle of *C. angulatus* (López Mañanes 2004, Pinoni and López Mañanes 2004, del Valle *et al*. 2012). DA appears to be a key primary chemical messenger, having multiple effects and playing a role in regulatory pathways involved in the control of adaptive responses to salinity in this crab (López Mañanes 2004, Pinoni and López Mañanes 2004, del Valle *et al*. 2012). The mechanisms of regulation (i.e. primary chemical messengers) or factors involved in the modulation of the activity and/or secretion of digestive enzymes in the hepatopancreas of crustaceans have been investigated little and are therefore very far from having been elucidated. The effect of DA on lipase activity in hepatopancreas of *C. angulatus* in vivo (Fig. 4) suggests that this biogenic amine is one primary chemical messenger involved in these mechanisms. Since the effect of DA on lipase activity occurred only in crabs acclimated to 35 psu, it can be speculated that the different response of this activity to salinity (Fig. 2) could be at least partially attributed to a differential activation of signaling pathways mediated by DA under osmoconforming and osmoregulating conditions. However, a role of other signaling pathways operating under osmoregulating conditions cannot be ruled out. Because the effect of DA on lipase activity was found in vivo, further studies are required to establish whether this effect involves its binding to specific receptors on hepatopancreas and/or on other target tissues inducing the secondary release of other messengers. The stimulation by DA of the release of glucose from hepatopancreas of *C. angulatus* in vitro, suggests its direct effect on this tissue (López Mañanes 2004). The effect of DA appeared to be specific on lipase activity since proteolytic activity was not affected at any salinity (Fig. 4). Work in our laboratory shows that maltase activity is also not affected (unpublished results). This suggests the occurrence of differential and specific mechanisms of modulation of the activity of digestive enzymes in the hepatopancreas of *C. angulatus*. In the American crayfish *Orconectes limosus*, in vitro experiments showed a differential release of digestive enzyme activity from the hepatopancreas stimulated by several vertebrate gastrointestinal hormones (Resch-Sedlmeir and Sedlmeir 1999). In the lobster *Panulirus argus* trypsin enzymes are regulated at the transcription and secretion level by distinct pandrial signals related to the quality of dietary proteins (Perera *et al*. 2012).

In conclusion, the results of this study show that lipase activity occurs in the hepatopancreas of *C. angulatus* and that lipase and proteolytic activity in the hepatopancreas are differentially affected upon acclimation to low and high salinity and DA injection, suggesting that they participate in the biochemical adaptation process to environmental salinity in this crab and that digestive enzymes have different modulation mechanisms. Whether these activities are involved in biochemical-physiological adjustments secondary to hyper-regulation and hypo-regulation (i.e. digestive adjustments leading to enhanced digestive capacity for lipids and proteins and for absorption of metabolites) remains to be investigated. Future studies will focus on establishing the exact physiological role of these responses in the hepatopancreas and other signaling pathways involved to provide a better understanding of the integrative responses and mechanisms of regulation underlying biochemical adaptation to salinity in *C. angulatus* in particular and in euryhaline crabs in general.

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