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Glucose homeostasis in the euryhaline crab *Cytograpsus angulatus*: Effects of the salinity in the amylase, maltase and sucrase activities in the hepatopancreas and in the carbohydrate reserves in different tissues

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ABSTRACT

We studied the existence, biochemical characteristics and response to different environmental salinities of amylase, maltase and sucrase activity in the intertidal euryhaline crab *Cyrtograpsus angulatus* (Dana, 1852) along with the response to distinct salinities of glycogen and free glucose content in storage organs. Amylase, maltase and sucrase activities were kept over a broad range of pH and temperature and exhibited Michaelis–Menten kinetics. Zymography showed the existence of two amylase forms in crabs exposed to 35 (osmoconformation) and low (6–10 psu; hyper-regulation) or high (40 psu) (hypo-regulation) salinities. Carbohydrases activity in the hepatopancreas and glycemia were not affected in crab exposed to different environmental salinities. In 6 and 40 psu, the glycogen content in anterior gills was lower than in 35 psu. In 6, 10 and 40 psu, glycogen concentration in hepatopancreas, muscle and posterior gills were similar to that in 35 psu. Free glucose concentration in chela muscle was higher in 6 and 40 psu than in 35 psu. The existence and biochemical characteristics of carbohydrases activity and the adjustments in concentration of glycogen in anterior gills and free glucose in chela muscle suggests the ability to perform complete hydrolysis of glycogenic substrates and to keep glucose homeostasis in relation to acclimation to different salinity conditions.

1. Introduction

In decapod crustaceans, glucose homeostasis is fundamental for maintaining organ functions and for compensation to numerous environmental stress factors (Verri et al., 2001; Lorenzon, 2005; Dutra et al., 2008). The carbohydrates digestion such as starch, glycogen, disaccharides as well as the absorption of glucose via the hepatopancreas are principal origin of hemolymphatic glucose (Verri et al., 2001; Obi et al., 2011). The hepatopancreas is multifunctional organ. It is the principal site of digestive enzymes synthesis and where intracellular digestion begins and is accomplished (Zeng et al., 2010; Ribeiro et al., 2014; Wang et al., 2014). The occurrence and level of key carbohydrases in the hepatopancreas has a fundamental role in the metabolism of glycogenic carbohydrates.

α-amylases (α-1,4 glucan-4-gluconohydrolase) are hydrolytic enzymes responsible for the hydrolysis of internal α-D-(1, 4) glycoside bonds of α-glucans (Janeček et al., 2014; Xie et al., 2014; Peng et al., 2015; Tiwari et al., 2015). In all animals, α-amylases have a central physiological importance due to their function in the initial steps of hydrolysis of dietary starch and of dietary and/or storage glycogen (Date et al., 2015; Saborowski, 2015). Amylase activity was detected in the hepatopancreas of various decapod crustaceans and has been found that several forms occur although with a high grade of interspecific variability (Le Moullac et al., 1997; Van Wormhoudt and Sellos, 2003; Johnston and Freeman, 2005; Perera et al., 2008a, 2008b; Coccia et al., 2011; Aragón-Axomulco et al., 2012; Castro et al., 2012; Rodríguez-Viera et al., 2016). However, very little information is available about the occurrence and biochemical characteristics of amylase in the hepatopancreas of intertidal euryhaline crabs (Blandamer and Beechey, 1966; Van Wormhoudt et al., 1995; Asaro et al., 2011, 2017). Maltase, which hydrolyzes α-1,4 glycosidic linkages from non-reducing ends, has a main role in glycogenic carbohydrates digestion by participating in the initial (assisting to α- amylase) and in the final steps (Lin et al., 2012, 2014, 2015; Dhital et al., 2013).The existence of specific

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disaccharidases in the hepatopancreas would also facilitate the potential use of glycogenic disaccharides (i.e. maltase, sucrose) as glucose sources. Reports about existence and biochemical characteristics of maltase and sucrase in the hepatopancreas of intertidal euryhaline crabs are still limited (McClintock et al., 1991; Asaro et al., 2011).

In estuaries and coastal lagoons, intertidal euryhaline crabs must deal with sharp and broad changes in environmental salinity. Biochemical adaptation to environmental salinity implies adjustments in different tissues (Michiels et al., 2013, 2015a; Pinoni et al., 2013, 2017; Larsen et al., 2014). In intertidal euryhaline crabs, simultaneous determinations of diverse parameters in different organs are needed for an integral analysis of the adaptation at biochemical level to different salinities (Romano and Zeng, 2012; Pinoni et al., 2013, 2015).

Cyrtograpsus angulatus (Dana, 1852) is a euryhaline crab common in intertidal areas of southwestern Atlantic coast from Rio de Janeiro (Brazil) to Patagonia (Argentina) and of the Pacific coast in Chile and Perù. *C. angulatus* is one of the dominant crabs in these regions (Boschi, 1964; Spivak, 1997). This crab is a predator-omnivorous-carrion (Spivak, 1997; Botto et al., 2005). The digestive battery at the biochemical level subjacent to its dietary behavior is not totally understood. We have shown the occurrence of different digestive enzymes activities (i.e. endo and ectoproteases and lipase) (Michiels et al., 2013; Michiels, 2015) in the hepatopancreas of *C. angulatus*, but studies on carbohydrase activities (i.e. amylase, maltase and sucrase) are still lacking. In Mar Chiquita coastal lagoon (Argentina) *C. angulatus* is exposed to sharp, recurrent and deeply changes in salinity (from 4 to 40 psu and from time to time reaching lower and higher values) (López Mañanes et al., 2002; Pinoni and López Mañanes, 2004, 2008; Michiels et al., 2013). We showed that biochemical acclimation to salinity implicates multiple and integral responses (López Mañanes et al., 2002; Pinoni and López Mañanes, 2004, 2008; Pinoni, 2009). The distinct regulation of proteolytic and lipase activities in the hepatopancreas suggest that different adjustments in proteins and lipids metabolism are triggered by exposure to high salinity (Michiels et al., 2013). Nothing is known yet about the occurrence of adjustments in components involved in glucose homeostasis at the biochemical level. The aims of this work were to determine i) the occurrence and biochemical characteristics of amylase, maltase and sucrase activities in the hepatopancreas of *C. angulatus* and ii) the effect of low and high salinity on these activities in the hepatopancreas and on the content of carbohydrate reserves in different tissues of this crab.

2. Methods

2.1. Collection and maintenance of animals

Crabs in intermolt (carapace width > 2.5 cm) (Drach and Tchernigovtzeff, 1967) were captured from the mudflat area of Mar Chiquita coastal lagoon (37°32′–37°45′S; 57°19′–57°26′W, Argentina) in autumn. Crabs were transported to the laboratory on the same day of capture in lagoon water. The salinity for all experiments was determined in practical salinity units (psu). The individuals of *C. angulatus* were distributed in 3 groups (4–8 individuals per condition): I) dilute seawater (6–10 psu), II) seawater (35 psu), and III) concentrated seawater (40 psu) for 10 days (Michiels et al., 2013). Dilute and concentrated seawater preparation and the maintenance of crabs in aquaria were done as previously described (Michiels et al., 2015a). The aquaria contained 36 L of water which was continuously aerated and filtered (Atman filter HF-0400). A regime of 12 h light/12 h dark was applied and the temperature was kept at 22 ± 2 °C. The animals were fed with commercial food (43% carbohydrates, 44% proteins, 13% lipids; 0.07 g per individual), three times a week ad libitum and were starved for one or two days before sampling (Michiels et al., 2013). No changes in the feeding behavior and no mortality occurred under the experimental conditions utilized. The regulations and statements of Ethics Committee CICUAL (OCA 1499/12) FCEyN Universidad Nacional de Mar del Plata were followed.

2.2. Sample procedures

Animals were weighed and anesthetized by cold for about 25 min. For glucose and osmolality determination a sample of hemolymph was taken to be used as described below. The hepatopancreas, chelae muscles, anterior (1–5) and posterior (6–8) gills were at once cut out and gently dried on a paper towel and weighed. Wet mass was measured to the nearest 0.01 g. Immediately after weighing, the hepatopancreas was homogenized in Tris/HCl buffer (0.1 M, pH 7.4; 4 mL g tissue^{-1}) (CAT homogenizer 9120, tool T10) and centrifuged at 10,000 × *g* for 15 min at 4 °C. A sample of the homogenate was kept for posterior determination of glycogen content. The supernatant was fractionated and stored at − 20 °C for further enzymatic assays. Glycerol (1.3% v v^{-1}) was added to supernatant samples before freezing (Ljungström et al., 1984).The chela muscle and anterior and posterior gills were mixed with homogenizing medium (0.25 M sucrose/0.5 mM EGTA-Tris, pH 7.4) (8 mL or 4 mL g tissue^{-1}, respectively) and homogenized on ice with homogenizer as described for hepatopancreas (chela muscle) or in a motor-driven hand-operated Teflon-glass homogenizer (Potter–Elvehjem, 1700 rpm) (anterior and posterior gills), the homogenates were fractionated and stored at − 20 °C until use.

2.3. Hemolymph osmolality

Hemolymph was sampled from the intrabranchial sinus at the base of the cheliped with a syringe flushed with an anticoagulant (sodium citrate buffer, 10% w v^{-1} pH 7.4) and put in an iced centrifuge tube to separate plasma (2,000 × *g* during 3 min at 0 °C) (IEC-Centra 7R). A cryoscopic osmometer (Osmomat 030, Gonotec) was used to determine the osmolality (mOsm kg^{-1}) of the hemolymph and external medium. The values are given as hemolymph osmolality (measurement of solutes concentration) defined as the number of osmoles (Osm) of solutes per kilogram of solvent and as osmoregulatory capacity which is calculated as the difference between the value of osmolality of the hemolymph and that of the medium (Lignot et al., 2000; Charmantier and Anger, 2011). Osmoregulatory capacity is a common parameter to analyze the osmoregulatory performance at a given salinity (Lignot et al., 2000; Charmantier and Anger, 2011).

2.4. Biochemical assays

2.4.1. Amylase activity

Amylase activity in hepatopancreas was measured according to Biesiot and Capuzzo (1990) using starch (15 mg mL^{-1}) as substrate, as we previously detailed (Asaro et al., 2011). Briefly, the sample was incubated for 15 min at 30 °C in the presence of starch in 50 mM phosphate buffer (pH 5.2), 1.5 mL of dinitrosalicylic acid reagent (Miller, 1959) was added for further incubation for 10 min at 100 °C. After cooling, the released maltose was assessed reading absorbance at 540 nm (ZL5000 PLUS, Zeltec). To determine the effect of varying pH, temperature and starch concentration, the activity was assayed at varying pH (5.2–7.0) (50 mM phosphate buffer), temperature (4–45 °C) and starch concentration (0.03–17.97 mg mL^{-1}) in the reaction mixture. Individuals acclimated to 35 psu were used in these experiments.

2.4.2. Maltase and sucrase activity

Maltase and sucrase activity in hepatopancreas were determined measuring the glucose released from the specific substrate as we detailed (Asaro et al., 2011). Briefly, the sample was incubated during 10 min at 37 °C with 42 mM of maltose or sucrose in 0.1 M maleate-NaOH buffer (pH 5.2). The reaction was arrested with 1.5 mL of a glycemia kit (glucose oxidase 10 kU L; peroxidase 1 kU; l,4-aminophenazone 0.5 mmol L^{-1}; phosphates pH 7.0100 mmol L^{-1},

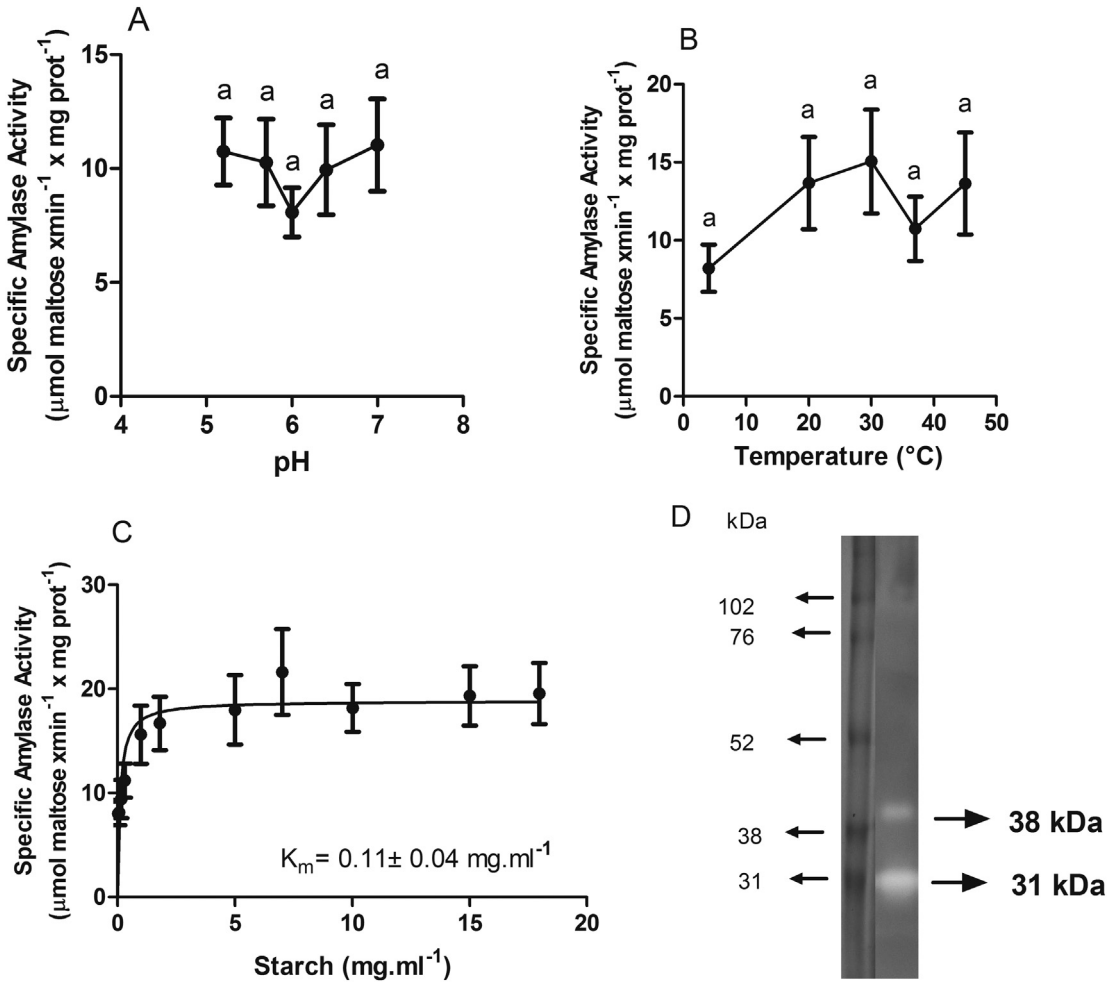

Fig. 1. Effect of pH, temperature and starch concentration on amylase activity in hepatopancreas of *C. angulatus* submitted to 35 psu. (A) Effect of pH (5.2–7.0). The activity was measured at 30 °C and in the presence of 15 mg mL^{-1} of starch (B) Effect of temperature (4–45 °C). The activity was measured at pH 5.2 and in the presence of 15 mg mL^{-1} starch. (C) Effect of starch concentration (0.03–17.97 mg mL^{-1}). The activity was measured at 30 °C and at pH 5.2. The curves are the ones which best fit the experimental data (GraphPad Prism 5.01). Data are the mean ± S.E. for five individuals. Different letters indicate significant differences (One-way ANOVA, $p < 0.05$). (D) Zymogram of amylolytic activity from hepatopancreas extract of *C. angulatus* acclimated to 35 psu. The right arrows indicate the bands of 31 and 38 kDa.

hydroxybenzoate12 mmol L^{-1}) (Wiener Lab Glicemia AA) and further incubated during 5 min at 37 °C. Glucose amount was quantified reading absorbance at 505 nm of the colored quinone complex. The effect of pH, temperature and substrate concentration disaccharidases activity was studied measuring the activity at various pH (3.5–8.3) (0.1 mM maleate buffer), temperatures (4–45 °C) and substrate concentration (0.56–42 mM) in the assay mixture. Individuals acclimated to 35 psu were used in these experiments.

2.4.3. Glycogen and free glucose content

Glycogen content in hepatopancreas, muscle, anterior gills and posterior gills was measured as glucose equivalent according to Pinoni et al. (2011). Free glucose was assessed from assay without α-amyloglucosidase. Released glucose from glycogen hydrolysis was calculated as the difference between the values obtained with and without α-amyloglucosidase.

2.4.4. Plasma glucose

Plasma glucose was determined at once after hemolymph sampling and centrifugation using glycemia kit as we previously described (Pinoni et al., 2011).

2.4.5. Protein concentration

The protein concentration in hepatopancreas was assayed according to Bradford (1976), using bovine serum albumin as standard.

2.5. Zymogram analysis of amylolytic activity in the hepatopancreas

Zymogram analysis (Substrate-SDS-PAGE) for amylolytic activity was carried out in the corresponding samples as described by Perera et al. (2008a), with modifications. The samples were not boiled or treated with mercaptoethanol before electrophoresis on 10% polyacrylamide gels. The gels were run at 15 mA and 4 °C in a vertical electrophoresis equipment (Hoeffer SE260, 8 × 10 × 0.75 cm). After electrophoresis, the gels were put into a starch solution (1%) at pH 6.0 for 90 min. After incubation, staining was carried out with iodine/KI solution (10 mM). Molecular weight markers (MWM) (12–250 kDa, Amersham full-range rainbow), in the absence of reducing agent, were utilized for apparent MW determination.

2.6. Statistical analysis

The statistic program Sigma-Stat 3.0 statistical package was used for all tests carried out. This program automatically performs a previous test for equal variance and normality. The effect of different pH or temperature on amylase, maltase and sucrase activities were analyzed using One Way ANOVA. A posteriori ANOVA (the Holm–Sidak) was used to identify differences. The effect of varying concentrations of

starch, maltose and sucrose were analyzed by means of non-linear regression analysis and Km values (Michaelis-Menten constant) were estimated by analysis of these curves by GraphPad Prism 5.01 software. $p < 0.05$ was considered to be significant. The difference between hemolymph and external osmolality (water of the aquarium) was analyzed using the parametric (*t*-test) or non-parametric test (Mann-Whitney). The difference between the osmoregulatory capacity between 6 and 10 psu was analyzed with the nonparametric test (Mann-Whitney). The differences in the activities of amylase, maltase and sucrase in hepatopancreas, glucose concentration in hemolymph, glycogen concentration or free glucose in different organs in relation to environmental salinity were analyzed using the parametric test (One Way ANOVA) or non-parametric (Kruskal-Wallis). A posteriori ANOVA or Kruskal–Wallis test (the Holm–Sidak or the Dunn's method, respectively) was used to identify differences (Zar, 2010).

3. Results

3.1. Carbohydrases activities in hepatopancreas: biochemical characteristics

Amylase activity in hepatopancreas was determined within the ranges of pH 5.2–7.0 and of temperature 4–45 °C (Fig. 1A and B). Amylase activity was similar within the ranges of pH and temperature studied ($F = 0.45$, $p = 0.77$; $F = 1.025$, $p = 0.48$, respectively). The Fig. 1C illustrates the effect of starch concentrations on amylase activity. The results showed that amylase activity of *C. angulatus* presented a Michaelis-Menten kinetics ($Km = 0.11 \pm 0.04$ mg mL^{-1}). The zymogram analysis showed that hepatopancreas of crabs acclimated to 35 psu exhibited two bands with amylolytic activity with molecular mass of about 38 and 31 kDa. The band of 31 kDa appeared to exhibit the highest activity (Fig. 1D).

Maltase activity in hepatopancreas was similar within the ranges of pH (3.5–8.3) and temperature (4–45 °C) ($F = 0.50$, $p = 0.77$; $F = 2.95$, $p = 0.055$, respectively) (Fig. 2A and B). The effect of maltose concentrations on maltase activity of hepatopancreas of *C. angulatus* is shown in Fig. 2C. Maltase activity showed Michaelis-Menten kinetics ($Km = 8.08 \pm 3.96$ mM).

Sucrase activity in hepatopancreas was assayed in the range of pH 3.5–8.3 (Fig. 3A). Sucrase activity was similar at pH 3.5–5.2. At pH 6.0 sucrase activity decreased, being about 44% lower than the activity at pH 5.2. At pH 6.4 and 6.8 the activity was similar to that found at pH 5.2. At pH 8.3, the activity was about 47% of the activity at pH 5.2 ($F = 3.26$, $p = 0.022$). Sucrase activity was maintained over a wide range of temperature (4–45 °C) ($F = 0.18$, $p = 0.94$) (Fig. 3B). The effect of sucrose concentrations on sucrase activity of hepatopancreas of *C. angulatus* is shown in Fig. 3C. Sucrase activity showed Michaelis-Menten kinetics ($Km = 14.26 \pm 5.81$ mM).

3.2. Hemolymph osmolality

Crabs exposed to low salinity (6 and 10 psu), exhibited values of hemolymph osmolality higher (around four and twofold) compared to the osmolality of the external medium ($T = 57$ $p = 0.002$; $T = 21$ $p < 0.001$), respectively. In 35 psu, hemolymph osmolality was not different from external medium while it was about 12% lower in 40 psu ($t = 4$, 19 $p = 0.0021$) (Fig. 4A). In 6 psu the difference between hemolymph and external medium osmolality values was higher compared to that in 10 psu ($T = 62$, $p = 0.029$) (Fig. 4B).

3.3. Carbohydrases activity in the hepatopancreas, plasma glucose and glycogen and free glucose content in storage tissues at different salinities

No significant differences between carbohydrases activity of individuals of *C. angulatus* acclimated to different environmental salinities (6, 10, 35 and 40 psu, $p > 0.05$) were found (Fig. 5A, C and D).

The zymogram analysis of amylolytic activity in hepatopancreas of crabs acclimated to 6, 10 and 40 psu showed a similar pattern to that found for individuals acclimated to 35 psu (Fig. 5B).

The concentration of glucose (mg l^{-1}) in the hemolymph of crabs maintained at 6, 10 and 40 psu was similar to 35 psu (6 psu: 55.95 ± 7.65; 10 psu: 54.63 ± 8.61, 35 psu: 77.04 ± 19.25, 40 psu: 69.98 ± 20.36) ($p > 0.05$). The glycogen content in hepatopancreas, muscle and posterior gills of crabs maintained at low and high salinity was similar to the corresponding values in 35 psu ($p > 0.05$).

The glycogen content in anterior gills in crabs acclimated in 6 psu and 40 psu was lower (55 and 70%, respectively) than in 35 psu ($F = 7.98$, $p = 0.001$). There were no significant differences between 10 and 35 psu in the glycogen concentration in anterior gills ($p > 0.05$) (Fig. 6A). Free glucose concentration in chela muscle was higher in both 6 psu and 40 psu (about three and two fold, respectively) than in 35 psu ($H = 8.45$, $p = 0.037$). Free glucose concentration in hepatopancreas, anterior and posterior gills was similar in all salinities tested ($p > 0.05$) (Fig. 6B).

4. Discussion

Our results show the occurrence of amylase, maltase and sucrase in hepatopancreas of the intertidal crab *C. angulatus* (Dana, 1852) which suggest the ability to perform complete starch degradation and the potential utilization of diverse types of carbohydrates (i.e. starch, glycogen, disaccharides) as glucose sources. Previous work of our lab showed the presence of proteases and lipase activities in

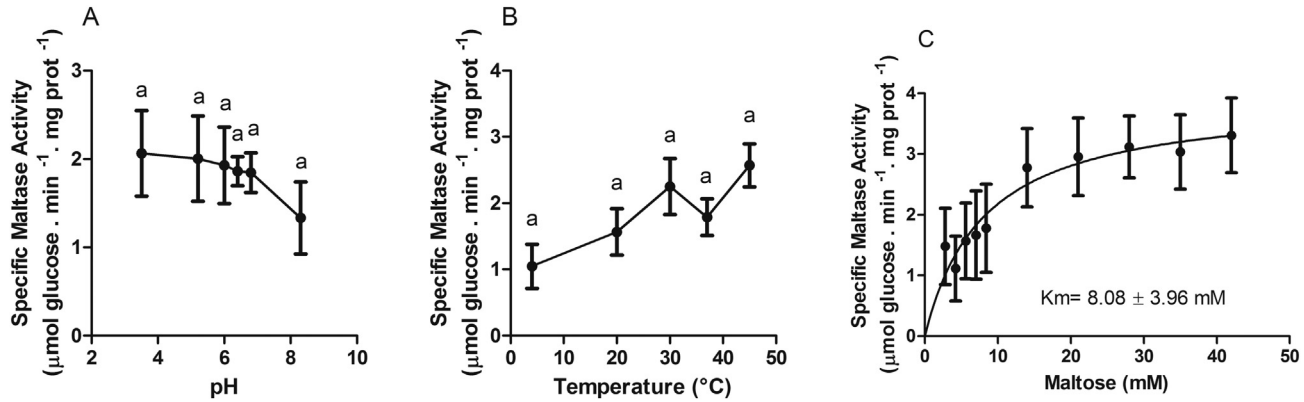

Fig. 2. Effect of pH, temperature and maltose concentration on maltase activity in hepatopancreas of *C. angulatus* submitted to 35 psu. (A) Effect of pH (3.5–8.3). The activity was measured at 30 °C and in the presence of 28 mM maltose. (B) Effect of temperature (4–45 °C). The activity was measured at pH 5.2 and in the presence of 28 mM maltose. (C) Effect of maltose concentrations (0.56–42 mM). The activity was measured at 30 °C and at pH 5.2. The curves are the ones which best fit the experimental data (GraphPad Prism 5.01). Data are the mean ± S.E. for five individuals. Different letters indicate significant differences (One-way ANOVA, $p < 0.05$).

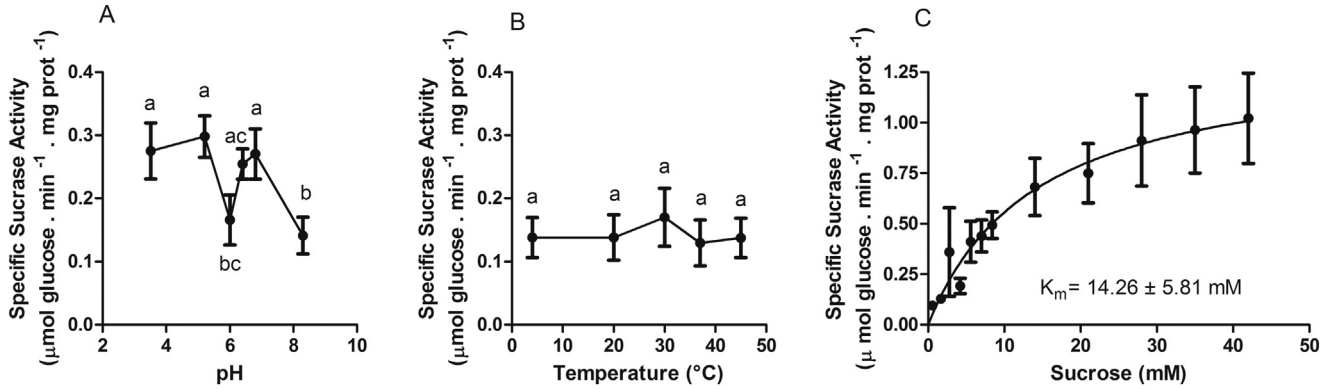

Fig. 3. Effect of pH, temperature and sucrose concentration on sacarase activity in hepatopancreas of *C. angulatus* submitted to 35 psu. (A) Effect of pH (3.5–8.3). The activity was measured at 30 °C and in the presence of 28 mM sucrose. (B) Effect of temperature (4–45 °C). The activity was measured at pH 5.2 and in the presence of 28 mM sucorse. (C) Effect of sucrose concentrations (0.56–42 mM). The activity was measured at 30 °C and at pH 5.2. The curves are the ones which best fit the experimental data (GraphPad Prism 5.01). Data are the mean ± S.E. for five individuals. Different letters indicate significant differences (One-way ANOVA, $p < 0.05$).

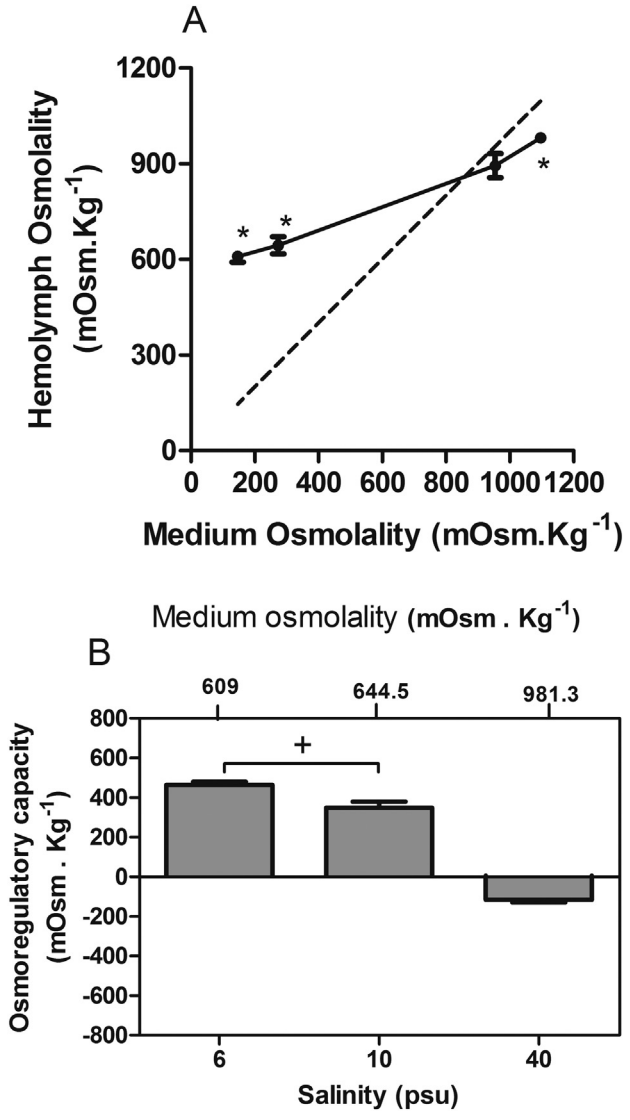

Fig. 4. (A) Hemolymph osmolality of individuals of *C. angulatus* acclimated to 6 (609 mOsm kg^{-1}), 10 (644 mOsm kg^{-1}), 35 (895 mOsm kg^{-1}) and 40 (981 mOsm kg^{-1}) psu. Dashed line: isosmotic line. Isosmotic point: 850 mOsm kg^{-1} (GraphPad Prism 5.01). * Significantly different from the medium osmolality (Mann Whitney test, $p < 0.05$). (B) Osmoregulatory capacity (difference between hemolymph and medium osmolality); + significantly different (*t*-test, $p < 0.05$). Data are the mean ± S.E. for six-eight individuals.

hepatopancreas of *C. angulatus* pointing out its ability to also perform lipid and protein degradation and potentially use them as an energy sources (Michiels et al., 2013, 2017). The occurrence of various specific digestive enzyme activities in the hepatopancreas is in agreement with the predator-omnivorous-carrion behavior of this crab in Mar Chiquita coastal lagoon (Spivak, 1997; Botto et al., 2005). The maintenance of amylase activity over a wide range of pH is similar to that we described in the euryhaline crab *Neohelice granulata* (Asaro et al., 2011) and to that found in *Scylla serrata* (Pavasovic et al., 2004) but quite different from amylase activity of other decapod crustaceans (Johnston, 2003; Perera et al., 2008a; Figueiredo and Anderson, 2009; Coccia et al., 2011; Castro et al., 2012). The hepatopancreas of decapod crustaceans is the main site of digestive enzyme synthesis and where intracellular digestion is started and carried out (Zeng et al., 2010; Ribeiro et al., 2014; Wang et al., 2014). When the internal reserves of a cell have to be mobilized, digestive enzymes would be intracellularly activated (Sánchez-Paz et al., 2006). Previous work of our lab supports the idea of a modulation of intracellular digestive enzymes activity in hepatopancreas of *C. angulatus* and *N. granulata* (Michiels et al., 2015a, 2015b, 2017). The conservation of amylase activity over a broad values of pH could support a high hydrolytic capacity intracellularly and/or after secretion from the hepatopancreas. The maintenance of activity at various temperatures is similar to that found in various decapod crustaceans (Pavasovic et al., 2004; Perera et al., 2008a; Coccia et al., 2011; Castro et al., 2012). Strikingly, amylase activity appeared to be high at low and high temperature. In Mar Chiquita coastal lagoon, *C. angulatus* is often exposed to a wide range of temperatures. Further experimental work is needed to establish whether an extreme tolerant temperature amylase in the hepatopancreas could be related to thermal acclimatization (i.e. maintenance of carbohydrate degradation to sustain energy supply) as we suggested for lipase activity (Michiels et al., 2013). The Michaelis–Menten behavior of amylase activity is similar to that described in the crayfish *Procambarus clarki* (Hammer et al., 2003) and the euryhaline crab *N. granulata* (Asaro et al., 2011). Zymogram analysis is a tool commonly used for the identification of active amylase forms in hepatopancreas of decapod crustaceans. A great interspecific variability occurs since up to 10 active amylase bands has been reported for some species while others appear to exhibit one form (Van Wormhoudt et al., 1995; Le Moullac et al., 1997; Van Wormhoudt and Sellos, 2003; Dutta et al., 2006; Perera et al., 2008a, 2008b; Castro et al., 2012; Rodríguez-Viera et al., 2016). The presence of two forms of amylase in the hepatopancreas of *C. angulatus* (with the band of 31 kDa with the highest activity) is similar to that found in *C. maenas* (Van Wormhoudt et al., 1995) but it is quite different from that we have recently found in the hepatopancreas of *N. granulata* in which at least five bands with amylolytic activity were detected (Asaro, 2016; Asaro et al., 2017).

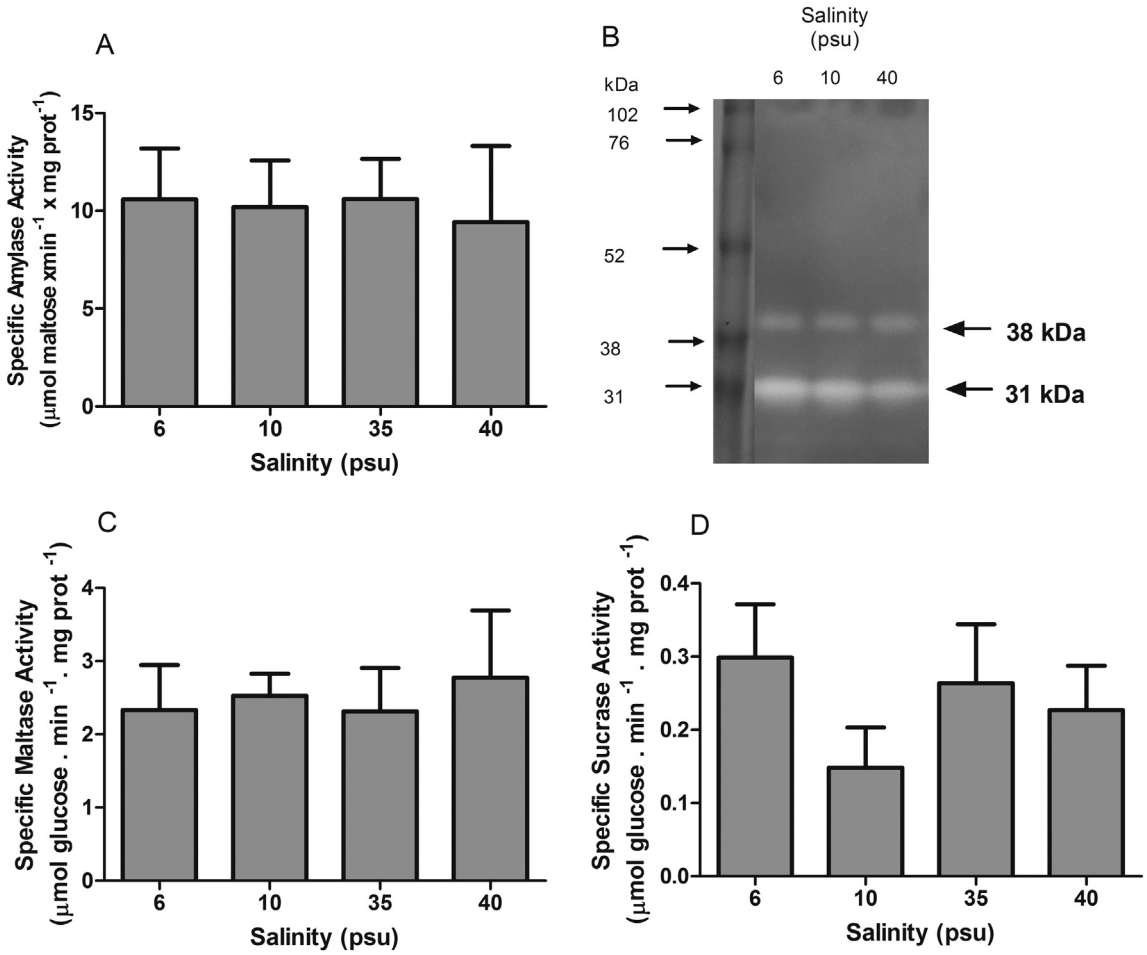

Fig. 5. (A) Amylase activity (B) Zymogram of amylolytic activity. The right arrows indicate the bands of 31 and 38 kDa. (C) Maltase and (D) Sucrase activities in hepatopancreas of individuals of *C. angulatus* acclimated to different salinities (6–40 psu). Data are the mean ± S.E. for four-seven crabs.

Similarly to amylase, maltase activity in hepatopancreas of *C. angulatus* was maintained over a broad range of pH and temperature and exhibited Michaelis-Menten kinetics. These biochemical characteristics of maltase activity detected in hepatopancreas of *C. angulatus* are in agreement with that we found in *N. granulata* (Asaro et al., 2011). Unlike *C. angulatus*, maltase activity in other decapod crustaceans is optimal within a narrow pH range (Figueiredo et al., 2001; Figueiredo and Anderson, 2009). The response of maltase activity to temperature further supports the maintenance of capability for digestion of key glycogenic substrates under low and high temperatures as we pointed out above for amylase activity. Concerning sucrase activity, to our knowledge, our work in *N. granulata* is the only report available about the existence and biochemical features of this activity in a euryhaline crab (Asaro et al., 2011). In fact, reports on sucrase activity in other crustaceans are also scarce (Saxena and Murthy, 1982). Similarly to *N. granulata*, sucrase activity in *C. angulatus* was maintained over a wide range of pHs and temperature and also exhibit Michaelis–Menten kinetics (Asaro et al., 2011).

C. angulatus exhibited hyper/hypo-regulatory ability since hemolymph osmolality values were higher and lower than those of the corresponding external medium when exposed to low salinities (6–10 psu) and high salinity (40 psu), respectively, while behave as osmoconformer in 35 psu (Michiels et al., 2013; this work). The comparison of osmoregulatory capacity of individuals exposed to various salinities allows to examine the existence of distinct performance in response to different degree and/or type of osmotic challenge. The higher osmoregulatory capacity value of *C. angulatus* in 6 psu compared to 10 psu indicates a greater hypo-osmoregulatory performance in relation to the degree of hypo-osmotic challenge. Compensation in response to different environmental salinities is usually, an energy-demanding process. Changes at molecular and biochemical levels such as those in enzymes and system transports components of the branchial osmoregulatory machine during biochemical adaptation to salinity often requires the mobilization of energy substrates to metabolically deal with salinity challenge (Pinoni et al., 2013; Larsen et al., 2014). This could lead to changes in carbohydrate, lipids, and/or protein metabolism and then to a differential utilization of energy reserves in various storage organs (Pinoni et al., 2013, 2017). The maintenance of glycemia in *C. angulatus* suggest that availability of glucose from the hemolymph would not be a constraint upon acclimation to different environmental salinities in this crab. As we pointed out above, a major part of hemolymphatic glucose comes from the digestion of glycogenic substrates and the absorption of glucose in the hepatopancreas (Obi et al., 2011; Wang et al., 2016). The maintenance of carbohydrases activity in the hepatopancreas suggests the conservation of the capacity for hydrolysis of glycogenic substrates in low and high salinities. Previously, we found that amylase activity was increased in low salinity in *N. granulata* (Asaro et al., 2011), therefore suggesting interspecific differences in digestive adjustments at the biochemical level in hepatopancreas. Whether this is due to the existence of different biochemical and/or regulatory pathways in both species is unknown. Since unlike to that we found for lipase and proteolytic activities (Michiels et al., 2013, 2017), amylase and disaccharidases activities in hepatopancreas of *C. angulatus* were not affected, differential and specific mechanisms of modulation of digestive enzymes appear to occur upon acclimation to different environmental salinities. Results of our lab illustrate that this is also the

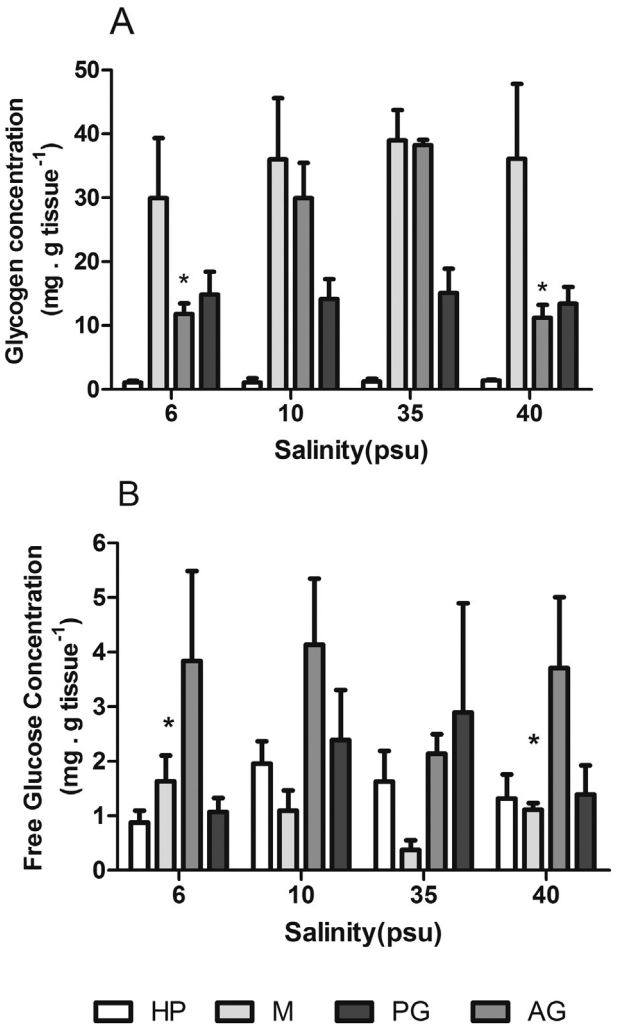

Fig. 6. (A) Glycogen (B) and free glucose concentration in hepatopancreas (HP), muscle (M), anterior gills (AG) and posterior gills (PG) of individuals of *C. angulatus* acclimated to different salinities (6–40 psu). Data are the mean ± SE for five-eight crabs. * Significantly different from the values of glycogen or free glucose in the same tissue of individuals acclimated to 35 psu (One-way ANOVA, $p < 0.05$).

case for *N. granulata* (Pinoni et al., 2013, 2015; Michiels et al., 2015a). The differences in the number of amylase forms in the hepatopancreas of various crustaceans were associated with changes in external and/or internal factors, although these responses exhibit great interspecific variability (Borowsky, 1984; Le Moullac et al., 1997; Perera et al., 2008b; Castro et al., 2012). The maintenance of the number and/or pattern of amylase forms in the hepatopancreas of *C. angulatus* is in accordance to that found for total amylase activity detected by biochemical analysis. This points out that amylase in the hepatopancreas would not be neither modulated at the molecular level under low or high salinity. Recent studies in our lab show that under exposure to 10 psu *N. granulata* exhibits an extra amylase form which further supports the occurrence of interspecific variability in amylase responses in hepatopancreas to low salinity in intertidal euryhaline crabs (Asaro, 2016; Asaro et al., 2017). Besides to be transported to the hemolymph, glucose resulting from the digestion of polysaccharides in the hepatopancreas of decapod crustaceans can also be stored as glycogen in this organ (Obi et al., 2011; Wang et al., 2016). The hepatopancreas of *C. angulatus* exhibited low glycogen content while free glucose levels were high. This suggests a role of this organ in carbohydrates metabolism such as keeping a suitable and constant glucose supply. Glucose could also be synthesized by gluconeogenic pathways since various decapod crustaceans appear to have key enzymes of this biochemical route in the hepatopancreas (Wang et al., 2016). Anterior gills have an important role in the metabolism of carbohydrates in various euryhaline crabs in which are sites of glycogen storage and/or utilization (Martins et al., 2011; Pinoni et al., 2013, 2017). The minor glycogen content in anterior gills of *C. angulatus* acclimated to 6 and 40 psu suggests the use of carbohydrate reserves in relation to both hypo- and hyper-regulation. Since no changes occurred in 10 psu, utilization of glycogen reserves in anterior gills of *C. angulatus* under hyper-regulatory conditions would depend on the degree of hypo-osmotic challenge. As we discussed above, *C. angulatus* exhibited higher osmoregulatory capacity in 6 psu when compared to 10 psu. We previously showed that anterior gills are involved in biochemical adaptation to low salinity in this crab. Different adjustments at the biochemical occur in anterior gills depending on the degree of hypo-osmotic stress (López Mañanes et al., 2002; Elhalem, 2003; Elhalem and López Mañanes, 2003, 2004). Further experimental work is needed to establish whether the lower glycogen content in anterior gills in 6 and 40 psu (while free glucose was maintained) is related to an immediate intracellular utilization of glucose product of glycogen hydrolysis to sustain energy-demanding osmoregulatory mechanisms. The chela muscle is a major site of glycogen storage in *C. angulatus* but since no changes in glycogen content occurred, mobilization of this reserve appeared not to occur upon acclimation to low or high salinity. This is in agreement to that we previously showed for *N. granulata* (Pinoni et al., 2013). However, the enhanced free glucose content in chela muscle in 6 and 40 psu suggests the occurrence of adjustments in carbohydrate pathways in chela muscle (i.e. up regulation of gluconeogenic pathways and/or of the uptake of glucose) underlying hyper- and hypo-osmoregulation. Similarly to anterior gills, the fact that free glucose content in chela muscle was affected in 6 psu but not in 10 psu further supports the idea of the occurrence of differential responses at the biochemical level depending on the extent of hypo-osmotic stress.

5. Conclusions

The existence and biochemical features of amylase, maltase and sucrase activities in the hepatopancreas of *C. angulatus* suggests the ability to perform complete hydrolysis of key glycogenic substrates of this crab. The maintenance of carbohydrase activities in the hepatopancreas and glycemia, the high free content of glucose in hepatopancreas and the differential adjustments in glycogen and free glucose content in anterior gills and chela muscle supports the idea of the ability to sustain glucose homeostasis in response to exposure to low and high salinity. Our results increase the knowledge on the differential and specific role of various digestive enzymes and energy storages in different organs in biochemical adaptation to low and high salinity of intertidal euryhaline hyper-hyporegulators crabs. Since biochemical routes of carbohydrate metabolism of *C. angulatus* are unknown, future studies focused in regulation pathways of the various components involved, will provide a better understanding of the mechanisms underlying the maintenance of glucose homeostasis upon different challenges.

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