

Research article

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## The influence of habitat on metabolic and digestive parameters in an intertidal crab from a SW Atlantic coastal lagoon

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**Abstract.** The hepatopancreas of decapod crustaceans is an organ which can act as indicator for digestive and metabolic parameters under different physiological and/or environmental conditions. However, biochemical studies on digestive and metabolic parameters of the hepatopancreas of euryhaline burrowing crabs such as *Neohelice granulata* from habitats with different diet compositions are still scarce. In the wild, adult males of *N. granulata* from Mar Chiquita Coastal Lagoon (Argentina) in mudflat habitat have diets with higher lipid and protein content than crabs from the saltmarsh, suggesting that diets could be an important factor influencing hepatopancreas activities. We tested this hypothesis here by exposing adult male crabs to a similar experimental diet and comparing hepatopancreas parameters for lipid components and protein metabolism between males from these two habitat types at different times (up to three months). At month 3, we noticed a decrease of the triglyceride concentration and lipase activity and an increase of protein concentration in crabs from the mudflat. In contrast, triglycerides and protein concentration did not change in crabs from the saltmarsh, while lipase activity decreased and levamisole insensitive AP increased at month 3. The results indicate that digestive and metabolic parameters in the hepatopancreas of crabs from habitats varying in diet content remain different, even if crabs are subsequently fed by a similar experimental diet. This suggests that specific intrinsic regulations of these hepatopancreas parameters could operate differently in each habitat and could not be changed by recent diet conditions.

**Keywords.** Intertidal crabs, hepatopancreas, energy reserves, lipase, alkaline phosphatase.

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### Introduction

The hepatopancreas of decapod crustaceans is a sensitive indicator organ for digestive and metabolic adjustments to distinct physiological and/or environmental conditions (BELGRAD & GRIFFEN 2016; PINONI *et al.* 2018; LÓPEZ MAÑANES *et al.* 2020). In various intertidal burrowing crabs, the modulation of specific digestive enzymes and/or energy reserves in the hepatopancreas is part of the responses involved in biochemical adaptation to key environmental challenges such as salinity and food availability

(MICHIELS *et al.* 2017; PINONI *et al.* 2018; LÓPEZ MAÑANES *et al.* 2020). In intertidal crabs of the same species successfully inhabiting different habitats in a same intertidal area (e.g., mudflat or saltmarsh) of coastal lagoons, the distinct and particular environmental conditions in each habitat may impose distinct challenges leading to intraspecific differences in responses, for example, at the biochemical level (PINONI *et al.* 2011; MUELLER 2017; LÓPEZ MAÑANES *et al.* 2020). This could imply the presence of different intrinsic regulatory mechanisms in crabs coming from different habitats that persist even when they are subsequently exposed to the same laboratory conditions (PINONI *et al.* 2013; LÓPEZ MAÑANES *et al.* 2020). This could indeed be the case for adult males of *Neohelice granulata* from mudflat and saltmarsh habitats of Mar Chiquita Coastal Lagoon (Buenos Aires Province, Argentina) which show intraspecific differences in lipid and protein metabolism components in the hepatopancreas under exposure to identical osmotic conditions (PINONI *et al.* 2013; LÓPEZ MAÑANES *et al.* 2020), but there are currently no other studies of this kind on the influence of key environmental parameters on the biochemistry of metabolic and digestive adjustments in intertidal crabs.

*Neohelice granulata* (Dana, 1851) (phylum Arthropoda, subphylum Crustacea, class Malacostraca) is a euryhaline burrowing crab that is found in intertidal areas of the southwestern Atlantic from southern Brazil to northern Argentinean Patagonia where it plays a key ecological role (LUPPI *et al.* 2013; ANGELETTI & CERVELLINI 2015; SPIVAK 2020). The same population of this crab can inhabit different habitats such as mudflats or saltmarshes (LUPPI *et al.* 2013; NUÑEZ *et al.* 2020). The modulation of key enzymes in the hepatopancreas such as lipase or levamisole-insensitive and -sensitive alkaline phosphatase (AP) is one of the possible responses at the biochemical level to external and internal factors in *N. granulata* (PINONI 2009; PINONI *et al.* 2015; MICHIELS *et al.* 2015a; LÓPEZ MAÑANES *et al.* 2020). In Mar Chiquita Coastal Lagoon, adult males of *N. granulata* occupy both the mudflat and the saltmarsh habitats (LUPPI *et al.* 2013). In the mudflat, *N. granulata* consumes more items of animal origin, thus sustaining a richer diet in lipids and proteins compared to crabs in the saltmarsh. In the saltmarsh, crabs consume more plant items, mainly *Spartina densiflora*, the main cordgrass in this habitat (PINONI *et al.* 2011, 2013; LUPPI *et al.* 2013; LANCIA *et al.* 2014). In autumn, adult males of *N. granulata* stay for up to 3 months in burrows of both mudflat and saltmarsh, where they feed, which leads to between-habitat differences in digestive and metabolic characteristics (e.g., levels and/or modulation of energy reserves and key enzymes in hepatopancreas) (PINONI *et al.* 2011; LÓPEZ MAÑANES *et al.* 2020). Varying triglyceride and protein concentrations in the hepatopancreas are part of biochemical adaptations to each habitat type (PINONI *et al.* 2011; LÓPEZ MAÑANES *et al.* 2020). Therefore, adult males of *N. granulata* provide a good model to compare the effect of diet on digestive and metabolic components at the biochemical level between habitats. We propose that diet should differently affect the content of energy reserves and key enzymes in the hepatopancreas of crabs from the mudflat and the saltmarsh, likely due to the particular digestive and metabolic characteristics of crabs in each habitat. (PINONI *et al.* 2011; LÓPEZ MAÑANES *et al.* 2020). The aim of our study was to test whether a similar diet under controlled conditions influences key components of lipid and protein metabolism in the hepatopancreas of adult males of crabs from mudflat and the saltmarsh habitats differently. For this purpose, we determined the concentration of triglycerides and protein reserves and lipase and levamisole-sensitive and -insensitive AP activity in the hepatopancreas along with the hepatosomatic index (liver weight as a percentage of the whole body weight) as parameters for physical condition (ALBANESI *et al.* 2020) in crabs from both the mudflat and the saltmarsh of Mar Chiquita Coastal Lagoon immediately after capture and after one and three months in the lab, respectively.

## Material and methods

### Animal collection and maintenance

Adult male crabs in intermolt (n=80) (carapace width greater than 2.5 cm) were live trapped in autumn (May 2013) from burrows of the open mudflat and saltmarsh habitats of the Argentinian Mar Chiquita

Coastal Lagoon (37°32'–37°45' S, 57°19'–57°26' W) (PINONI *et al.* 2011, 2013) (Fig. 1). Variations in key environmental conditions such as temperature (T) and salinity (S) are wider in burrows from the mudflat than in the saltmarsh (mudflat:  $\Delta T^{\circ}\text{C}$  10.8,  $\Delta\text{S}$ : 17.2; saltmarsh  $\Delta$ :  $T^{\circ}\text{C}$  5.8,  $\Delta\text{S}$ : 10.5) (LUPPI *et al.* 2013). The chosen sampling time of the crabs and the experimental period are based on the fact that the movement of adult male crabs between the two habitat types is highly reduced during autumn as male crabs remain up to 3 months inside their burrows (LUPPI *et al.* 2013; personal observations). Captured animals were transported to the laboratory in the water of the collection site under continuous aeration which took about 30 minutes. One group of crabs from both the mudflat and the saltmarsh each was immediately investigated with biochemical assays without any acclimation at time zero ( $t_0$ ). Other groups of crabs from each habitat type were maintained in separate aquaria (2 aquaria with 30 individuals per aquarium) under controlled conditions: 35 psu; salinity at which adult males from both sites from Mar Chiquita Coastal Lagoon exhibit the same hemolymph osmotic concentration as that of the external medium; therefore they are osmo-conform (PINONI *et al.* 2013); 12-h light/12-h dark;  $20 \pm 2^{\circ}\text{C}$ , and pH 7.4. 36 L of water were continuously aerated, filtered with Atman filters (HF-0400) and partially changed every second day as previously described (PINONI *et al.* 2013; MICHIELS *et al.*

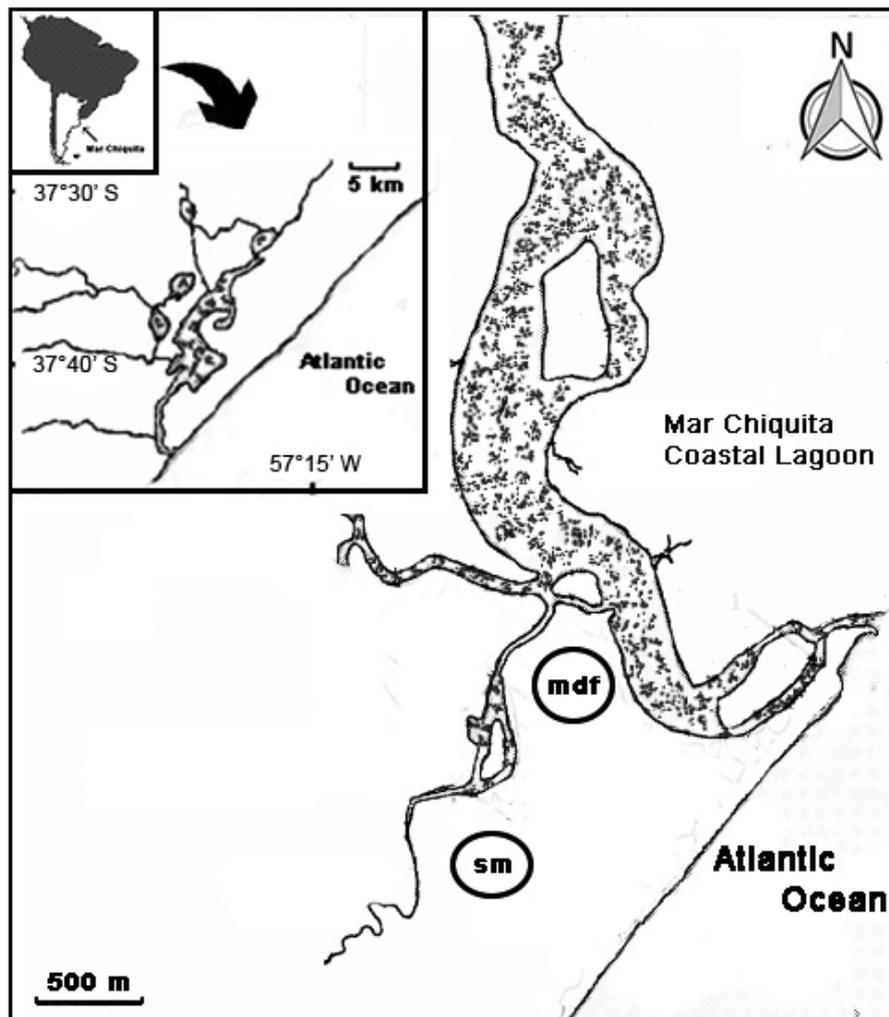


Fig. 1 – Map of the Argentinian Mar Chiquita Coastal Lagoon with exact position of the sampling sites and the two habitat types, mudflat (mdf) and saltmarsh (sm). Inset: geographical position of the Mar Chiquita Coastal Lagoon.

2015a). Crabs were fed three times a week with commercial fish food from TetraPond (containing 62% carbohydrates; 31% proteins; 5% lipids) throughout the experimental period (up to 3 months); all examined individual were starved 24 h prior to biochemical assays. At months 1 and 3, triglyceride and protein content and digestive enzymes activities were determined in the hepatopancreas as described below. The feeding behavior was monitored as described by MÉNDEZ *et al.* (2019) while crabs were individually fed (0.07 g). Only animals which consumed the total amount of offered food, which took up to 5 min, were used for subsequent analyses (MÉNDEZ *et al.* 2019).

### Preparation of hepatopancreas extracts

The hepatopancreas extracts were prepared as described in literature (PINONI *et al.* 2013; MICHIELS *et al.* 2020). Briefly, crabs were weighed and cold-anaesthetized for about 20 min. The hepatopancreas was homogenized in 50 mM Tris/HCl pH 7.4 (4 ml g tissue<sup>-1</sup>) (CAT homogenizer XI20, tool T10) and centrifuged at 10,000g for 15 min (Sorval, rotor SS34, at 4° C). Before centrifugation, an aliquot of homogenate was removed to determine the triglyceride and protein content. The supernatant was separated into aliquots and glycerol (1.3% v v<sup>-1</sup>) was added before freezing (LJUNGSTRÖM *et al.* 1984) until the samples were later used for enzymatic assays.

### Biochemical assays

Triglycerides (TG) were measured by the colorimetric method of glycerol phosphate oxidase with a commercial kit (TG Wiener Lab AA cod. 861110001) as described (PINONI *et al.* 2013; MICHIELS *et al.* 2015b). Each sample was incubated with chlorophenol, lipoprotein lipase (LPL), glycerol kinase (GK), glycerol phosphate oxidase (GPO), peroxidase (POD), adenosine triphosphate (ATP) and 4-aminophenazone (4-AF) in buffer 'Good' (pH 6,8) (Reactive A of the kit) for 5 min at 37°C. The amount of released glycerol was determined by estimating the absorbance of the colored quinonimine complex at 505 nm (spectrophotometer ZL5000 PLUS, Zeltec).

Protein assays were conducted according to BRADFORD (1976) monitoring the increase in absorption at 595 nm produced by the binding of the dye Coomassie Brilliant Blue G-50 to proteins (spectrophotometer ZL5000 PLUS, Zeltec). Bovine serum albumin was used as protein concentration standard to build the corresponding standard curve and to calculate the protein concentrations of the samples (PINONI *et al.* 2013; MICHIELS *et al.* 2015a, 2017).

All biochemical parameters were always determined from a single sample that had not been defrosted prior to analyses. Previous work in our laboratory showed that the freezing procedure did not alter the estimated activities as values were stable for at least up to eight months of freezing (MÉNDEZ *et al.* 2019; LÓPEZ MAÑANES *et al.* 2020). The assay conditions used had been determined as optimal to measure activity in the hepatopancreas of *N. granulata* (PINONI 2009; MICHIELS *et al.* 2015a, 2015b).

Lipase activity was determined by measuring pNP-palmitate hydrolysis (MARKWEG *et al.* 1995) with some modifications as described by MICHIELS *et al.* (2015a). The reaction was initiated by adding pNP-palmitate (final concentration 0.7 mM) to a reaction mixture containing the corresponding sample in 50 mM Tris–HCl buffer (pH 8.5)/4 µl of Tween 80. Incubation was carried out at 37°C for 3 min. The reaction was stopped by addition 0.5 ml of 0.2 % w v<sup>-1</sup> of trichloroacetic acid (TCA). The amount of released p-nitrophenol (pNP) was determined by absorbance at 410 nm (spectrophotometer ZL5000 PLUS, Zeltec).

Levamisole-insensitive and -sensitive alkaline phosphatases (AP) activities were determined as in PINONI *et al.* (2005) and PINONI & LÓPEZ MAÑANES (2008). AP (EC 3.1.3.1) are membrane-bound

glycoproteins that are widely found in animals which hydrolyse phosphate from a variety of molecules at a range of optimal pH above 7.0 (LALLÈS 2019). Levamisole is an inhibitor used to discriminate between different AP isoforms (CHAN & STINSON 1986; PINONI *et al.* 2005, 2015; MOTA *et al.* 2008; DIEZ-ZAERA *et al.* 2011; LALLÈS 2019). Levamisole-insensitive AP activity was determined by measuring pNPP hydrolysis in a reaction medium containing 4 mM MgSO<sub>4</sub> in 0.1 M Tris-HCl buffer (pH 7.7) 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 4mM MgSO<sub>4</sub> in 100 mM Tris-HCl buffer (pH 8.5) in the absence (total AP activity) and in the presence of 16 mM levamisole. The reaction was initiated by the addition of pNPP (final concentration 9.5 mM) and incubation was carried out at 37°C for 30 min. The reaction was stopped by adding 2 mL of 0.1 M KOH. The amount of released pNP was determined by absorbance at 410 nm (spectrophotometer ZL5000 PLUS, Zeltec).

### Hepatosomatic index determination

To determine the hepatosomatic index (HSI), each crab and also its hepatopancreas was weighed. The hepatosomatic index (HSI,%)=hepatopancreatic weight/(body weight - hepatopancreatic weight) \* 100 was calculated as in ALBANESI *et al.* (2020) and DA SILVA VIANNA *et al.* (2020). This index is commonly used as parameter for the maintenance of physical conditions (ALBANESI *et al.* 2020; DA SILVA VIANNA *et al.* 2020; LEÃO *et al.* 2021).

### Statistical analysis

Statistical analyses were conducted using the Sigma-Stat 4.0 statistical package for Windows, which automatically performs a test for equal variance and normality among sample groups before subsequently doing parametric tests like ANOVA or t-test. If samples do not pass this test, non-parametric tests are used instead. We chose the most suitable statistical analysis depending on the level of comparison for the same biochemical parameter:

- a) One Way Repeated Measure ANOVA was used when analysing the same biochemical parameter at different exposure times to experimental diet of crabs from each site,  $p < 0.05$  was considered to be significant. This allowed us to test the temporal dynamics of each biochemical parameter. The Bonferroni correction was used to reduce the instances of false positives in multiple comparisons;
- b) A t-test was used to compare the values of the same biochemical parameter of crabs from different sites (mudflat versus saltmarsh) at the same exposure time to the experimental diet. The t-test is commonly used to compare the means for the same parameter between two groups (ZAR 1999).

## Results

### Hepatopancreas triglyceride concentrations

In crabs from the mudflat, triglyceride concentrations in the hepatopancreas at month 1 were similar to those at  $t_0$ . After 3 months (Fig. 2A), we noticed a significant decrease compared to  $t_0$  (about 60%; ( $F_{(8,2)} = 40.5$ ,  $t = 9$ ,  $p = 0.002$ ) and month 1 (about 65%; ( $F_{(8,2)} = 40.5$ ,  $t = 13.9$ ,  $p < 0.001$ ). In crabs from the saltmarsh, triglyceride concentrations did not change throughout the experimental period (Fig. 2B).

At  $t_0$  and month 1, triglyceride concentrations in the hepatopancreas of individuals from the saltmarsh were significant lower (about 80%,  $p < 0.001$ ; and 90%,  $p = 0.008$ , respectively) compared to the corresponding values in crabs from the mudflat. No significant differences were observed between the two habitat types at month 3 (Fig. 2).

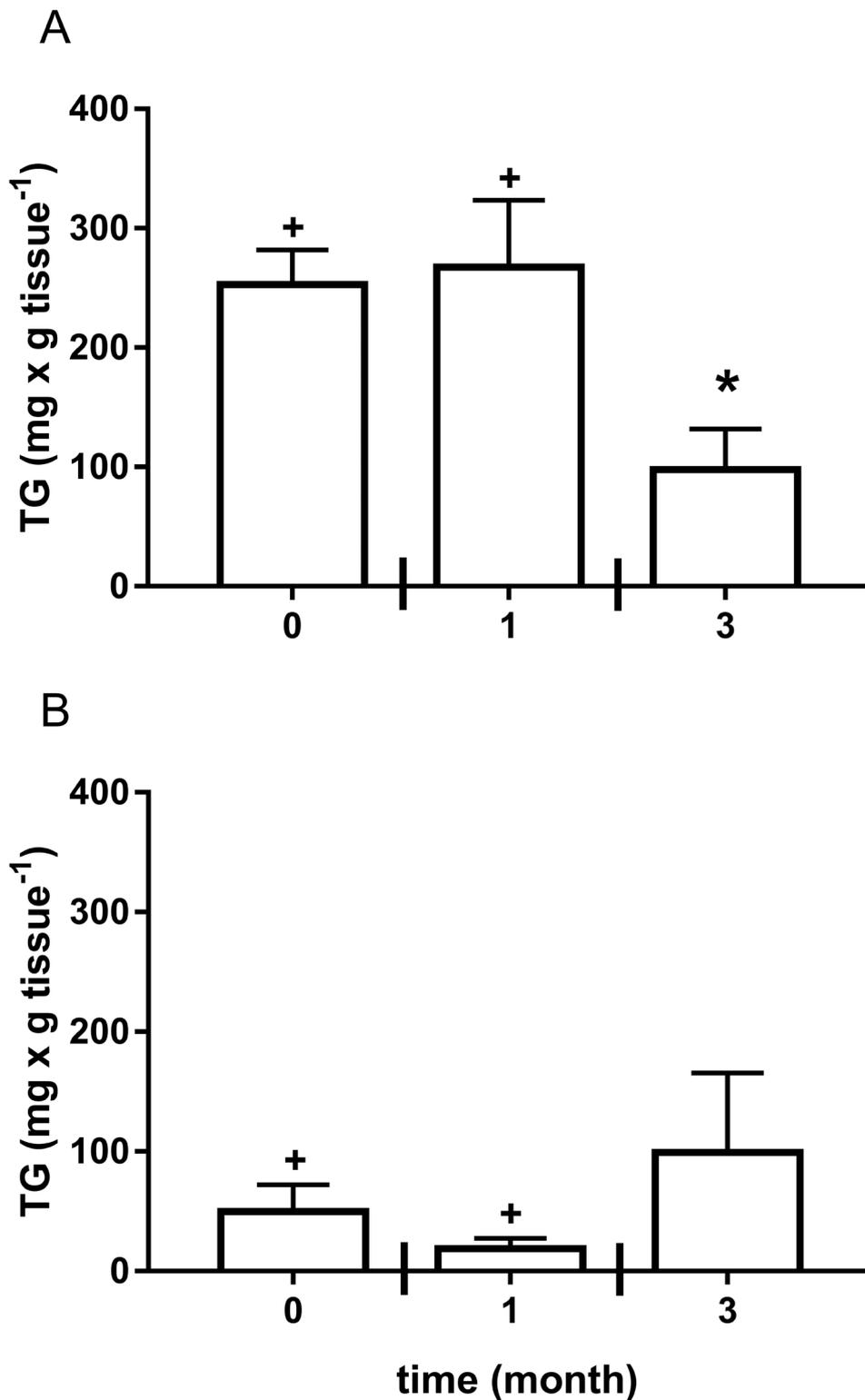


Fig. 2 – Triglyceride (TG) concentration in the hepatopancreas of *N. granulata* from the mudflat (A) and the saltmarsh (B) immediately after capture ( $t_0$ ) and at months 1 and 3 with the same experimental diet. \* indicates significant differences from the corresponding value at  $t_0$ . (One-way RM ANOVA,  $p < 0.05$ ). + indicates significant differences between crabs from the mudflat and the saltmarsh at  $t_0$  and at the same time of exposure under a similar diet (t-test,  $p < 0.05$ ). Data are the mean of five crabs per treatment.

### Protein concentrations

In crabs from the mudflat, protein concentrations in the hepatopancreas had not changed by month 1. At month 3, protein concentrations were significantly higher than the values at  $t_0$  (increase of about 140%;  $F_{(8,2)} = 101.34$ ,  $t = 23.28$ ,  $p < 0.001$ ) and at month 1 (increase of about 80%;  $F_{(8,2)} = 101.34$ ,  $t = 8.04$ ,  $p = 0.004$ ) (Fig. 3A). Protein concentrations in the hepatopancreas of crabs from the saltmarsh did not change throughout the experimental period (Fig. 3B).

When comparing protein concentrations between crabs from the two habitat types, we only observed a difference at month 1 when protein concentrations were lower (about 20%) in crabs from the saltmarsh than from the mudflat ( $p = 0.012$ ) (Fig. 3) but not at  $t_0$  or after 3 months (Fig. 3).

### Lipase activity

At month 1, lipase activity in hepatopancreas of crabs from the mudflat was similar to  $t_0$  while it had decreased about 45% by month 3 ( $F_{(8,2)} = 18.59$ ;  $t = 6.33$ ;  $p = 0.01$ ) (Fig. 4A). A decrease in hepatopancreas lipase activity by month 3 (about 40%) also occurred in crabs from the saltmarsh ( $F_{(8,2)} = 6.27$ ;  $t = 7.9$ ;  $p = 0.004$ ) (Fig. 4B).

No significant differences in lipase activity were observed at  $t_0$  and month 1 between crabs from the different habitats while at month 3, lipase activity was significantly higher (about 80%) in crabs from the saltmarsh ( $p < 0.001$ ) (Fig. 4).

### Levamisole-sensitive and -insensitive AP activity

In crabs from the mudflat, levamisole-sensitive AP activity increased (about 4000%) from  $t_0$  to month 1 ( $F_{(8,2)} = 9.66$ ;  $t = 5.11$ ;  $p = 0.011$ ). At month 3, the opposite pattern was observed, namely a significant decrease in levamisole-sensitive AP activity (about 3900%) as compared to month 1 ( $F_{(8,2)} = 29.66$ ;  $t = 6.17$ ;  $p = 0.005$ ) (Fig. 5A) reaching values similar to  $t_0$ . No significant changes in levamisole-sensitive AP occurred in hepatopancreas of crabs from the saltmarsh (Fig. 5C) through time.

Levamisole-sensitive AP activity differed significantly between individuals from the two habitat types only at  $t_0$ , with higher activities in crabs from the saltmarsh (about 6000%;  $p = 0.004$ ; Fig. 5A, C).

In crabs from the mudflat, levamisole-insensitive AP activity in hepatopancreas did not change significantly throughout the experimental period (Fig. 5B). In the hepatopancreas of crabs from the saltmarsh, the levamisole-insensitive AP activity was significantly higher (about 300%) at month 3 compared to  $t_0$  ( $F_{(10,2)} = 26.38$ ;  $t = 5.81$ ,  $p = 0.006$ ,) and to month 1 ( $F_{(10,2)} = 26.38$ ;  $t = 5.54$ ;  $p = 0.008$ ) (Fig. 5D).

No differences were observed between crabs from the two habitat types at  $t_0$  or at month 1 while at month 3, the levamisole-insensitive AP activity was significantly higher (about 400%) in individuals from the saltmarsh compared to the mudflat ( $p < 0.001$ ) (Fig. 5B, D).

### Behavioral and physical crab parameters under exposure to a similar diet

Crabs from both habitat types did not exhibit any differences in the feeding behavior throughout the experimental period. No mortality occurred in any group.

The hepatosomatic index (HIS) was similar throughout the entire experimental period in crabs from both habitats, the mudflat and the saltmarsh, without any significant differences (Fig. 6). Also, no significant differences of the HIS were found between crabs from the two habitat types at the same time (Fig. 6).

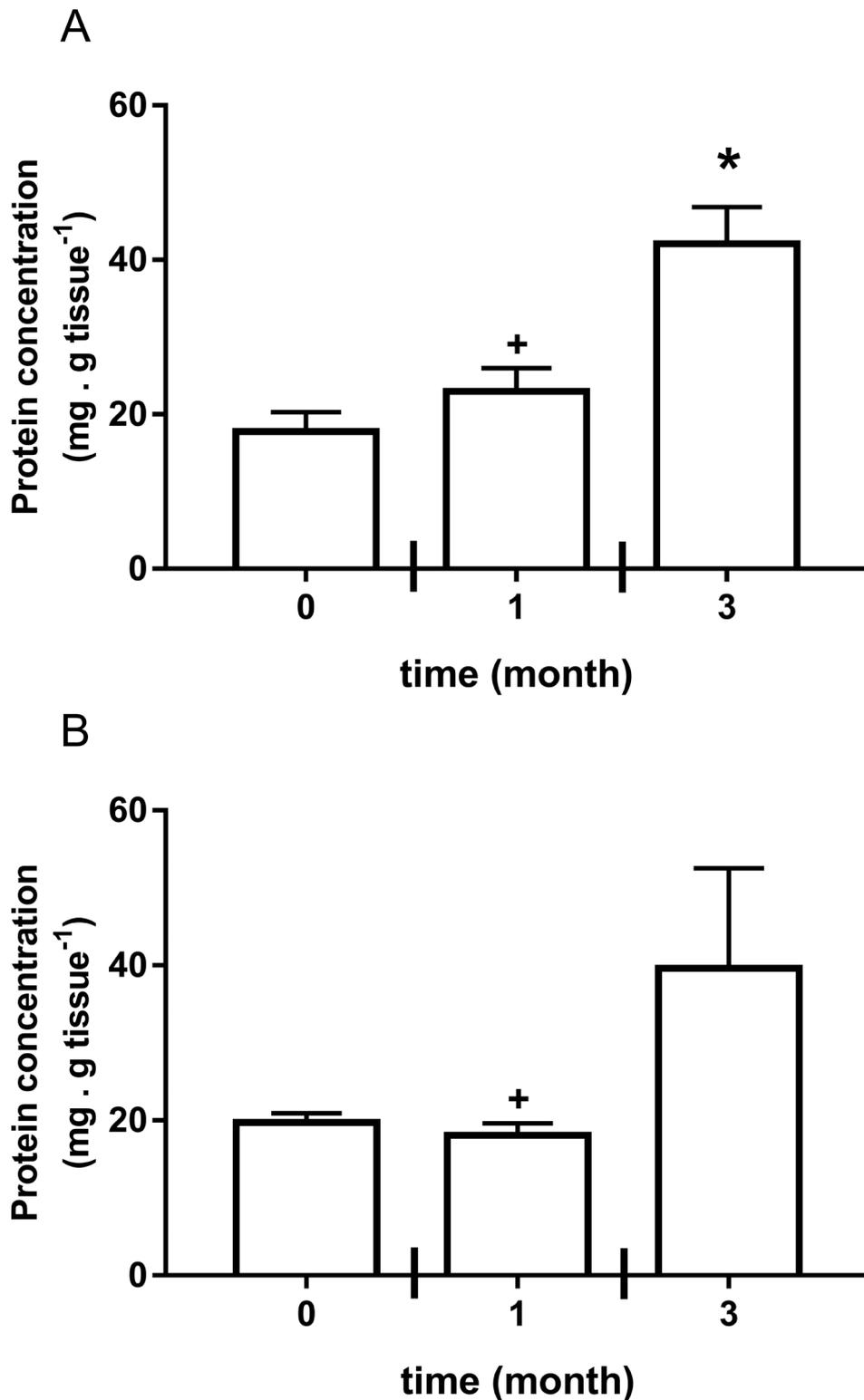


Fig. 3 – Protein concentration in the hepatopancreas of *N. granulata* from the mudflat (A) and the saltmarsh (B) immediately after capture ( $t_0$ ) and at month 1 and 3 with the same experimental diet. \* indicates significant differences from the corresponding value at  $t_0$ . (One-way RM ANOVA,  $p < 0.05$ ). + indicates significant differences between crabs from the mudflat and the saltmarsh at  $t_0$  and at the same time of exposure under a similar diet (t-test,  $p < 0.05$ ). Data are the mean of five crabs per treatment.

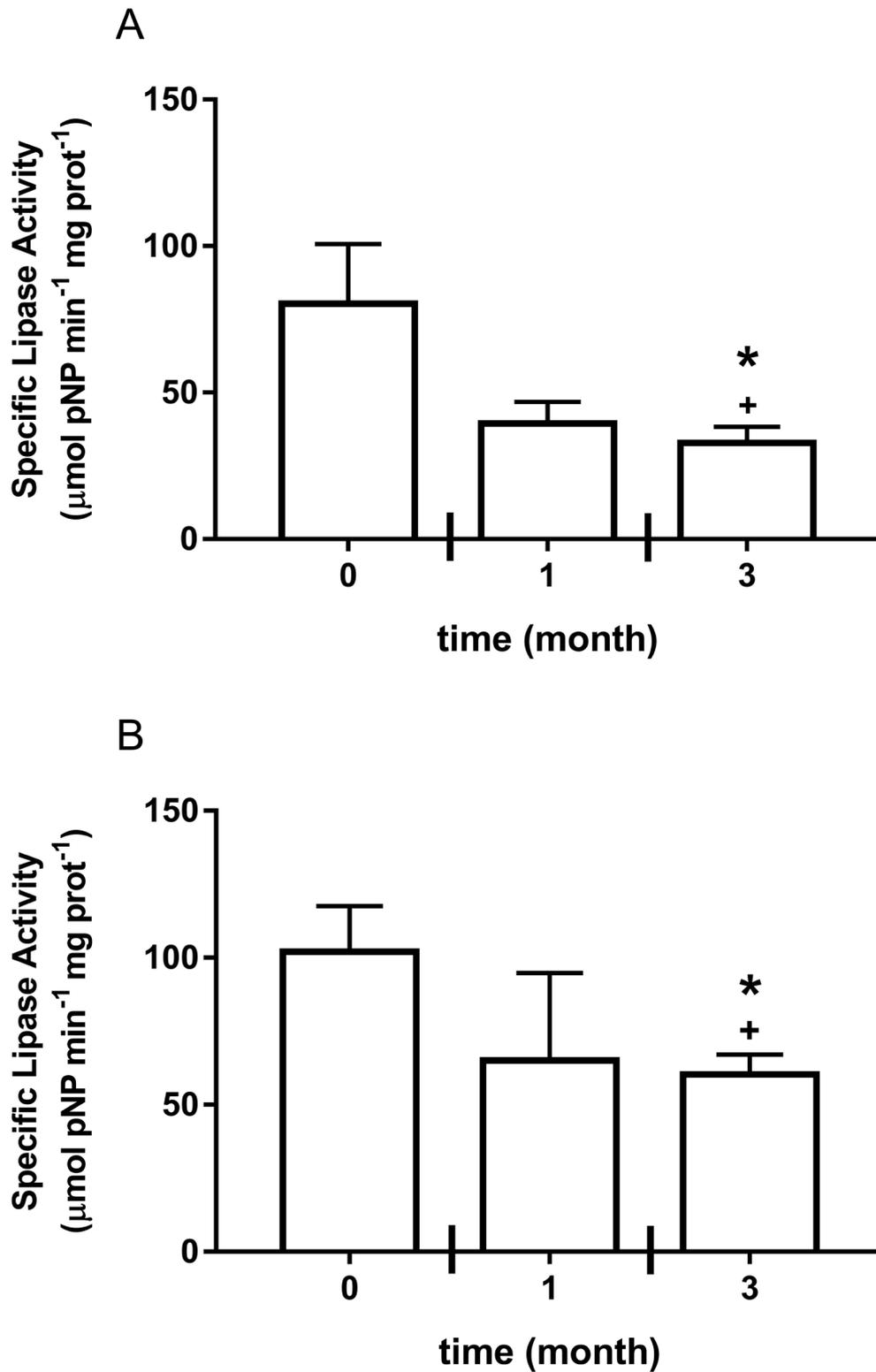


Fig. 4 – Lipase Activity in the hepatopancreas of *N. granulata* from the mudflat (A) and the saltmarsh (B) immediately after capture ( $t_0$ ) and at month 1 and 3 with the same experimental diet. \* indicates significant differences from the corresponding value at  $t_0$ . (One-way RM ANOVA,  $p < 0.05$ ). + indicates significant differences between crabs from the mudflat and the saltmarsh at  $t_0$  and at the same time of exposure under a similar diet (t-test,  $p < 0.05$ ). Data are the mean of five crabs per treatment.

## Discussion

### Lipid metabolism components in the hepatopancreas of *N. granulata*

#### Triglyceride concentrations

Crabs build lipid stores through anabolism mainly by direct uptake of lipids from diet (SABOROWSKI 2015). Digested lipids are mainly stored as triglycerides and in most species, such as *N. granulata*, the hepatopancreas represents the primary storage site, particularly for long-term energy reserve (PINONI *et al.* 2011; ŠTRUS *et al.* 2019; LÓPEZ MAÑANES *et al.* 2020). In the mudflat of Mar Chiquita Coastal Lagoon, *N. granulata* sustains a diet with high lipid and protein content by consuming mostly animal items (BAS *et al.* 2014; LANCIA *et al.* 2014). The higher availability of dietary lipids should be detectable as higher triglyceride concentrations in the hepatopancreas of crabs from the mudflat as compared to saltmarsh in autumn at the time of capture ( $t_0$ ), which is indeed the case (Fig. 2). In crabs from the

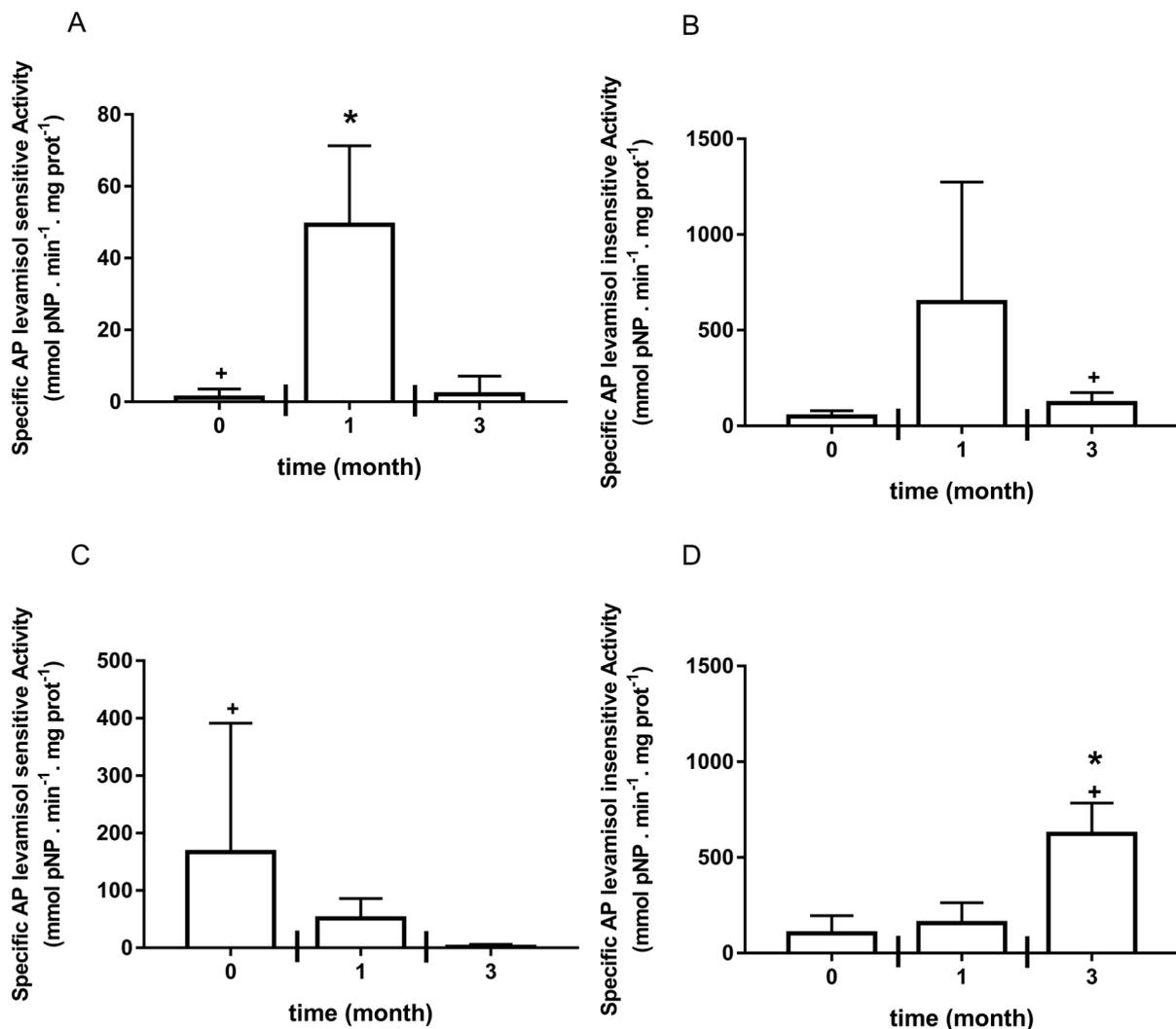


Fig. 5 – Levamisole-sensitive and -insensitive AP activities in the hepatopancreas of *N. granulata* from the mudflat (A–B) and the saltmarsh (C–D), immediately after capture ( $t_0$ ) and at month 1 and 3 with the same experimental diet. \* indicates significant differences from the corresponding value at  $t_0$ . (One-way RM ANOVA,  $p < 0.05$ ). + indicates significant differences between crabs from the mudflat and the saltmarsh at  $t_0$  and at the same time of exposure under a similar diet (t-test,  $p < 0.05$ ). Data are the mean for six (mudflat) and five (saltmarsh) crabs per treatment.

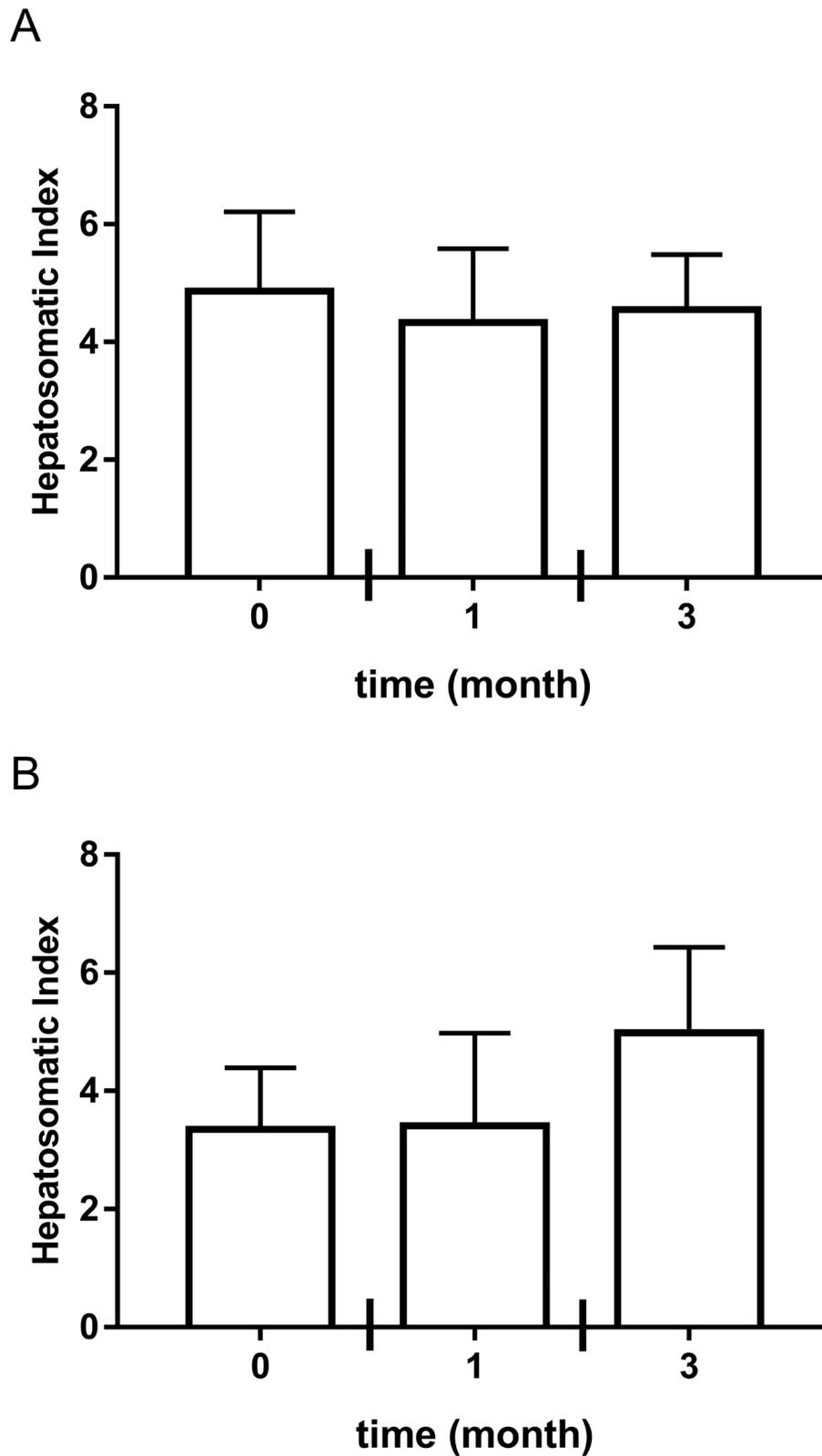


Fig. 6 – Hepatosomatic index of *N. granulata* from the mudflat (A) and the saltmarsh (B) immediately after capture ( $t_0$ ) and at month 1 and 3 with the same experimental diet. Data are the mean for six (mudflat) and five (saltmarsh) crabs per treatment.

mudflat, the significant decrease in triglyceride concentrations at month 3 under an experimental diet of lower lipid content than in the wild (Fig. 2A) supports our hypothesis that the availability of dietary lipids indeed influences the building/degradation of this reserve. Since digestion–absorption routes are still unknown in *N. granulata*, further experiments are needed to establish the link between decreased digestion of dietary lipids and concentration of triglycerides in the hepatopancreas of crabs from the mudflat. In the euryhaline crab *Cyrtograpsus angulatus* (Dana, 1852), regulation of intracellular lipase activity by external and internal factors is linked to variations in the triglycerides concentrations in the hepatopancreas (MICHIELS *et al.* 2013).

Adult males from the saltmarsh appeared to be metabolically adapted to the more stable environmental conditions throughout time of this habitat including the kind of available food (LÓPEZ MAÑANES *et al.* 2020). Digestive and metabolic components of lipid and protein metabolism at the biochemical level in the hepatopancreas appeared to be less sensitive to changes in key environmental factors compared to crabs from the mudflat (PINONI *et al.* 2013; LÓPEZ MAÑANES *et al.* 2020). The low consumption of animal dietary items, particularly during autumn, could explain the lower triglycerides content in the hepatopancreas compared to crabs in the mudflat at the time of capture ( $t_0$ ) (Fig. 2) as we described above. Lipids are limited dietary components for adult males in the saltmarsh (BAS *et al.* 2014; LANCIA *et al.* 2014). Crabs in this habitat exhibit digestive and metabolic characteristics suggesting their biochemical adaptation to a diet of low lipids content (LÓPEZ MAÑANES *et al.* 2020). Our finding that triglyceride concentrations in the hepatopancreas did not change up to month 3 in males from the salt marsh under exposure to an experimental diet with higher proportions of lipids than in the wild, supports this idea (Fig. 2B).

The lack of any statistical difference in hepatopancreas triglyceride concentrations between crabs from the two different habitats sites at 3 months could be attributed to the decrease of this reserve in individuals from the mudflat (Fig. 2).

### Lipase activity

Changes of digestive enzyme activity in the digestive tract can lead to modifications in the digestive capacity for specific nutrients (DEL VALLE & LÓPEZ MAÑANES 2011, 2012; ROMANO & ZENG 2012; KARASOV & DOUGLAS 2013). In various animals, digestive enzyme activity and/or expression changes in direct correlation to the content of specific substrate in the diet (KARASOV & DOUGLAS 2013). In general, levels of digestive enzymes in the digestive tract of vertebrates correlate positively with those of the corresponding substrate in the diet (MELO *et al.* 2012; KARASOV & DOUGLAS 2013). We would also expect this to be the case for lipase activity in hepatopancreas of *N. granulata* from the mudflat since this activity significantly decreased with an experimental diet having lower lipid content than in the wild (Fig. 4A). Furthermore, the simultaneous decrease in both lipase (Fig. 4B) and triglyceride content (Figs 2A, 4A) supports our hypothesis that key components of the lipid metabolism are indeed influenced by diet in crabs from the mudflat.

Some animals exhibit an inverse relationship between levels of digestive enzymes in the digestive tract and specific substrate in the diet, making them an exception to the general hypothesis of a direct relationship as we pointed out above (KARASOV & DOUGLAS 2013; XIE *et al.* 2017). This appears to be the case for lipase activity in the hepatopancreas of *N. granulata* from the saltmarsh (Fig. 4), which was lower under a diet with higher lipid content than in the wild.

Our result that between-habitat, intraspecific differences in lipid metabolism indeed occurred with the same experimental diet, is further supported by our findings that in crabs from the saltmarsh, lipase activity was higher at month 3 compared to crabs from the mudflat (Fig. 4), and that modulation of

lipase activity was not linked to variations in triglyceride reserves (Figs 2B, 4B) unlike for crabs from the mudflat.

### **Protein concentration in the hepatopancreas of *N. granulata***

In intertidal crabs, protein metabolism plays a key role in biochemical adaptation to various environmental parameters such as variations in diet, salinity and temperature (PINONI *et al.* 2013, 2018). The hepatopancreas is a site for initial synthesis of macromolecules as for example proteins (ŠTRUS *et al.* 2019; VOGT 2019). The increase in the protein concentration, which we observed (Fig. 3A) at month 3 indicates that diet also affects the protein metabolism of the hepatopancreas of crabs from the mudflat. Whether the increase in protein concentration can compensate for the observed decrease in triglyceride (see above), thereby allowing the maintenance of metabolic and physical conditions (Fig. 6A) requires further investigation.

In the saltmarsh, *N. granulata* are biochemically adapted to a diet with low protein content (LÓPEZ MAÑANES *et al.* 2020). Under controlled conditions, digestive and metabolic parameters in the hepatopancreas of crabs from the saltmarsh seem to be less affected by variations in key environmental factors such as salinity (PINONI *et al.* 2013; LÓPEZ MAÑANES *et al.* 2020). This also appears to be the case for protein concentrations of the hepatopancreas as these did not change throughout the study period with an experimental diet (Fig. 3B), implying that adjustments in protein metabolism (i.e., synthesis/degradation pathways) are most likely not involved in biochemical adaptations of *N. granulata* from the saltmarsh to dietary conditions.

### **AP activity in the hepatopancreas of *N. granulata***

In the hepatopancreas of decapod crustaceans, AP are key enzymes due to their role in the synthesis and secretion of digestive enzymes and the absorption of digestive products (e.g., lipids) (VERRI *et al.* 2001; WANG *et al.* 2014). Diet-driven changes of levamisole-sensitive AP activity (Fig. 5A) in the hepatopancreas of crabs from the mudflat could possibly be linked to variations in energy reserve content (Figs 2A, 3A). In *N. granulata*, AP activity is involved in postprandial adjustments of protein metabolism in the hepatopancreas (PINONI *et al.* 2015; PINONI & LÓPEZ MAÑANES 2016). Postprandial changes in AP and proteolytic activities have been described in *N. granulata* from the mudflat under controlled conditions (PINONI *et al.* 2015). The simultaneous decrease of levamisole-sensitive AP and lipase activity (Figs 4A, 5A) in the hepatopancreas of crabs from the mudflat suggests a coordinated variation of these activities. A diet with reduced nutrients seemed to lead to lower digestion and absorption of lipids and to a decrease in triglyceride reserves (Fig. 2A). Further studies (e.g., at the cellular level) are needed to establish the possible physiological link between levamisole-sensitive AP and lipase activities in crabs from the mudflat.

Diet appeared to drive levamisole-insensitive AP activity (Fig. 5D) in hepatopancreas of crabs from the saltmarsh (Fig. 5D). Whether the observed, enhanced levamisole-insensitive AP activity is indeed involved in the maintenance of triglycerides and proteins in crabs from the saltmarsh as could be expected from our results (Figs 2B, 3B), requires further investigation. In the closely related euryhaline crab *C. angulatus*, levamisole-insensitive AP activity has a role in dietary adjustments (PINONI & LÓPEZ MAÑANES 2016).

In various decapod crustaceans, AP has been involved in macromolecule synthesis (e.g., lipids and proteins) taking place in the hepatopancreas after feeding (MCGAW & CURTIS 2013; CARTER & MENTE 2014; WANG *et al.* 2014; SUN *et al.* 2021). When energy reserves must be changed in hepatopancreatic

cells, levamisole-insensitive AP activity appears to be regulated intracellularly in a coordinated fashion to the changes of these reserves (LÓPEZ MAÑANES *et al.* 2020).

### Conclusions

The different responses of energy reserves and key enzymes to a similar experimental diet in the hepatopancreas of *N. granulata* from the mudflat and saltmarsh illustrate that the specific influence of diet is depending on the habitat of origin.

In the wild, differential adjustments in lipid and protein metabolism (at the level of key digestive enzymes activities and utilization of energy reserves) occur in crabs from the mudflat and the saltmarsh (PINONI *et al.* 2011; LÓPEZ MAÑANES *et al.* 2020). Distinct and specific intrinsic mechanisms of regulation (e.g., chemical messengers) could be operating being triggered by the particular environmental characteristics of each site (e.g., food resources). We found that energy reserves and enzyme activities, which are regulated in relation to habitat in the wild, are also differentially affected in crabs from two different habitats during exposure to a similar experimental diet. Our results suggest that these distinct intrinsic mechanisms could continue to be operating during our experiment of three months, leading to a differential use of digestive and metabolic pathways in crabs depending on their habitat of origin.

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### Ethics

This project was performed following the regulations and statements of Ethics Committee CICUAL (OCA 1499/12), FCEyN Universidad Nacional de Mar del Plata.

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