Journal of the Marine Biological Association of the United Kingdom, page 1 of 11.
doi:10.1017/S0025315417000601

The euryhaline crab *Uca tangeri* showed metabolic differences to sex and environmental salinity

SILVINA A. PINONI[1]*, ISMAEL JEREZ-CEPA[2]*, ALEJANDRA A. LÓPEZ MAÑANES[1]† AND JUAN MIGUEL MANCERA ROMERO[2]†

[1]Instituto de Investigaciones Marinas y Costeras (IIMyC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Universidad Nacional de Mar del Plata, Mar del Plata, Argentina, [2]Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Puerto Real, Spain

*These authors have contributed equally to this work.

*†Senior authors have contributed equally to this work.

This study constitutes a first attempt to investigate sex differences in osmoregulatory capacity and metabolic responses in relation to hyper- and hypo-osmoregulation in the intertidal euryhaline crab Uca tangeri. *Adult male and female specimens from Cadiz Bay, Spain (36°23′–37′N 6°8′–15′W), were acclimated to three different environmental salinities (12, 33 and 55 psu) during 7 days, and several parameters were assessed in haemolymph (osmolality, glucose, amino acids, triglycerides and lactate) as well as in metabolic key organs (hepatopancreas, anterior and posterior gills: glycogen, free glucose, amino acids and triglycerides). Specimens from both sex exhibited high and similar hyper- and hypo-osmoregulatory capacities. However, metabolite levels were differentially affected upon acclimation to low and high salinity in several metabolic organs and haemolymph of male and females: (i) glycogen in gills, (ii) free glucose in gills and hepatopancreas, (iii) amino acids in hepatopancreas, (iv) triglycerides in haemolymph, hepatopancreas and posterior gills, and (v) lactate in haemolymph. The results suggest the occurrence of differential metabolic adjustments upon hyper- and hypo-osmoregulation related to sex in the intertidal euryhaline crab* U. tangeri.

Keywords: euryhaline crab, *Uca tangeri*, salinity, hepatopancreas, gills, haemolymph

Submitted 19 October 2016; accepted 28 March 2017

INTRODUCTION

Uca tangeri (Brachyura: Ocypodidae; Eydoux, 1835) is an intertidal euryhaline burrowing crab which is found from southern Spain and Portugal (~37°N) through the west coast of Africa, down to Angola (~16°S) (Spivak & Cuesta, 2009; de Gibert *et al.*, 2013; Rodríguez-Tovar *et al.*, 2014). *Uca tangeri* is the only fiddler crab species to inhabit Europe. In spite of its ecological and economical importance, few studies are available about physiological aspects of this crab (Drews & Graszynski, 1983; Krippeit-Drews *et al.*, 1989; Mourente *et al.*, 1994). Moreover, studies on metabolic adjustments in responses to key environmental factors, such as salinity, and possible sex differences are lacking. Recently the importance of taking into account the potential sex differences in physiological studies of the euryhaline crab *Carcinus maenas* has been pointed out (Pennoyer *et al.*, 2016).

The spatial and temporal changes in environmental salinity faced by intertidal euryhaline crabs require different strategies for controlling movements of water and ions between the individuals and their medium (McNamara & Faria, 2012; Romano & Zeng, 2012; Havird *et al.*, 2016). Phenotypic flexibility implies reversible modifications within individual phenotypic traits (from molecular to organisms) which can increase the chances of survival for animals facing changes in environmental conditions (Piersma & Drent, 2003; Pfenning *et al.*, 2010; Kelly *et al.*, 2012). The ability to osmoregulate is a primary physiological determinant for euryhalinity and for the occupancy of different zones within an intertidal area (Pinoni *et al.*, 2013; Larsen *et al.*, 2014; Havird *et al.*, 2016). Hyper- and hypo-osmoregulation abilities allow maintenance of the osmotic concentration of the haemolymph within a stable range, above or below that of the aquatic environment in low and high salinities, respectively (McNamara & Faria, 2012; Romano & Zeng, 2012). Comparisons of osmoregulatory capacity (the difference between haemolymph and aquatic environment osmotic pressures at a given salinity, Lignot *et al.*, 2000) of male and females will allow evaluation of the possible differential performance upon distinct osmotic challenges depending on sex.

Compensation in response to different environmental conditions is, usually, an energy-demanding process which requires the mobilization of energy substrates to metabolically deal with challenge. Carbohydrate, lipid and protein storage and metabolites in body fluids can be altered by changing environmental conditions. Decapod crustaceans use carbohydrates, lipids and proteins as principal energy molecules but the extent of use and the relative importance of the different

Corresponding author:
A.A. López Mañanes
Email: mananes@mdp.edu.ar

metabolic sources involved vary depending on species and type of challenge (Pinoni *et al.*, 2013; Jimenez & Kinsey, 2015; Wang *et al.*, 2016). In males of various species of decapod crustaceans, adaptation to environmental salinity is a complex process involving adjustments at the biochemical level in several tissues and organs and a metabolic reorganization (Michiels *et al.*, 2013, 2015; Pinoni *et al.*, 2013; Chen *et al.*, 2015). Modifications in various haemolymph metabolites have been reported (Romano *et al.*, 2012, 2014; Larsen *et al.*, 2014; Prymaczok *et al.*, 2016). The differential patterns of physiological challenges faced by males and females could result in sex differences concerning the regulation of osmotic balance and the possible associated use and distribution of energy resources. Comparative studies on metabolic adjustments at different levels in response to low and high salinity in adult male and females of a single species of euryhaline crab are scarce. The hepatopancreas, due to its central role for storage of energy substrates and synthesis of macromolecules, is a sensitive indicator for metabolism modifications related to osmoregulatory processes (Zeng *et al.*, 2010; Ribeiro *et al.*, 2014; Wang *et al.*, 2014). Anterior and posterior gills also have a role as energy storage in various osmoregulating crabs although with differential degree of importance depending on species, type of energy substrate and salinity challenge (Pinoni *et al.*, 2013).

In Rio San Pedro, a shallow inlet of the salt marsh zone in the eastern shore of the Cádiz Bay, Spain (36°23′–37′N 6°8′–15′W), adult specimens of *U. tangeri* are exposed to a wide range of environmental salinity ranging from 12 up to 46 psu, although frequently higher values can be reached (González-Gordillo *et al.*, 2003; Ferrón *et al.*, 2009; personal observations). In this context, the aims of this work were to study in both male and female specimens of *U. tangeri* from the Rio San Pedro population: (i) osmoregulatory pattern and capacity, and (ii) the influence of different environmental salinities (12, 33 and 55 psu) implying distinct osmoregulatory responses (hyper-osmoregulation; osmoconformation; hypo-osmoregulation) on metabolic parameters in the haemolymph as well as on energy reserves content in key storage organs (hepatopancreas and gills). We determined osmolality, glucose, amino acids, triglycerides and lactate concentration in haemolymph, as well as glycogen, free glucose, amino acid and triglyceride values in hepatopancreas and anterior and posterior gills. This work will increase the knowledge on the metabolic response to changes in environmental salinity of osmoregulating crabs as well as the variability in relation to sex.

MATERIALS AND METHODS

Animal collection and maintenance

Adult intermoult males and females (non-ovigerous) of *U. tangeri* were caught during early autumn (September 2013) by hand at low tides in Rio San Pedro, a shallow inlet of the salt marsh zone in the eastern shore of the Cádiz Bay, Spain (36°23′–37′N 6°8′–15′W). The bay is under a strong tidal and wind regime, and in summer air temperatures can reach up to 35°C. In these conditions, and in some places where the water exchange is low (such as growing fish ponds), we can find 55 psu-water and also regular populations of this burrowing crab. Crabs were placed in 50 l buckets with some stones to allow them the possibility to emerge from water. The immersion pattern of crabs was not affected by salinity. Crabs were transported on the day of collection in the same water of the site of collection under continuous aeration to the wet laboratory at the Department of Biology (Faculty of Marine and Environmental Sciences, University of Cádiz). Specimens were acclimated to different environmental salinities: (i) diluted seawater (12 psu, 287 mOsm kg^{-1}), (ii) seawater (33 psu, 765 mOsm kg^{-1}) or (iii) concentrated seawater (55 psu, 1386 mOsm kg^{-1}) for one week before sampling. A period of one week of acclimation is standard for biochemical and metabolic studies in various crabs (i.e. Figuereido & Anderson, 2009; Jin *et al.*, 2011, 2012; Ninlanon, 2011; Cheng *et al.*, 2013; Maciel *et al.*, 2014). The experimental salinities were obtained either by mixing full-strength seawater with dechlorinated tap water or by mixing full-strength seawater with natural marine salt (Salina de La Tapa, Puerto de Santa María, Cádiz, Spain). Salinity, expressed in practical salinity units (psu), was measured with a refractometer (Master Refractometer Manual ATAGO®, Master-S10M Cat. No. 2473) and the corresponding osmotic pressure with a cryoscopic osmometer (Fiske One-Ten Osmometer, FISKE USA). Aquaria water was continuously aerated and filtered, and partially changed every 2 days to keep constant physicochemical parameters. Animals were maintained under natural photoperiod (October 2013; latitude 36°31′34″N) and water temperature (20 ± 2°C) and were fed three times a week with commercial fish food (Élite-Skretting, about 0.07 g $ind.^{-1}$), but they were starved 24 h prior to sampling. No differences in the feeding behaviour were observed in the different experimental salinities tested. No mortality of individuals occurred at any salinity throughout the experimental period. All experimental procedures complied with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals.

Sampling procedures

Crabs were weighed and cold-anaesthetized by placing them into ice for about 15 min. A sample of haemolymph (about 500 µl) was withdrawn from the intrabranchial sinus by means of a syringe previously rinsed with heparin (Sigma H6279, 25 000 units per 3 ml 0.6% NaCl saline solution), at the base of the cheliped. It was centrifuged at 13 030 g for 3 min (Microcentrifugette ALC® 4204) and split into 100–200 µl aliquots (Allender *et al.*, 2008). The hepatopancreas as well as anterior (1–5 pairs) and posterior (6–8 pairs) gills were immediately excised, rapidly and gently dried by putting them on paper towel frozen on liquid nitrogen and then stored at −80°C until use in the preparation of extracts (see below).

Extracts preparations

To assess metabolite levels, around 0.2 g of gills and hepatopancreas were homogenized by ultrasonic disruption (Ultrasonic Cell Disruptor XL MICROSON ™, Misonix Incorporated) in 7.5 v/w ice-cold 0.6 N perchloric acid. Homogenates were neutralized with the same volume of 1 M potassium bicarbonate and centrifuged at 4°C and 3220 g during 30 min. The supernatants were stored in different aliquots at −80°C until use for the metabolite assays.

Haemolymph osmolality

Osmolality (mOsm kg^{-1}) was measured in an aliquot of 10 µl of haemolymph and water medium with a cryoscopic osmometer (Fiske One-Ten Osmometer, FISKE USA).

Haemolymph and organ parameters

Glucose, triglycerides (TAG) and lactate concentrations in haemolymph and organs were measured with commercial kits (SPINREACT, Spain). Total protein levels were measured using commercial kits ('PIERCE® BCA Protein Assay Kit', THERMO) based on the bicinchoninic acid assay. Total α-amino acid levels in haemolymph, gills and hepatopancreas were determined using the ninhydrin method described by Moore (1968). Glycogen levels in gills and hepatopancreas were determined by hydrolysing organ extracts with amyloglucosidase (AGS, Sigma A7420) during 120 min in a thermostatic bath at 40°C as Keppler & Decker (1974) described. Glucose obtained after glycogen breakdown (subtracting free glucose levels) was determined with the same kits used to determine haemolymph glucose levels. Metabolite levels were determined spectrophotometrically in a 96-well microplate, and performed with a 'PowerWave™ 340' spectrophotometer (BIO-TEK, USA) using KC Junior™ Data Analysis Software for Microsoft Windows XP®.

Statistical analysis

Statistical analyses were performed using the Sigma-Stat 3.0 statistical package for Windows operating system, which automatically performs a preliminary test for equal variance and normality to check parametric assumptions. A parametric (*t*-test or one-way ANOVA) or non-parametric (Mann–Whitney or Kruskal–Wallis, respectively) analysis of variance was used to estimate the statistical significance of the differences, and $P < 0.05$ was considered to be significant. *A posteriori* ANOVA or Kruskal–Wallis test (the Holm–Sidak or the Dunn's method, respectively) was used to identify differences (Zar, 1999).

RESULTS

Haemolymph parameters

Haemolymph osmolality of male and female crabs acclimated to 33 psu was similar to that of the aquatic environment (Figure 1A, B). However, at 12 psu haemolymph osmolality was about 2.5-fold higher while at 55 psu it was 2.0-fold lower with respect to the aquatic environment (Figure 1A, B). Our results indicated that the difference between environment and haemolymph osmolality at 55 psu appeared to be higher than at 12 psu (Figure 1C). In addition, no differences in osmoregulatory capacity were found between male and female specimens in either 12 or 55 psu (Figure 1C).

No differences were observed for any haemolymph metabolite assessed (glucose, lactate, triglycerides, protein and amino acids) in male specimens acclimated to different environmental salinities (12, 33 and 55 psu) (Figure 2). A similar situation was observed in female specimens for glucose and protein values (Figure 2A, B). However, amino acids and lactate levels in 12 and 55 psu acclimated specimens were lower (about 25 and 70%, respectively) than in 33 psu (Figure 2C, E). Triglycerides level enhanced (about 34%) at 55 psu with respect to 33 psu (Figure 2D). Glucose and lactate concentrations in the haemolymph of females acclimated to 33 psu were higher (about 20% and 2.5-fold, respectively) than the corresponding value in males (Figure 2A, E).

Hepatopancreas parameters

In males and females, glycogen concentration was not affected by acclimation to different environmental salinities (Figure 3A). However, free glucose values in males were higher (about 4-fold) at both 12 and 55 psu compared with 33 psu (Figure 3B). A similar situation was observed in females acclimated at 55 psu, but not at 12 psu (Figure 3B).

Male specimens acclimated to 12 psu presented higher (about 35%) amino acids concentration with respect to specimens kept at 33 psu. However, in females no differences were found in relation to environmental salinity (Figure 3C).

Finally, triglycerides values decreased (about 50%) in males acclimated to both 12 and 55 psu, with respect to 33 psu, while no differences related to salinity acclimation were observed in lactate concentration (Figure 3D).

Anterior and posterior gills parameters

In both male and female specimens, all of the metabolites assessed in anterior gills showed statistically significant differences related to environmental salinity (Figure 4). Only free glucose values in females acclimated to 12 psu were lower (about 67%) than at 33 psu, while they were similar at 55 psu (Figure 4B). In addition, in females acclimated to 33 psu, amino acids concentration in anterior gills was higher (about 45%) than the corresponding value in males (Figure 4C). However, more changes related to sex and environmental salinity were detected in posterior gills. In both males and females, glycogen concentration at 12 psu was similar to that at 33 psu and decreased at 55 psu (Figure 4A). Females acclimated to 33 psu exhibited higher glycogen and free glucose concentrations in posterior gills (about 40 and 58%, respectively) than the corresponding values in males (Figure 4A, B). Free glucose levels in male specimens acclimated to 12 psu were about 3-fold higher than in 33 psu, while no differences were found in females (Figure 4B). In male specimens acclimation to 12 psu enhanced triglycerides values with respect to those in 33 (about 2-fold) and 55 psu (about 1.5-fold). In 33 psu, triglycerides concentration in posterior gills of females was ~48% higher than males (Figure 4D).

DISCUSSION

Our results show that adult males and females of *U. tangeri* from Rio San Pedro, Cadiz, Spain exhibit similar hyper- and hypo-osmoregulatory capacity upon acclimation to low and high salinity, respectively. However, they exhibited distinct responses in metabolic parameters suggesting the occurrence of differential adjustments in relation to sex. We determined the haemolymph osmolality of male and females of *U. tangeri* acclimated to different salinities as a tool to evaluate and compare the osmoregulatory pattern and capacity. Males and females behaved as hyper/hypo-osmoregulators since they exhibited haemolymph osmolality values higher and lower

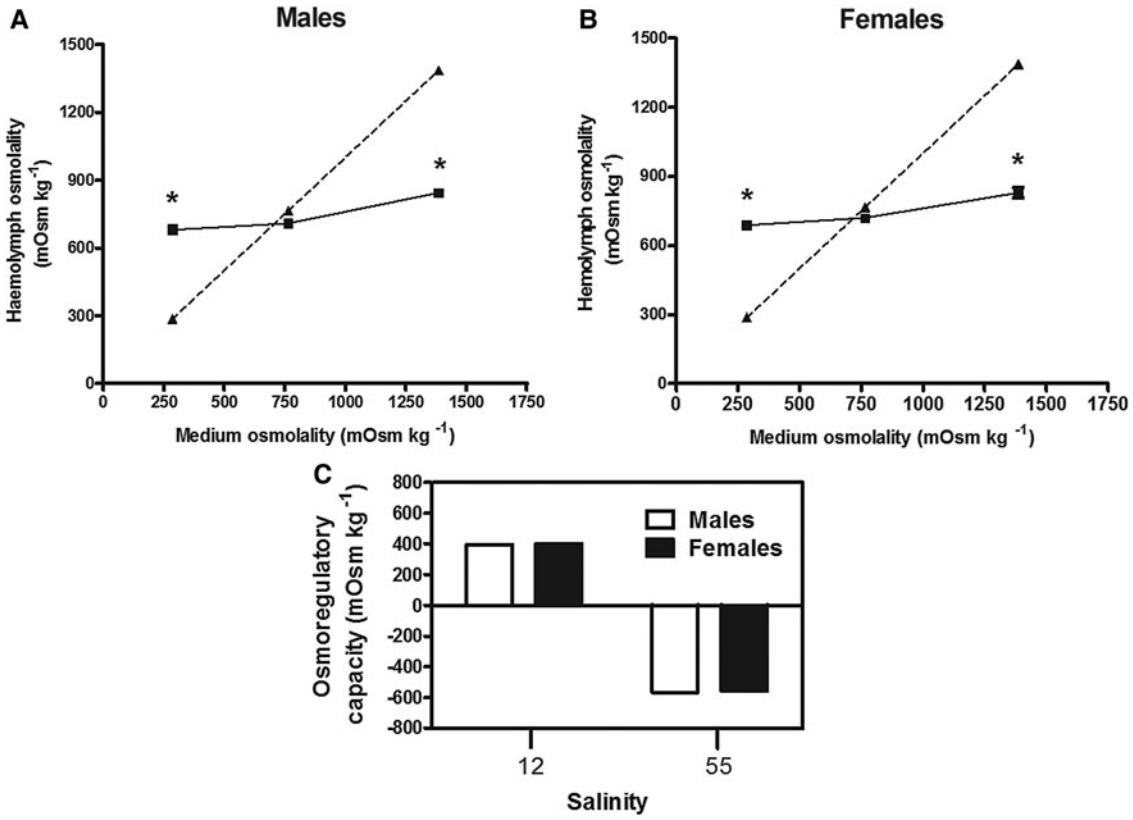

Fig. 1. Haemolymph osmolality of male and female of *U. tangeri* acclimated to 12 (287 mOsm × kg^{-1}), 33 (765 mOsm × kg^{-1}) and 55 (1386 mOsm × kg^{-1}) psu (A, B). Dashed line: isoosmotic line. Isoosmotic point: Males = 692 mOsm × kg^{-1}; Females = 724 mOsm × kg^{-1}. C: Osmoregulatory capacity (difference between haemolymph and medium osmolality). In some cases, error bars were smaller than the symbols used. * Indicate different from the medium osmolality (*t* test, $P < 0.05$). Data are the mean ± SEM (N = 8–12).

than those of the corresponding aquatic environment upon acclimation to low (12 psu) and high (55 psu) salinity, respectively, while osmoconforming at 33 psu (Figure 1A, B). This differs to recent results in females of the euryhaline crab *Carcinus maenas* which showed an increased ability to maintain their internal osmolality compared with males upon exposure to low salinity (Pennoyer *et al.*, 2016). The higher osmoregulatory capacity in both males and females of *U. tangeri* at high salinity (55 psu) compared with that in low salinity (12 psu), suggests a higher ability for hyper-osmoregulation. Whether this is linked with the fact that in Rio San Pedro, freshwater input from Cadiz Bay is relatively low so the salinity of the water is more usually high (Bautista- Chamizo *et al.*, 2016; Pereira *et al.*, 2016) remains to be investigated. Flexibility in osmoregulatory behaviour of euryhaline crabs determines a successful and/or differential occupancy of distinct areas in estuarine, inlets and coastal lagoons habitats that exhibit wide changes in environmental salinity.

Molecular and biochemical changes such as those in enzymes and system transport components of the branchial osmoregulatory machine need energy (McNamara & Faria, 2012; Romano & Zeng, 2012). Biochemical adaptation to low and high salinity in males of various euryhaline crabs implies a metabolic reorganization which could lead to a mobilization of energy reserves from different storage sites. However, the responses (i.e. type of reserves, storage organs involved) are species-specific and depend on the type of osmotic challenge (Romano & Zeng, 2012; Pinoni *et al.*, 2013; Romano *et al.*, 2014). Hyper and hypo-osmoregulation can require different mechanisms which lead to differential adjustments in carbohydrates, protein and lipid metabolism (Michiels *et al.*, 2013, 2015; Pinoni *et al.*, 2013, 2015; Romano *et al.*, 2014). The possible occurrence of differential metabolic adjustments related to sex is poorly studied. Glucose is the principal monosaccharide in the haemolymph of decapod crustaceans. Glucose homeostasis is essential for supporting the regular functions of various organs and in responses to environmental factors (Lorenzon *et al.*, 2005, 2007; Jimenez & Kinsey, 2015). The similar values of glycaemia in males and females of *U. tangeri* upon acclimation to low and high salinity indicates that availability of glucose from the haemolymph would not be a constraint upon hyper and hypo-osmoregulation (Figure 2A).

The hepatopancreas of decapod crustaceans is a key metabolic organ constituting a main site for intracellular digestion and absorption of nutrients and storage of energy reserves (Carter & Mente, 2014; Lignot & Charmantier, 2015; Saborowski, 2015). A major part of haemolymphatic glucose comes from the hepatopancreas (Wang *et al.*, 2016). Glucose produced from the digestion of polysaccharides in the hepatopancreas can be transported to the haemolymph or can be stored as glycogen. Glucose can also be synthesized by gluconeogenic pathways (Martins *et al.*, 2011; Jimenez & Kinsey, 2015; Wang *et al.*, 2016). The higher levels of free glucose in the hepatopancreas of males of *U. tangeri* in low and high salinity, and of females in 55 psu (Figure 3B), suggest the occurrence of adjustments in carbohydrate

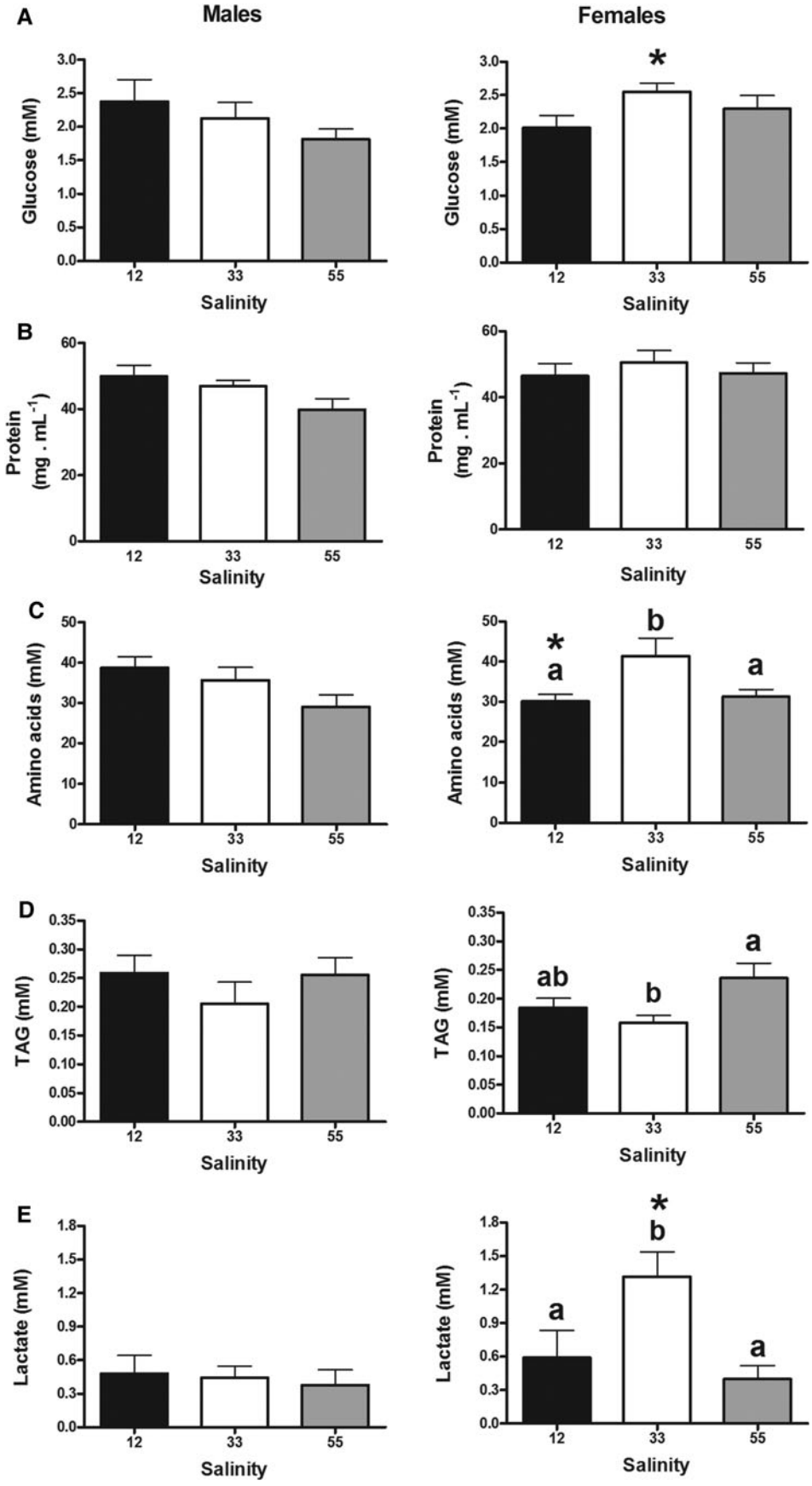

Fig. 2. Glucose (A), proteins (B), amino acids (C), triglycerides (D) and lactate (E) concentrations in the haemolymph of male (left) and female (right) of *U. tangeri* acclimated to 12, 33 and 55 psu. Different letters indicate significant differences between salinities (one-way ANOVA, $P < 0.05$). * Indicate different from the corresponding value of males in 33 psu (*t*-test, $P < 0.05$). Data are the mean ± SEM for eight individuals.

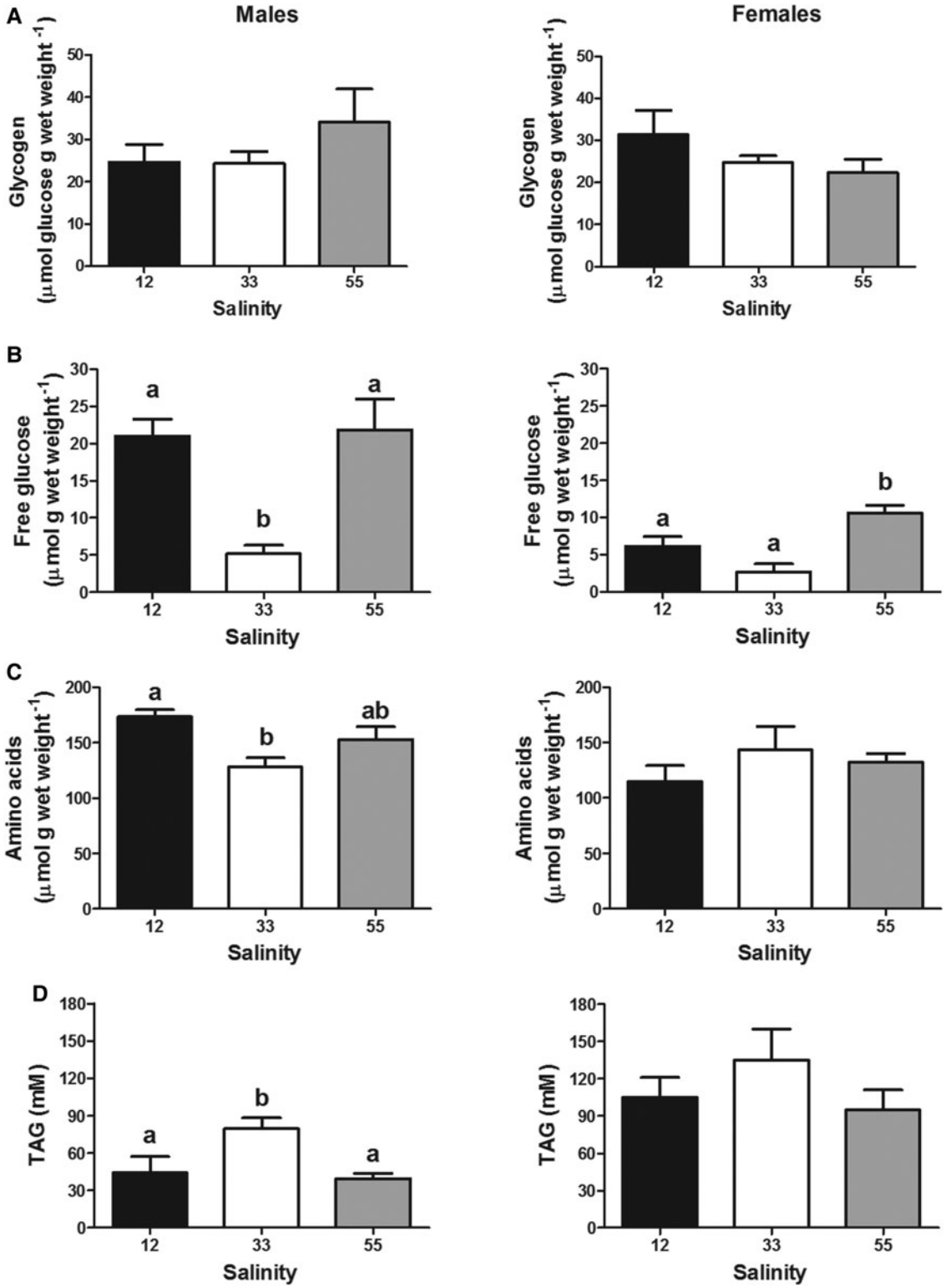

Fig. 3. Glycogen (A), free glucose (B), amino acids (C) and triglycerides (TAG) (D) concentrations in hepatopancreas of males and females of *U. tangeri* acclimated to 12, 33 and 55 psu. Different letters indicate significant differences between salinities (one-way ANOVA, $P < 0.05$). Data are the mean ± SEM for eight individuals.

metabolism likely to maintain glycaemia upon hyper and hypo-osmoregulation. The increased free glucose while glycogen concentrations was not affected, points to possible adjustments in gluconeogenic pathways. Most crustaceans studied so far appear to have key enzymes of the gluconeogenesis pathway in the hepatopancreas (Martins *et al.*, 2011; Wang *et al.*, 2016). The fact that in females the content of free glucose in the hepatopancreas was not affected in low salinity (while increased in males) (Figure 3B) suggests different adjustments in carbohydrate metabolism upon hyper-regulation depending on sex. On the other hand, the increase of free glucose content in the hepatopancreas in 55 psu

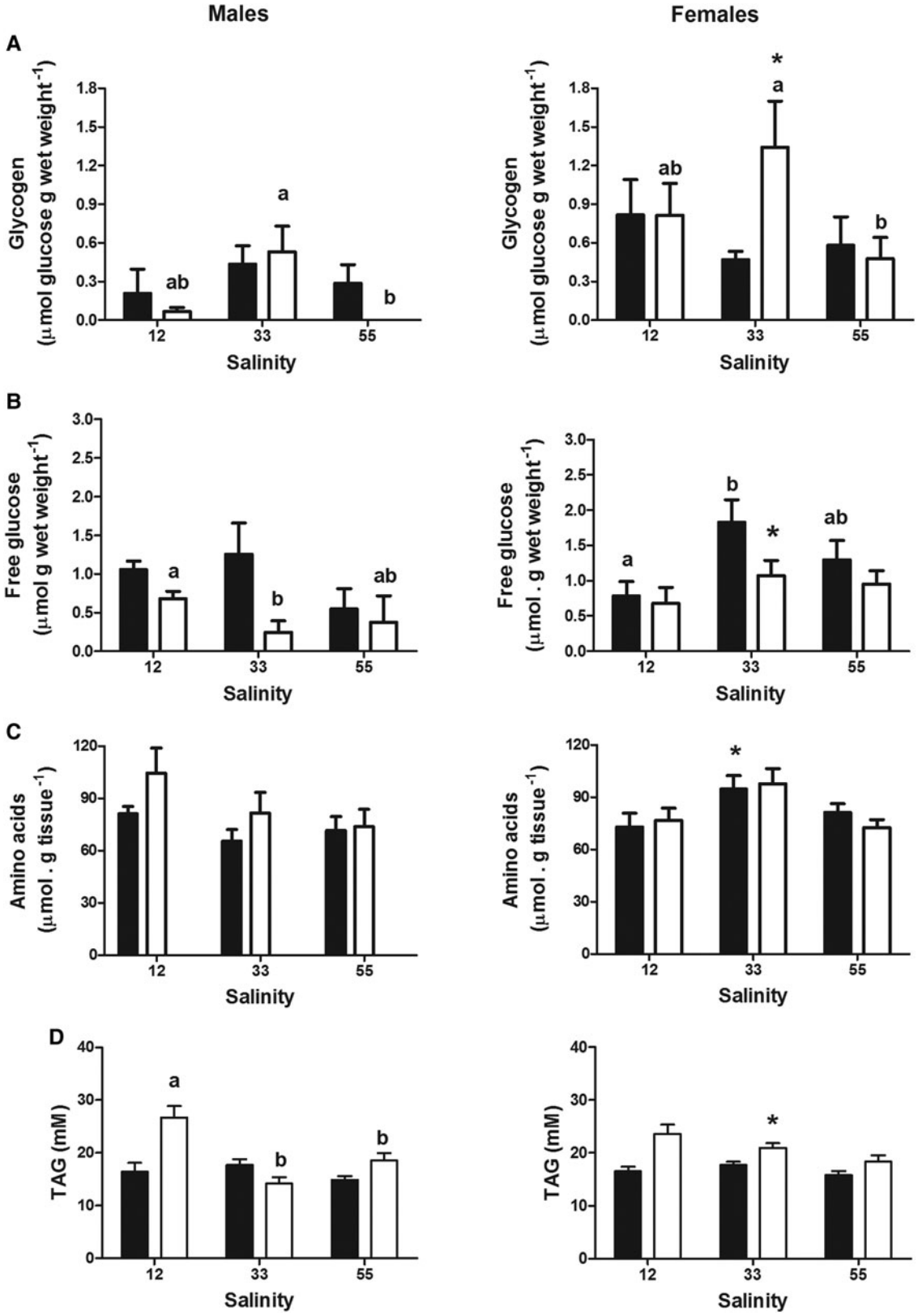

Fig. 4. Glycogen (A), free glucose (B), amino acids (C) and TAG (D) concentrations in anterior (black bars) and posterior (open bars) gills of males and females of *U. tangeri* acclimated to 12, 33 and 55 psu. Different letters indicate significant differences between salinities (one-way ANOVA, $P < 0.05$). * Indicate different from the corresponding value of males in 33 salinity (*t* test, $P < 0.05$). Data are the mean ± SEM for eight individuals.

suggests the occurrence of different responses in carbohydrate metabolism in relation to the type of osmotic challenge in females (Figure 3B). Hypo- and hyper-osmotic stresses *in vitro* on hepatopancreas sections from males of the euryhaline crab *Neohelice granulata* induce different adjustments in gluconeogenesis pathways (Martins *et al.*, 2011). Further

investigation is necessary to determine whether this is the case for females of *U. tangeri*. Anterior and posterior gills have an important role in the metabolism of carbohydrates in males of various euryhaline crabs in which they are sites of glycogen storage and utilization (Martins *et al.*, 2011; Pinoni *et al.*, 2011, 2013). The high concentration of glycogen under osmoconforming conditions (33 psu) (Figure 4B) indicates that the anterior and posterior gills of males and females of *U. tangeri* constitute sites of glycogen storage. The higher content of glycogen in posterior gills of females compared with males (Figure 4B) supports the idea of a difference in carbohydrate metabolism of gills between sexes. In anterior and posterior gills of males of various species of euryhaline crabs, components of carbohydrate metabolism such as glycogen reserves are differently modulated depending on the type and degree of the osmotic challenge (Martins *et al.*, 2011; Pinoni *et al.*, 2011, 2013). Most animals utilize stored glycogen when coping with different stresses and changes in key environmental factors (Watford, 2015; Wang *et al.*, 2016). Carbohydrates can also support energy demands of various crustaceans during stressful conditions (Wang *et al.*, 2016). The fact that no changes in glycogen content in anterior gills occurred in males or females of *U. tangeri* (Figure 4A) suggests that mobilization of this reserve would not be involved in biochemical adaptation to low (12 psu) or high (55 psu) salinity. This differs to that we previously found in males of the euryhaline crabs *N. granulata* and *Cyrtograpsus angulatus* from Mar Chiquita coastal lagoon (Argentina) (Pinoni *et al.*, 2013; unpublished results). However, the lower free glucose level in anterior gills of females of *U. tangeri* acclimated to low salinity (12 psu) compared with 33 psu (osmoconformation) suggests, as we discussed above for hepatopancreas, the occurrence of specific adjustments in females in carbohydrate pathways (i.e. down-regulation of gluconeogenic pathways, intracellular utilization) underlying hypo-osmoregulation. Gills contain enzymes involved in gluconeogenesis in some species of decapod crustaceans (Bianchini *et al.*, 2008; Wang *et al.*, 2016). The variations found in glycogen and free glucose content in posterior gills of males and females of *U. tangeri* in response to low and high salinity indicate the role of these gills in metabolic reorganization (i.e. carbohydrate metabolism) in response to salinity and, furthermore, points to sex differences and the dependence on the type of osmotic challenge (Figure 4A, B). A further experimental approach is needed to determine whether the specific increase of free glucose in posterior gills of males in low salinity (Figure 4B), while no changes occurred in glycogen concentration (Figure 4A), is related with the activation of gluconeogenesis pathways under hyper-regulation. On the other hand, our results suggest the utilization by both sexes of glycogen reserves in posterior gills in high salinity (55 psu) since this reserve was not detected in males and it was decreased in females (Figure 4A). The maintenance of free glucose content in posterior gills in both sexes in 55 psu (Figure 4B) supports the idea that a rapid intracellular utilization of glucose resulting in glycogen degradation could occur, for instance, for provision of energy needed for the osmoregulatory machine as suggested for other species (Romano & Zeng, 2012). In decapod crustaceans, lactate has multiple metabolic fates (Henry *et al.*, 1994; Maciel *et al.*, 2008; Pellegrino *et al.*, 2008). The lower haemolymph lactate values in females of *U. tangeri* in low and high salinity suggests its potential utilization as substrate for gluconeogenic pathways which could lead to an increase in free glucose in anterior gills and hepatopancreas (anterior gills in low salinity and hepatopancreas in high salinity). This idea is further supported by the fact that glycogen content in hepatopancreas and anterior gills was not affected by environmental salinity (Figures 3 & 4A). In several tissues and organs of *N. granulata*, gluconeogenesis from lactate occurs under different physiological conditions (Pellegrino *et al.*, 2008). The fact that lactate concentration in haemolymph of females in 33 psu was higher than that in males (Figure 2E) could indicate a differential degree of reliance on and a major potential ability for anaerobic energy production of females.

In several crustaceans, protein metabolism plays a key role in biochemical adaptation to environmental salinity by supporting an adequate provision of amino acids for fuelling osmoregulation (Shinji *et al.*, 2012; Romano & Zeng, 2012). However, in some species of euryhaline crab such as males of *N. granulata* from different populations, salinity affects protein metabolism differentially (i.e. no variations, or distinct tissues and organs involved depending on type of osmotic challenge) (Bianchini *et al.*, 2008; Pinoni *et al.*, 2013). Some species enhance free amino acids in the haemolymph (i.e. via breakdown of proteins in the haemolymph or storage organs) in high salinity, but this is not a universal response (Romano & Zeng, 2012). Similarly to that described above for carbohydrate metabolism, *U. tangeri* appears to exhibit sex-specific adjustments in protein metabolism parameters in response to low and high salinity. The higher amino acids levels in hepatopancreas of males in low salinity (12 psu) compared with those in 33 psu (osmoconformation) (Figure 3C) with no concomitant changes in haemolymph proteins or amino acid values (Figure 2B, C), suggests that intracellular adjustments in protein metabolism (i.e. modulation of synthesis/degradation pathways) could be occurring. Gluconeogenesis in the hepatopancreas of the euryhaline crab *N. granulata* from Brazilian populations is involved in osmoregulatory adjustments to hypo-osmotic stress (Oliveira & Da Silva, 2000). Experiments *in vitro* with hepatopancreas sections of males of *N. granulata* suggests that changes in the carbon amino acid flux between gluconeogenesis and lipid synthesis pathways are among the strategies to respond to hypo-osmotic medium (Martins *et al.*, 2011). Whether this is the case for males of *U. tangeri* (which could, for instance, explain in part the higher hepatopancreas free glucose described above) requires further investigation. Since no changes occurred in 55 psu (hyper-osmoregulation), variations in amino acid levels in hepatopancreas of males of *U. tangeri* appears to be a specific adjustment underlying hypo-osmoregulation (Figure 3C). *In vitro* experiments with hepatopancreas sections of males of *N. granulata* showed that adjustments in protein metabolism components are dependent on the degree and type of osmotic challenge (Bianchini *et al.*, 2008). The decrease in haemolymph amino acids concentration in females of *U. tangeri* in low and high salinity with no changes in haemolymph protein concentration (Figure 2B, C) suggests, contrary to males, a utilization or a diminished provision of circulating amino acids from different sites under osmoregulation. As we described above, the hepatopancreas is the main site for absorption of products of digestion (i.e. amino acids) (Carter & Mente, 2014; Lignot & Charmantier, 2015; Saborowski, 2015). The similar amino acid levels in the hepatopancreas of females in low and high salinity with respect to those in 33 psu, suggest that adjustments in the hepatopancreas (i.e. a

minor absorption or protein digestion) would not account for the decrease in haemolymphatic pool. Furthermore, it indicates the occurrence of sex-specific adjustments in protein metabolism in response to environmental salinity (Figures 2 & 3). The fact that no changes occurred in amino acid content in gills of females suggests that other destination sites could be using amino acids from haemolymph.

In crustaceans, lipids are mainly stored as triglycerides (over 80–90%) which are a major source of energy in various species. The hepatopancreas is the main site for this storage (Wright & Ahearn, 1997; Sánchez-Paz *et al.*, 2006; Dima *et al.*, 2009; Pinoni *et al.*, 2013). In males of various euryhaline crabs, acclimation to different environmental salinities induces changes in lipids concentration or mobilization in the hepatopancreas and gills suggesting that increased lipid oxidation is associated with hyper- and hypo-osmotic regulation. However, these responses are species-dependent (Luvizotto-Santos *et al.*, 2003; Pinoni *et al.*, 2013; Jimenez & Kinsey, 2015). The decreased triglycerides concentration in the hepatopancreas of males of *U. tangeri* in low and high salinity (Figure 3D) suggests that a mobilization of this reserve is also one component of biochemical adaptation upon hyper- and hypo-osmoregulation conditions. This differs to that found in males of *N. granulata* in which no changes in hepatopancreas triglycerides or total lipids content occurred under acclimation to low salinity (Luvizotto-Santos *et al.*, 2003; Pinoni *et al.*, 2013). Whether the decrease in triglyceride content in hepatopancreas of males of *U. tangeri* is linked to the use of stored lipid in gluconeogenesis pathways leading to the concomitant increase in free glucose level (Figure 3B) remains to be investigated. This is further supported by the fact that no changes in haemolymph triglycerides levels (Figure 2D). As we pointed out above, *in vitro* hypo- and hyper-osmotic stresses on hepatopancreas sections of *N. granulata* induce different adjustments in gluconeogenesis pathways (Martins *et al.*, 2011). Again, sex differences occurred in response to salinity in lipid metabolism parameters in *U. tangeri*. In females, low and high salinity had no effect on hepatopancreas triglyceride values (Figure 3D), suggesting that utilization of this lipid reserve would not be involved in biochemical adaptation to salinity. Triglycerides in the hepatopancreas of females could be conserved to be used as an energy source for other energy-demanding physiological processes (e.g. reproduction) as suggested for various crustacean species (Lautier & Lagarrigue, 1988; Mourente *et al.*, 1994; Ituarte *et al.*, 2006). The triglycerides concentration enhancement in haemolymph of females acclimated to 55 psu indicates that other adjustments in lipid metabolism could be occurring upon hypo-osmoregulation. Anterior and posterior gills are sites of triglycerides storage in some species of crustaceans (Buckup *et al.*, 2008) while not in others (Pinoni *et al.*, 2013). The high content of triglycerides in gills of males and females of *U. tangeri* indicates their role as potential site of storage or utilization of this reserve under different environmental challenges. However, adjustments in triglyceride content in gills occurred only in males, in which the higher triglycerides content in posterior gills in 12 psu (Figure 4D) suggest the modulation of lipid metabolism (i.e. anabolism) under hyper-osmoregulation. This agrees with the reported increases in lipid content in gills of juveniles of the mud crab *Scylla serrata* under low salinities (Romano *et al.*, 2014). The fact that in males acclimated to high salinity (55 psu), triglyceride values were affected in the hepatopancreas but not in the posterior gills, unlike the values found in 12 psu (Figures 3 & 4D), suggests the occurrence of differential pathways to site-specific storage and of distinct mechanisms of regulation of this reserve depending on osmotic challenge direction. Since, the lipid metabolic routes are unknown in *U. tangeri*, a further experimental approach is needed to establish the components involved and the mechanisms of regulation of triglycerides metabolism (i.e. catabolism/anabolism) in the hepatopancreas and posterior gills.

In conclusion, the results of this work show that concentrations of (i) glycogen in gills, (ii) free glucose in gills and hepatopancreas, (iii) amino acids in hepatopancreas, (iv) triglycerides in haemolymph, hepatopancreas and posterior gills, and (v) lactate in the haemolymph, are differentially affected upon acclimation to low and high salinity (12 and 55 psu, respectively) in male and female *U. tangeri*, suggesting the occurrence of differential metabolism upon hyper- and hypo-osmoregulation related to sex. Future studies should be focused on the mechanisms of regulation involved to provide a better understanding of differences between sexes of the complex responses underlying biochemical adaptation to salinity in *U. tangeri*, in particular, and in euryhaline crabs in general.

FINANCIAL SUPPORT

This study was partly supported by grant AGL2013-48835-C2-1-R (Ministerio de Economía y Competitividad, Spain) to Juan Miguel Mancera. SAP and ALM were working at Dr Mancera lab in the context of a working commission allowed by CONICET and the University of Mar del Plata, Argentina.

REFERENCES

Allender M.C., Schumacher J., Milam J., George R., Cox S. and Martín-Jiménez T. (2008) Pharmacokinetics of intravascular itraconazole in the American horseshoe crab (*Limulus polyphemus*). *Journal of Veterinary Pharmacology and Therapeutics* 31, 83–86.

Bautista- Chamizo E., De Orte M.R., Del Valls A. and Riba I. (2016) Simulating CO_2 leakages from CCS to determine Zn toxicity using the marine microalgae *Pleurochrysis roscoffensis*. *Chemosphere* 144, 955–965.

Bianchini A., Machado Lauer M., Nery L., Pinto Colares E., Monserrat J.M. and dos Santos Filho E.A. (2008) Biochemical and physiological adaptations in the estuarine crab *Neohelice granulata* during salinity acclimation. *Comparative and Biochemical Physiology A* 151, 423–436.

Buckup L., Dutra B., Ribarcki F., Fernandes F., Noro C., Oliveira G. and Vinagre A. (2008) Seasonal variations in the biochemical composition of the crayfish *Parastacus defossus* (Crustacea, Decapoda) in its natural environment. *Comparative and Biochemical Physiology A* 149, 59–67.

Carter C.G. and Mente E. (2014) Protein synthesis in crustaceans: a review focused on feeding and nutrition. *Central European Journal of Biology* 9, 1–10.

Chen K., Li E., Li T., Xu C., Wang X., Lin H. and Chen L. (2015) Transcriptome and molecular pathway analysis of the hepatopancreas in the Pacific white shrimp *Litopenaeus vannamei* under chronic

low-salinity stress. *PLoS ONE* 10, e0131503. doi: 10.1371/journal.pone.0131503.

Cheng L., Jin X.K., Li W.W., Li S., Guo X.N., Wang J., Gong Y.N., He L. and Wang Q. (2013) Fatty acid binding proteins FABP9 and FABP10 participate in antibacterial responses in Chinese mitten crab. *Eriocheir sinensis*. *PLoS ONE*. 8, e54053. doi: 10.1371/journal.pone.0054053

de Gibert J.M., Muñiz F., Belaústegui Z. and Hyžný M. (2013) Fossil and modern fiddler crabs (*Uca tangeri*: Ocypodidae) and their burrows from SW Spain: ichnologic and biogeographic implications. *Journal of Crustacean Biology* 33, 537–551.

Dima J.M., De Vido N.A., Leal G.A. and Barón P.J. (2009) Fluctuations in the biochemical composition of the Patagonian stone crab *Platyxanthus patagonicus* A. Milne Edwards, 1879 (Platyxanthidae: Brachyura) throughout its reproductive cycle. *Scientia Marina* 73, 423–430.

Drews G. and Graszynski K. (1983) Ökophysiologische Untersuchung zur osmoregulatorischen Leistung der euryhalinen Winkerkrabbe *Uca tangeri* (Eydoux 1835) in ihrem Lebensraum und der Atlantikküste Südwest-Spaniens. *Verhandlungen der Deutschen Zoologischen Gesellschaft in Hamburg* 76, 225.

Ferrón S., Alonso Pérez F., Ortega T. and Forja J.M. (2009) Benthic respiration on the northeastern shelf of the Gulf of Cádiz (SW Iberian Peninsula). *Marine Ecology Progress Series* 392, 69–80.

Figueiredo M.S.R.B. and Anderson A.J. (2009) Digestive enzyme spectra in crustacean decapods (Paleomonidae, Portunidae and Penaeidae) feeding in the natural habitat https://en.wikipedia.org/wiki/Aquaculture_Research. *Aquaculture Research* 40, 282–291.

González-Gordillo J.I., Arias A.M., Rodríguez A. and Drake P. (2003) Recruitment patterns of decapod crustacean megalopae in a shallow inlet (SW Spain) related to life history strategies. *Estuarine, Coastal and Shelf Science* 56, 593–607.

Havird J.C., Mitchell R.T., Henry R.P. and Santos S.R. (2016) Salinity-induced changes in gene expression from anterior and posterior gills of *Callinectes sapidus* (Crustacea: Portunidae) with implications for crustacean ecological genomics. *Comparative Biochemistry and Physiology D* 19, 34–44.

Henry R., Booth C.E., Lallier F.H. and Walsh P.J. (1994) Post-exercise lactate production and metabolism in three species of aquatic and terrestrial decapod crustaceans. *Journal of Experimental Biology* 186, 215–234.

Ituarte R., Bas C., Luppi T. and Spivak E. (2006) Interpopulational differences in the female reproductive cycle of the southwestern Atlantic estuarine crab *Chasmagnathus granulatus* Dana, 1851 (Brachyura: Grapsoidea: Varunidae). *Scientia Marina* 70, 709–718.

Jimenez A.G. and Kinsey S.T. (2015) Energetics and metabolic regulation. *The Natural History of the Crustacea* 4, 391–419.

Jin X.K., Li W.W., Cheng L., Li S., Guo X.N., Yu A.Q., Wu M.H., He L. and Wang Q. (2012) Two novel short C-type lectin from Chinese mitten crab, Eriocheir sinensis, are induced in response to LPS challenged. *Fish and Shellfish Immunology* 33, 1149–1158.

Jin X.K., Li W.W., He L., Lu W., Cheng L.L., Wang Y., Jiang H. and Wang Q. (2011) Molecular cloning, characterization and expression analysis of two apoptosis genes, caspase and nm23, involved in the antibacterial response in Chinese mitten crab. *Eriocheir sinensis*. *Fish and Shellfish Immunology* 30, 263–272.

Kelly S.A., Panhuis T.M. and Stoehr A.M. (2012) Phenotypic plasticity: molecular mechanisms and adaptive significance. *Comparative Physiology* 2, 1417–1439.

Keppler D. and Decker K. (1974) Glycogen. Determination with amyloglucosidase. In Bergmeyer H.U. (ed.) *Methods of enzymatic analysis*. New York, NY: Academic Press, pp. 1127–1131.

Krippeit-Drews P., Drews G. and Graszynski K. (1989) Effects of ion substitution on the transepithelial potential difference of the gills of the fiddler crab *Uca tangeri*: evidence for a H^+-pump in the apical membrane. *Journal of Comparative Physiology B* 159, 43–49.

Larsen E.H., Deaton L.E., Onken H., O'Donnell M., Grosell M., Dantzler W.H. and Weihrauch D. (2014) Osmoregulation and excretion. *Comprehensive Physiology* 4, 405–573.

Lautier J. and Lagarrigue J.G. (1988) Lipid metabolism of the crab *Pachygrasalinitys marmoratus* during vitellogenesis. *Biochemical Systematics and Ecology* 16, 203–212.

Lignot J.H. and Charmantier G. (2015) Osmoregulation and excretion. In Chang E.S. and Thiel M. (eds) *The natural history of Crustacea*. Volume 4. New York, NY: Oxford University Press, pp. 249–284.

Lignot J.H., Spanings-Pierrot C. and Charmantier G. (2000) Osmoregulatory capacity as a tool in monitoring the physiological condition and the effect of stress in crustaceans. *Aquaculture* 191, 209–245.

Lorenzon S., Edomi P., Giulianini P.G., Mettulio R. and Ferrero E.A. (2005) Role of biogenic amines and cHH in the crustacean hyperglycemic stress response. *Journal of Experimental Biology* 208, 3341–3347.

Lorenzon S., Giulianini P.G., Martinis M. and Ferrero E.A. (2007) Stress effect of different temperatures and air exposure during transport on physiological profiles in the American lobster *Homarus americanus*. *Comparative Biochemistry Physiology A* 147, 94–102.

Luvizotto-Santos R., Lee J., Branco Z., Bianchini A. and Nery L. (2003) Lipids as energy source during salinity acclimation in the euryhaline crab *Chasmagnathus granulata* Dana, 1851 (Crustacea-Grapsidae). *Journal of Experimental Zoology A* 295, 200–205.

Maciel F.E., Geihs M.A., Cruz B.P., Vargas M.A., Allodi S., Marins L.F. and Nery L.E.M. (2014) Melatonin as a signaling molecule for metabolism regulation in response to hypoxia in the crab *Neohelice granulata*. *International Journal of Molecular Sciences* 15, 22405–22420.

Maciel F.S., Valle S., Kucharski L.C. and Da Silva R.M.S. (2008) Lactate metabolism in the muscle of the crab *Chasmagnathus granulatus* during hypoxia and post-hypoxia recovery. *Comparative Biochemistry Physiology A* 151, 61–65.

Martins T.L., Chittó A.L.F., Rossetti C.R., Brondani C.K., Kucharski L.C. and Da Silva R.S.M. (2011) Effects of hypo- or hyperosmotic stress on lipid synthesis and gluconeogenic activity in tissues of the crab *Neohelice granulata*. *Comparative Biochemistry Physiology A* 158, 400–405.

McNamara J.C. and Faria S.C. (2012) Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: a review. *Journal of Comparative Physiology B* 182, 997–1014.

Michiels M.S., del Valle J.C. and López Mañanes A.A. (2013) Effect of environmental salinity and dopamine injections on key digestive enzymes in hepatopancreas of the euryhaline crab *Cyrtograpsus angulatus* (Decapoda: Brachyura: Varunidae). *Scientia Marina* 77, 129–136.

Michiels M.S., del Valle J.C. and López Mañanes A.A. (2015) Biochemical characteristics and modulation by external and internal factors of aminopeptidase-N activity in the hepatopancreas of a euryhaline burrowing crab. *Journal of Comparative Physiology B* 185, 501–510.

Moore S. (1968) Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the nynhidrin reaction. *Journal of Biological Chemistry* 1242, 6281–6283.

Mourente G., Medina A., González S. and Rodríguez A. (1994) Changes in lipid class and fatty acid contents in the ovary and midgut gland of

the female fiddler crab *Uca tangeri* (Decapoda, Ocypodiadae) during maturation. *Marine Biology* 121, 187–197.

Ninlanon W. (2011) Effect of pre-cooling on muscle yield of mud crab, *Scylla serrata*, during emersion storage. *2nd International Conference on Biotechnology and Food Science IPCBEE* 7, 126–129.

Oliveira G.T. and Da Silva R.S.M. (2000) Hepatopancreas gluconeogenesis during hyposmotic stress in *Chamagnathus granulata* crabs maintained on high-protein or carbohydrate-rich diets. *Comparative Biochemistry Physiology B* 127, 375–381.

Pellegrino R., Kucharski L.C. and Da Silva R.S.M. (2008) Effect of fasting and refeeding on gluconeogenesis and glyconeogenesis in the muscle of the crab *Chasmagnathus granulatus* previously fed a protein- or carbohydrate-rich diet. *Journal of Experimental Marine Biology and Ecology* 358, 144–150.

Pennoyer K., Himes A.R. and Frederich M. (2016) Effects of sex and color phase on ion regulation in the invasive European green crab, *Carcinus maenas*. *Marine Biology* 163, 137.

Pereira K.C., Costa P.M., Costa M.H., Luque A., DelValls T.A. and Riba López I. (2016) Effects of the increase of temperature and CO_2 concentration on polychaetae *Nereis diversicolor*: simulating extreme scenarios of climate change in marine sediments. *Hydrobiologia*. 772, 161–174. doi: 10.1007/s10750-016-2656-3.

Pfenning D.W., Wund M.A., Snell-Rood E.C., Cruickshank T., Schlichting C.D. and Moczek A.P. (2010) Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology and Evolution* 25, 459–467.

Piersma T. and Drent J. (2003) Phenotypic flexibility and the evolution of organismal design. *Trends in Ecology and Evolution* 18, 228–233.

Pinoni S., Méndez E. and López Mañanes A.A. (2015) Digestive flexibility in a euryhaline crab from a SW Atlantic coastal lagoon: alkaline phosphatase activity sensitive to salinity in the hepatopancreas. *Journal of the Marine Biological Association of the United Kingdom* 95, 1133–1140.

Pinoni S.A., Iribarne O. and López Mañanes A.A. (2011) Between-habitat comparison of digestive enzymes activities and energy reserves in the SW Atlantic euryhaline burrowing crab *Neohelice granulata*. *Comparative Biochemistry Physiology A* 158, 552–559.

Pinoni S.A., Michiels M.S. and López Mañanes A.A. (2013) Phenotypic flexibility in response to environmental salinity in the euryhaline crab *Neohelice granulata* from the mudflat and the saltmarsh of a SW coastal lagoon. *Marine Biology* 160, 2647–2661.

Prymaczok N.C., Pasqualino V.M., Viau V.E., Rodríguez E.M. and Medesani D.A. (2016) Involvement of the crustacean hyperglycemic hormone (CHH) in the physiological compensation of the freshwater crayfish *Cherax quadricarinatus* to low temperature and high salinity stress. *Journal of Comparative Physiology B* 186, 181–191.

Ribeiro K., Papa L.P., Vicentini C.A. and Franceschini Vicentini I.B. (2014) The ultrastructural evaluation of digestive cells in the hepatopancreas of the Amazon River prawn, *Macrobrachium amazonicum*. *Aquaculture Research* 2014, 1–9.

Rodríguez-Tovar F.J., Mayoral E. and Santos A. (2014) Influence of physicochemical parameters on burrowing activities of the fiddler crab *Uca tangeri* at the Huelva Coast (Southwest Spain): palaeoichnological implications. *Ichnos* 21, 147–157.

Romano N., Wu X., Zeng C., Genodepa J. and Elliman J. (2014) Growth, osmoregulatory responses and changes to the lipid and fatty acid composition of organs from the mud crab, *Scylla serrata*, over a road salinity range. *Marine Biology Research* 10, 460–471.

Romano N. and Zeng C. (2012) Osmoregulation in decapod crustaceans: implications to aquaculture productivity, methods for potential improvement and interactions with elevated ammonia exposure. *Aquaculture* 334–337, 12–23.

Romano N., Zeng C., Mat Noordin N. and Ng W.-K. (2012) Improving the survival, growth and hemolymph ion maintenance of early juvenile blue swimmer crabs, *Portunus pelagicus*, at hypo- and hyper-osmotic conditions through dietary long chain PUFA supplementation. *Aquaculture* 342–343, 24–30.

Saborowski R. (2015) Nutrition and digestion in physiology. In Chang E.S. and Thiel M. (eds) *The natural history of Crustacea*. Volume 4. New York, NY: Oxford University Press, pp. 285–319.

Sánchez-Paz A., García-Carreño F., Muhlia-Almazan A., Peregrino-Uriarte A., Hernández-López J. and Yepiz-Plascencia G. (2006) Usage of energy reserves in crustaceans during starvation: status and future directions. *Insect Biochemistry Molecular Biology* 36, 241–249.

Shinji J., Kang B., Okutsu T., Banzai K., Ohira T., Tsutsui N. and Wilder M. (2012) Changes in crustacean hyperglycemic hormones in Pacific whiteleg shrimp *Litopenaeus vannamei* subjected to air exposure and low-salinity stresses. *Fish Science* 78, 833–840.

Spivak E.D. and Cuesta J.A. (2009) The effect of salinity on larval development of *Uca tangeri* (Eydoux, 1835) (Brachyura: Ocypodidae) and new findings of the zoeal morphology. *Scientia Marina* 73, 297–305.

Wang X., Li E. and Chen L. (2016) A review of carbohydrate nutrition and metabolism in crustaceans. *North American Journal of Aquaculture* 78, 178–187.

Wang Z.-J., Lee J., Sia Y.X., Wanga W., Yang J.M., Yina S.J., Qiana G.Y. and Park Y.D. (2014) A folding study of Antarctic krill (*Euphausia superba*) alkaline phosphatase using denaturants. *International Journal of Biological Macromolecules* 70, 266–274.

Watford M. (2015) Starvation: metabolic changes. In eLS (Encyclopedia of Life Sciences) [online publication] Hoboken, NJ: Wiley, pp. 17.

Wright S.H. and Ahearn G.A. (1997) Nutrient absorption in invertebrates. In Dantzler W.H. (ed.) *Handbook of physiology, section 13: comparative physiology*. New York, NY: Oxford University Press, pp. 1137–1206.

Zar J.H. (1999) *Biostatistical analysis*. Mahwah, NJ: Prentice-Hall, Inc.

and

Zeng H., Ye H., Li S., Wang G., Huang J. (2010) Hepatopancreas cell cultures from mud crab, *Scylla paramamosain*. *In Vitro Cellular & Developmental Biology – Animal* 46, 431–437.

Correspondence should be addressed to:
A.A. López Mañanes
Instituto de Investigaciones Marinas y Costeras (IIMyC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Universidad Nacional de Mar del Plata, Mar del Plata, Argentina
email: mananes@mdp.edu.ar