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Osmoregulation in larvae and juveniles of two recently separated *Macrobrachium* species: Expression patterns of ion transporter genes

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Abstract :

In this comparative study, osmoregulatory mechanisms were analyzed in two closely related species of palaemonid shrimp from Brazil, *Macrobrachium pantanalense* and *Macrobrachium amazonicum*. A previous investigation showed that all postembryonic stages of *M. pantanalense* from inland waters of the Pantanal are able to hyper-osmoregulate in fresh water, while this species was not able to hypo-osmoregulate at high salinities. In *M. amazonicum* originating from the Amazon estuary, in contrast, all stages are able to hypo-osmoregulate, but only first-stage larvae, late juveniles and adults are able to hyper-osmoregulate in fresh water. The underlying molecular mechanisms of these physiological differences have not been known. We therefore investigated the expression patterns of three ion transporters (NKA α-subunit, VHA B-subunit and NHE3) following differential salinity acclimation in different ontogenetic stages (stage-V larvae, juveniles) of both species. Larval NKAα expression was at both salinities significantly higher in *M. pantanalense* than in *M. amazonicum*, whereas no difference was noted in juveniles. VHA was also more expressed in larvae of *M. pantanalense* than in those of *M. amazonicum*. When NHE3 expression is compared between the larvae of the two species, further salinity-related differences were observed, with generally higher expression in the inland species. Overall, a high expression of ion pumps in *M. pantanalense* suggests an evolutionary key role of these transporters in freshwater invasion.

Keywords : Salinity, Crustaceans, Branchial chamber, NHE3 transporter, V-type H+-ATPase, Na+/K+-ATPase

Introduction

Palaemonid shrimps are thought to originate from an ancestral tropical marine clade, which has shown a world-wide evolutionary tendency to adapt to non-marine conditions and successfully invade estuarine and limnic environments (review in Anger, 2013; Freire et al., 2003; Bauer, 2004; Augusto et al., 2009). Among those belonging to the genus *Macrobrachium*, *M. amazonicum* (Heller, 1862) has been considered as a particularly successful invader of South American freshwater habitats, showing an extremely wide range of geographic distribution in various hydrologically separated drainage systems. In recent years, however, a growing body of evidence suggested that "*M. amazonicum*" actually comprises at least two geographically and genetically distinct cryptic species (review in Weiss et al. 2015). One of these lives near the Amazon estuary, which is the type locality of *M. amazonicum*, another one is hololimnetic, living in landlocked inland waters (including the wetlands of the Pantanal region in southwestern Brazil) which drain to the La Plata River. Recent studies comparing shrimps from these two regions revealed biologically significant differences in various reproductive, developmental, morphometric, and ecological traits (Porto, 2004; Anger and Hayd, 2010; Urzúa and Anger, 2011; Hayd and Anger, 2013). Corresponding to their different life styles (estuarine *versus* hololimnic), significant differences were also found in adaptive physiological traits, especially in the osmoregulatory capabilities and structures of successive ontogenetic stages (Charmantier and Anger, 2011; Boudour-Boucheker et al., 2013). In hololimnetic shrimp from the Pantanal, all life-history stages are capable of hyper-osmoregulation in fresh water, while the function of hypo-osmoregulation at high salt concentrations is completely absent (Charmantier and Anger, 2011). Pantanal shrimp, which previously had been considered as a population of *M. amazonicum*, were described as a separate species, *M. pantanalense* (dos Santos et al., 2013).

Aquatic species living in or near to estuarine waters have to cope with wide salinity fluctuations. An elaborate mechanism of membrane transport processes facilitates at high salinities rapid removal of excess ions from the hemolymph, and at low salinities active ion absorption (Charmantier et al., 2009). The gills of marine and brackish-water crustaceans play a key role in the exchange of osmotically active substances between the environment and the hemolymph, thanks to the presence of specialized ion transporting cells (Lucu and Siebers, 1986). In palaemonid shrimps, ion transporters and channels are located in two different cell types within gill lamellae: septal and pillar cells (McNamara and Faria, 2012). Na^+/K^+-ATPase (NKA), one of the driving forces facilitating transepithelial transport mechanisms, is located in the intralamellar septal cells (Boudour-Boucheker et al., 2013). The other driving force, particularly at low salinity, might be the V-type H^+-ATPase (VHA) located in pillar

cells, particularly in the apical plasma membrane and membrane vesicles (Boudour-Boucheker et al., 2014).

Studies on different crustacean species have produced a variety of ion transport models (Lucu, 1990; Towle, 1990; Taylor and Taylor, 1992; Péqueux, 1995; Onken and Riestenpatt, 1998). Current models in palaemonid shrimps suggest that primary active excretion of H^+ *via* VHA drives secondary Na^+ absorption *via* an apical epithelial Na^+ Channel (ENaC) (McNamara and Faria, 2012). Other models in crustaceans propose the $Na^+/K^+/2Cl^-$ symporter or a Na^+/H^+ exchanger (NHE) as possible routes for apical Na^+ entry (McNamara and Faria, 2012). In palaemonid shrimps, septal cells express Na^+/K^+-ATPase (McNamara and Torres, 1999; Boudour-Boucheker et al., 2013) to drive Na^+ uptake, possibly sustained through Na^+ transporters or channels in the pillar cells that have not been identified. Na^+/H^+ exchangers (NHE) have been reported to be involved in pH homeostasis, cell volume regulation as well as transepithelial Na^+ transport (Wheatly and Gao, 2004). In teleost gills, Na^+/H^+ exchangers appear to have similar functions to those of other vertebrates, namely exchanging one Na^+ for one H^+ (Claiborne et al., 2002). Na^+/H^+ exchangers have also been studied in many invertebrate organisms and organs including crustacean crab gills (*Carcinus maenas*, Shetlar and Towle, 1989), lobster and prawn hepatopancreas (*Homarus americanus*, Ahearn and Clay, 1989; *Macrobrachium rosenbergii*, Ahearn et al., 1990). These studies suggest the presence of an electrogenic Na^+/H^+ exchanger displaying a transport stoichiometry of 2 Na^+/1 H^+ resulting in a polarization of membrane potential (Ahearn et al., 1990). This physiological property differs from those of vertebrate NHE where Na^+ uptake is achieved by electroneutral transport.

A NHE cDNA has been amplified and sequenced from gills of two crab species, *Carcinus maenas* and *Callinectes sapidus* (Newton et al., 1996; Towle et al., 1997). It is closely related to the vertebrate NHE isoforms previously sequenced, notably NHE3, but it is unknown whether this NHE is similar to the electrogenic exchanger described in membrane vesicles from crustacean epithelia (Shetlar and Towle, 1989; Pullikuth et al., 2003). In *C. maenas*, NHE is strongly expressed in gills, showing much lower abundance in all the other tissues examined: These findings suggest a role in acid-base regulation, volume regulation, or Na^+ uptake (Towle et al., 1997). In decapodids, the presence of a branchial Na^+/H^+ exchanger, possibly NHE3, might be important for palaemonid shrimps living in freshwater environments. Recurrent observations of conspicuously contrasting life-history traits suggest diversification in the two *Macrobrachium* species (Boudour-Boucheker et al., 2013; shortly before the taxonomical separation by dos Santos et al. (2013) referred to as different populations of *M. amazonicum*). These observations stimulated the present comparative study

on gene expression patterns during ontogeny. In order to understand if there are different ion transport mechanisms involved, we have investigated the expression patterns of the NKA α-subunit, the VHA B-subunit and the NHE3 following salinity acclimation and in different ontogenetic stages of *M. amazonicum* and *M. pantanalense*.

Materials and methods

Origin and maintenance of animals

Adult *Macrobrachium amazonicum* and *M. pantanalense* were obtained from the State University of Mato Grosso do Sul in Aquidauana (Mato Grosso do Sul, MS, Brazil). Shrimps were transported in cooling boxes to the Helgoland Marine Biological Laboratory (Germany), where they were kept in fresh water (FW; total ion concentration: 0.2 mg/l; 24°C; for more details, see Anger and Hayd, 2010; Charmantier and Anger, 2011). Adult shrimps were fed frozen pieces of marine isopods (*Idotea sp*.) and commercial aquarium food (Novo Tab, JBL). Ovigerous females were maintained in aerated flow-through aquaria with 30 l of FW. Newly hatched larvae were collected in sieves (0.3 mm mesh size) receiving the overflowing water and subsequently reared at 10 or 5 ppt salinity (*M. amazonicum* and *M. pantanalense*, respectively), in aerated 1 l beakers kept at 29°C and a 12:12 h light:dark cycle. Except for the zoea I stage, which is fully lecithotrophic, the larvae were fed freshly hatched *Artemia* sp. nauplii (Anger and Hayd, 2010).

Salinity acclimations

Intermediate and late larval stages (zoea V, decapodids) were acclimated for 24 h either to brackish water (5 ppt) or to slightly diluted seawater (DSW) (25 ppt). Three-week-old juveniles (referred to as early juveniles) were acclimated for 48 h either to 5 ppt or to 25 ppt. Five-month-old juveniles (referred to as late juveniles) were stepwise transferred to FW (0.2 ppt) or to DSW (salinities 20 and 25 ppt) in which they were maintained for 2 weeks. After acclimation, all shrimps were anesthetized by speed freezing, then dissected and maintained in Trizol at -80°C until analysis (see below).

The developmental stages and experimental salinities used in this study were chosen on the basis of previously observed differences in osmoregulatory capacities and survival rates (Charmantier and Anger, 2011), so that survival was always high enough to guarantee an availability of sufficient materials for sampling and measurements. In larvae, a minimal experimental salinity of 5 ppt was chosen because an acclimation to fresh water would have

resulted in high mortalities; this medium was used only for late juveniles. Salinities of 20 and 25 ppt were selected to compare ion transport mechanisms in two species with different osmoregulatory strategies (hypoosmoregulator *vs* osmoconformer).

RNA extraction and cDNA synthesis

Based on microscopic examinations of uropods, juveniles in stage C (intermolt stage) were selected. The small size and short molt cycle of larvae prevented microscopical routine moult-staging; however, larvae were sampled in the middle of each developmental stage, maximising the probability to select animals in stage C (see Hayd et al., 2008). For the extractions, whole larvae were used due to their small size. For early juveniles, only the cephalothorax with the gills, and for late juveniles only the gills were used. The tissues were cut in small pieces with sterile scalpels. Total RNA was extracted using Trizol reagent (Invitrogen, CergyPontoise, Val d'Oise, France) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Invitrogen) to remove any genomic DNA contamination. Quantification of total RNA was performed with a NanoDrop® ND-1000 V3300 Spectrophotometer (Nanodrop Technology Inc., Wilmington, Delaware, USA). Reverse transcription of 250 ng of RNA was performed using M-MLV reverse transcriptase and random primers (Invitrogen) following the manufacturer's instructions. The resulting cDNA was stored at -20°C.

NHE3 and EF1α partial sequencing

Degenerate primers (Table 1) were designed from Bioedit sequence alignments using *nhe3* sequences of several species including: *Aedes aegypti* (AF187723), *Homo sapiens* (NM_004174), *Rattus norvegicus* (NM_012654 XM_346529), *Lepisosteus oculatus* (JF19071), *Eriocheir sinensis* (AF301159), and *Carcinus maenas* (U09274). For Elongation Factor EF1α, a reference gene, the alignment included: *Daphnia magna* (AB734039.1), *Alpheus thomasi* (AF310826.1), *Danio rerio* (DQ083545.1), *Palaemonetes varians* (FJ654544.1), *Litopenaeus vannamei* (GU136229.1), *Saccharomyces cerevisiae* (M10992.1), *Homo sapiens* (NM_001402.5), and *Mus musculus* (NM_010106.2). For EF1α, degenerate primers previously tested on palaemonid shrimps were used (Ituarte et al., in press). Fifty µl of PCR product was then purified (Invitrogen) and sequenced using a BigDye® Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All sequencing was performed on the Génotypage-Séquençage platform of IFR 119 (Montpellier, France) with an ABI Prism 3130 XL 16 capillary Genetic Analyzer instrument (Applied Biosystems). From these partial sequences, specific primers were designed for qPCR (Table 1). Dilution series of a cDNA

mixture of several analyzed samples were performed in order to generate a standard curve and determine the efficiencies of each primer pair used in qPCR (Table 1).

Quantification of NHE3, NKA and VHA expression by quantitative real-time PCR (qPCR)

The specific forward and reverse primers for the target genes, NKA α-subunit and VHA B-subunit were designed based on the published sequences in *M. amazonicum* (Faleiros et al., 2010). For NHE3 and EF1α, specific primers were designed from our sequenced fragments (Table 1). The qPCR analyses were performed with a Light-CyclerTM system version 3.5 (Roche, Mannheim, Baden-Württemberg, Germany), using 2.5 µl of the LightCycler-FastStart DNA Master SYBR Green ITM Mix (Roche), 0.75 µL of each primer (reverse and forward at 0.5 µM) and 1 µL of diluted cDNA at 1/16 corresponding to 0.74 ng equivalent RNA. The qPCR conditions were denaturation at 95°C for 10 min, repeat of amplification (95°C, 15 s), hybridization (58°C, 5 s) and elongation (72°C, 10 s) 40 times, melting curve program at 60°C for 1 min and final cooling step at 40°C for 30 s. For each primer couple, the efficiency was determined and the melting curve was analyzed in order to check the presence of only one amplification product. For each reaction, the crossing point (CP) was determined. All samples were analyzed in triplicate and the mean CP was calculated. The results were normalized with the reference gene EF1α. Ultra-pure water was used as a no-template control in the qPCR. The relative expression of NHE3, NKA and VHA were calculated for each condition (species, salinities and stages) using the advanced relative quantification method (*eg.* E-Method) of the ROCHE Lightcycler 480 software. This method has been used with the real efficiencies calculated before and including a standard in each run. Statistical analyses were performed on 13 animals per condition with one reading per individual.

Statistical analysis

Pairwise comparisons using Wilcoxon rank sum test were performed to check the stability of the reference gene (EF1α). To compare gene expression between salinities, stages and species, we used two-way analysis of variance (ANOVA). A significance level of $p<0.05$ was chosen.

Results

Partial sequencing of EF1 α and NHE3

PCR amplification using degenerate primers resulted in a single amplification product of 262 bp and 495 bp for EF1α and NHE3. The PCR product for each target gene was purified and

sequenced. The respective nucleotide sequences were translated to ORFs that yielded high-scoring BLAST matches to known amino acid sequences of EF1α and NHE3 (Table 2). *Macrobrachium amazonicum* EF1α and NHE3 sequences are available in GenBank under the accession numbers KU158862 and KU158863. The specific primers amplified a sequence of 163 bp for EF1α and 121 bp for NHE3 that were used for relative gene expressions (Table 1).

Gene expression in larval and juvenile stages (NKA-α, VHA and NHE3)

NKA α-subunit expression

In the estuarine species *Macrobrachium amazonicum*, no significant salinity-related difference in the relative expression of NKA-α was found in the two larval stages studied (zoea V, decapodid), nor in early (Fig. 1) or late juveniles (Fig. 2).

In the inland species *M. pantanalense*, the zoea V stage showed, likewise, no difference in the relative expression of NKA-α at different salinities; in the decapodid and juvenile stages, however, a significant increase in NKA-α expression was observed after a direct exposure to 25 ppt salinity (Fig. 1A, B).

Interestingly, NKA-α expression was in all larval stages consistently higher in the inland than in the estuarine species, regardless of salinity, whereas no difference between species was noted in juveniles (Fig. 1B).

VHA B-subunit expression

In larval stages, VHA was slightly but consistently more expressed in *M. pantanalense* compared to *M. amazonicum*, except for the zoea V stage exposed to 25 ppt. No difference between salinities was noted, except for the zoea V of the in inland species, where VHA was more strongly expressed at 5 ppt salinity compared to 25 ppt (Fig. 1C).

In juveniles, VHA was in *M. amazonicum*, but not in *M. pantanalense*, more strongly expressed at 5 ppt compared to 25 ppt. Moreover, *M. amazonicum*, had higher VHA expression at 5 ppt than the inland species (Fig. 1D).

In late juveniles, VHA expression was in the estuarine species higher in FW than at 25 ppt salinity (Fig. 2).

NHE3 expression

In the zoea V stage of *M. amazonicum*, no difference in NHE3 expression was noted between salinities (Fig. 1E). In *M. pantanalense*, by contrast, the same larval stage showed a higher

expression at 5 ppt salinity compared to 25 ppt. A comparison between the two species shows an overall higher expression in the inland species exposed to 5 ppt. In the decapodid stage, the estuarine species had a higher NHE3 expression at 5 ppt salinity compared to 25 ppt whereas the opposite pattern was observed in the inland species, with a stronger expression at 25 ppt. A difference was noted between the decapodids of the two species exposed to 25 ppt, with higher NHE3 expression in the inland species (Fig. 1E).
Early and late juveniles showed no significant salinity-dependent or species-specific differences in NHE3 expression (Figs. 1F, 2).

Comparing the expression of the three analyzed transporters in late juveniles of *M. amazonicum*, VHA was significantly higher expressed than NKA-α and NHE3, by about 4-fold in FW and 2.5-fold at 25 ppt.

Discussion

Na^+/K^+-ATPase (NKA)

In the present study, we measured a consistently higher NKA-α expression in *M. pantanalense* (inland species) than in *M. amazonicum* (estuarine species) in the analyzed larval stages (zoea V and decapodid) at both salinities (5 and 25 ppt). However in the juvenile stage there was no difference between species. This result could be related to the developmental differences previously described between these two species: The larvae of the inland species show an accelerated gill development, along with an earlier development of ion transporting cells expressing NKA; these traits have been interpreted as an adaptation allowing for complete development in fresh water (Boudour-Boucheker et al., 2013). The branchial NKA is probably involved in Na^+ absorption from fresh water, which results in the possibility for this species to survive and live in this environment. In the estuarine species, the less expressed NKA, previously shown to be localized in the branchiostegites essentially, would be sufficient to drive ion transport in brackish water environments, but insufficient in FW.

In the inland species, differences in NKA-α expression between salinities were measured in decapodid and juvenile stages with higher expression at 25 ppt compared to 5 ppt salinity. Salinity-induced variations in NKA-α expression also occur in crab gill tissues (Lucu and Flik, 1999; Masui et al., 2005; Lovett et al., 2006). In the crab *Chasmagnatus granulata*, the transfer from a hyper-osmotic to a hypo-osmotic medium induced an increase in NKA-α

277 expression (Luquet et al., 2005). However, NKA-α expression varies little in *M. olfersi*
278 (Mendonça et al., 2007), *Callinectes sapidus* (Towle et al., 2001) and *Scylla paramamosain*
279 (Chung and Lin, 2006), despite considerable alterations in specific activity of gill NKA. A
280 discrepancy between transcript levels and protein activity suggests that post-transcriptional
281 mechanisms are involved. Increased NKA activity and no change in transcript levels could
282 thus be linked to mechanisms such as subunit assembly, membrane trafficking, or cell
283 signaling. Faleiros et al. (2010) have also studied NKA α-subunit expression in addition to
284 enzyme activity following salinity transfer in an inland shrimp population referred to as "*M.*
285 *amazonicum*". Their material was collected from an artificial lake in the state of São Paulo,
286 Brazil (Paraná River system, draining to the La Plata, as in *M. pantanalense*). This species has
287 thus probably the same hololimnic lifestyle as *M. pantanalense*. The relative expression of
288 NKA-α in the gills of adult shrimps increased markedly during short term (1 h, 5 h, 1 day)
289 acclimation to 25 ppt salinity. This pattern of salinity-induced variations in NKA-α expression
290 has been observed in this study in the inland species after exposures during one day for larval
291 stages and two days for juveniles. We can thus hypothesize that the regulation of NKA occurs
292 at a transcriptional level after a short time and from the larval decapodid stage on; it is
293 probably accomplished by hormonal modulation of NKA-α expression, a mechanism that is
294 well documented in euryhaline fish species (McCormick, 1995) but less in crustaceans. In
295 crustaceans, neuro-endocrine control of osmoregulation, demonstrated mostly in decapods
296 (reviews in Mantel and Farmer, 1983; Péqueux, 1995; Charmantier et al., 2009), is probably
297 effected by CHH, the Crustacean Hyperglycemic Hormone (Charmantier-Daures et al., 1994;
298 Spannings-Pierrot et al., 2000), along with dopamine, and cAMP probably involved as second
299 messenger, both able to modulate NKA activity (Liu et al., 2008; review in Charmantier et al.
300 2009). These results imply that the energetic costs of hypo-osmoregulation under stressful
301 conditions are met, at least in part, by enhanced NKA-α expression. The increase in NKA-α
302 expression in decapodid and early juvenile stages exposed to high salinity could also be
303 related to the synthesis of new enzymes necessary for the preparation and successful
304 transition during the metamorphic molt. According to Faleiros et al. (2010), following a long-
305 term (5-10 days) salinity acclimation to 25 ppt, branchial NKA activity is lower than in fresh
306 water, while expression values are comparable to those in fresh water. From these results, as
307 well as from ours, we can suggest that in the hololimnic inland species, NKA-α expression
308 increases following a direct exposure to high salinities and decreases afterwards to values
309 observed in fresh water. NKA is thus essential for both hyper- and hypo-ionic regulation as
310 shown in many euryhaline crustaceans (Charmantier et al., 2009). In the estuarine species, by
311 contrast, no effect of salinity on NKA-α expression has been found during ontogeny. Leone et

al., 2014 have demonstrated changes in NKA activity during the ontogeny of *M. amazonicum* (estuarine species). They suggested that the kinetic behavior of NKA may be stage-specific, possibly correlating with the biochemical adjustment of each ontogenetic stage to the optimal salinity found in its natural environment. We hypothesize that in the estuarine species, unlike inland species, NKA regulation occurs essentially at post-translational level. This difference in gene expression between both species could be due to different adaptive strategies linked to different habitats. Indeed, 25 ppt salinity represents for the inland species an osmotic challenge that it never faces in its natural habitat. The estuarine species, however, is more adapted to salinity variations owing to its diadromous migrations.

V-type H^+-ATPase (VHA)

The V-type H^+-ATPase appears as a key enzyme for osmoregulation in many organisms (Beyenbach, 2001; Kirschner, 2004; Covi and Hand, 2005). In hyper-osmoregulating freshwater crustaceans, amphibians and fish, VHA seems to be involved in driving ion uptake (Wieczorek et al., 1999; Nelson and Harvey, 1999; Kirschner, 2004; Beyenbach and Wieczorek, 2006), as also shown by studies on gene expression and protein activities (Faleiros et al., 2010; Lee et al., 2011; Towle et al., 2011). In *Eurytemora affinis*, a copepod known as a rapid freshwater invader, freshwater populations exhibited a higher increase in VHA activity in fresh water versus 15 ppt compared to saline populations (Lee et al., 2011).
Higher VHA expression in the larval stages of *M. pantanalense*, which develop in fresh water under natural conditions, may be related to an increased need to energize active ion uptake in fresh water by excreting H^+ to the external medium. The high larval capacity of this inland species to hyper-osmoregulate in fresh water (Charmantier and Anger, 2011) could be sustained by the capacity of expressing higher amounts of VHA than in estuarine larvae. In the zoea V stage of *M. pantanalense*, in which gill lamellae have developed, VHA expression is higher at 5 ppt than at 25 ppt salinity. In adult *M. amazonicum* gills, a striking decrease in VHA activity and expression has been observed 10 days after acclimation of the shrimps from FW to 21 ppt (Santos et al., 2007; Faleiros et al., 2010). In decapodid and juvenile stages of *M. pantanalense*, the absence of salinity-induced differences in VHA expression may be related to the short acclimation time (24h for decapodids and 48h for juveniles). Longer-term salinity acclimation is prevented by the short duration of the early life-history stages.
In the estuarine species, *M. amazonicum*, VHA expression did not vary between larval stages. These stages cannot survive in fresh water, probably partly due to the absence of VHA gene transcription following salinity change. This VHA expression pattern may originates from an

346 incomplete gill development reported in a previous study (Boudour-Boucheker et al., 2013).
347 In contrast, in both early and late juveniles, the gills are well developed, and VHA is more
348 expressed at 5 ppt and FW compared to 25 ppt. Interestingly, VHA seems of particular
349 importance in these more advanced developmental stages of *M. amazonicum*, since its
350 expression is 2.5 and 4 times higher than the other analyzed genes at 25 ppt and in FW. This
351 result supports the importance of branchial VHA in hyper-osmoregulation and salinity
352 adaptation, in relation to the ecology of the estuarine species, whose juvenile stages live in
353 fresh water and thus need an efficient ion uptake, possibly *via* transporters such as NHE or the
354 epithelial Na^+ channel ENaC.
355
356 **Na^+/H^+ exchanger (NHE3)**
357 Due to the functional distinction in the stoichiometry ($2Na^+/1H^+$ *vs* $1Na^+/1H^+$) of exchange in
358 the crustacean forms, further molecular studies are required on the electrogenic and
359 electroneutral Na^+/H^+ exchanger (NHE) of decapods (Towle et al., 1997). To our knowledge,
360 this is the first report of NHE3 in a palaemonid shrimp. In crustaceans, NHEs have been
361 detected in the hepatopancreas of lobsters (Ahearn and Clay, 1989) and shrimp, in *Peneaus*
362 *japonicus* (Vilella et al., 1998), and also in the gills of crabs, *Callinectes sapidus* (Shetlar and
363 Towle, 1989) and *Carcinus meanas* (Towle et al., 1997). Studies on crustacean and
364 echinoderm brush border membrane vesicles of gut, kidney and gill epithelia suggest the
365 presence of an electrogenic Na^+/H^+ ion exchanger with a transport stoichiometry of $2Na^+/1H^+$
366 (Ahearn and Clay, 1989; Shetlar and Towle, 1989; Ahearn and Franco, 1990 and 1991;
367 Ahearn et al., 1990). Using crustacean gut cells, the presence of two external cation-binding
368 sites with dissimilar binding properties was shown kinetically through external inhibition of
369 Na^+ transport (Ahearn and Clay, 1989; Ahearn and Franco, 1990 and 1991). It has also been
370 reported that this electrogenic system transports Ca^{2+}. The transport of Ca^{2+} and Na^+,
371 considered as competitive inhibitors, was blocked by amiloride, an inhibitor of NHE (Ahearn
372 and Franco, 1990). In the present study, species-specific differences in NHE3 expression
373 occur in larval *Macrobrachium* exposed to different salinities, with generally stronger
374 expression in the inland species, *M. pantanalense*. These results do not permit to confirm the
375 involvement of NHE3 in Na^+ uptake in low salinity media. Other secondary transporters such
376 as ENaC might conduct VHA-driven Na^+ uptake.

377 As a shrimp approaches molting, part of the Ca^{2+} that is contained in the exoskeleton is
378 transferred to the hemolymph across the gills and other permeable sites, or stored in epithelial
379 cells (Greenaway, 1985). Following ecdysis, the stored Ca^{2+} is again transferred to

hemolymph then to the tegument and the newly formed exoskeleton, possibly through NHE3 (Neufeld and Cameron, 1993; Flik and Haond, 2000). As the molting stage was not precisely controlled in larvae, different molting stages among the animals could partly account for the differences observed in NHE3 expression. Other factors such as intracellular pH could also affect gene expression levels (Wheatly and Gao, 2004). Brett et al. (2005) identified a new family of related genes called NHA. Future investigations on regulatory mechanisms, structural components and cellular localization should clarify the precise physiological role of Na^+/H^+ antiporters in *Macrobrachium* shrimps.

In conclusion, a functional difference between *M. amazonicum* and *M. pantanalense* is reported in this study, regarding genes involved in ion transport and their regulatory level. The expression of the main ion pumps NKA, VHA, and to a lesser extent NHE3, is higher in the larval stages of *M. pantanalense* than in *M. amazonicum*, in relation to their different life styles: The former spends its entire life cycle in fresh water while early development stages of the latter are exposed to estuarine salinity variations. The difference in expression of transporters also reflects developmental differences between the two species (Boudour-Boucheker et al., 2013). The strategy of rapid development of functional gills expressing the main ion pumps in the larvae of *M. pantanalense* is probably the major factor allowing this species to survive in fresh water, where its entire life cycle occurs. In addition, the capacity to produce hypotonic urine demonstrated in juveniles of both species and resulting from ionic reabsorption in the excretory antennal glands (Charmantier, unpublished results) probably contributed to the acquisition of hyper-osmoregulation in all larval stages of *M. pantanalense*. Selection pressure operating through successive generations led to a loss of hypo-osmoregulation in salt water, which is no longer needed by a hololimnetic species. In the estuarine species, in contrast, this function is still essential during the larval development in estuarine salt waters.

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591

Figure legends

Fig. 1: Relative expression of NKA-α (A, B), VHA (C, D) and NHE3 (E, F) in larvae (zoea V, decapodid) (A, C, E) and juveniles (B, D, F) of *Macrobrachium amazonicum* (A; estuarine species) and *M. pantanalense* (P; inland species) acclimated to 5 and 25 ppt salinity. Results are expressed as mean ± SEM; different letters indicate significant differences. ZV: zoea V; D: decapodid; JB: juvenile branchial chamber.

Fig. 2: Relative expression of NKA- *α*, VHA and NHE3 in late juveniles of *Macrobrachium amazonicum* (estuarine species) acclimated to fresh water (FW) and 25 ppt salinity. Results are expressed as the mean ± SEM. Different letters indicate significant differences.

Table legends

Table 1: Primer sequences used in this study. CS : primers used for cloning and sequencing ; d : degenerate primers ; F : forward, qPCR : primers used for qPCR ; R : reverse. The sequences used standard IUPAC code : R : A/G, Y : C/T, W : A/T.

Table 2: Percentage amino acid homology of *Macrobrachium amazonicum* EF1α and NHE3 partial sequences. Homologies were calculated according to the blastp algorithm.

Fig. 1

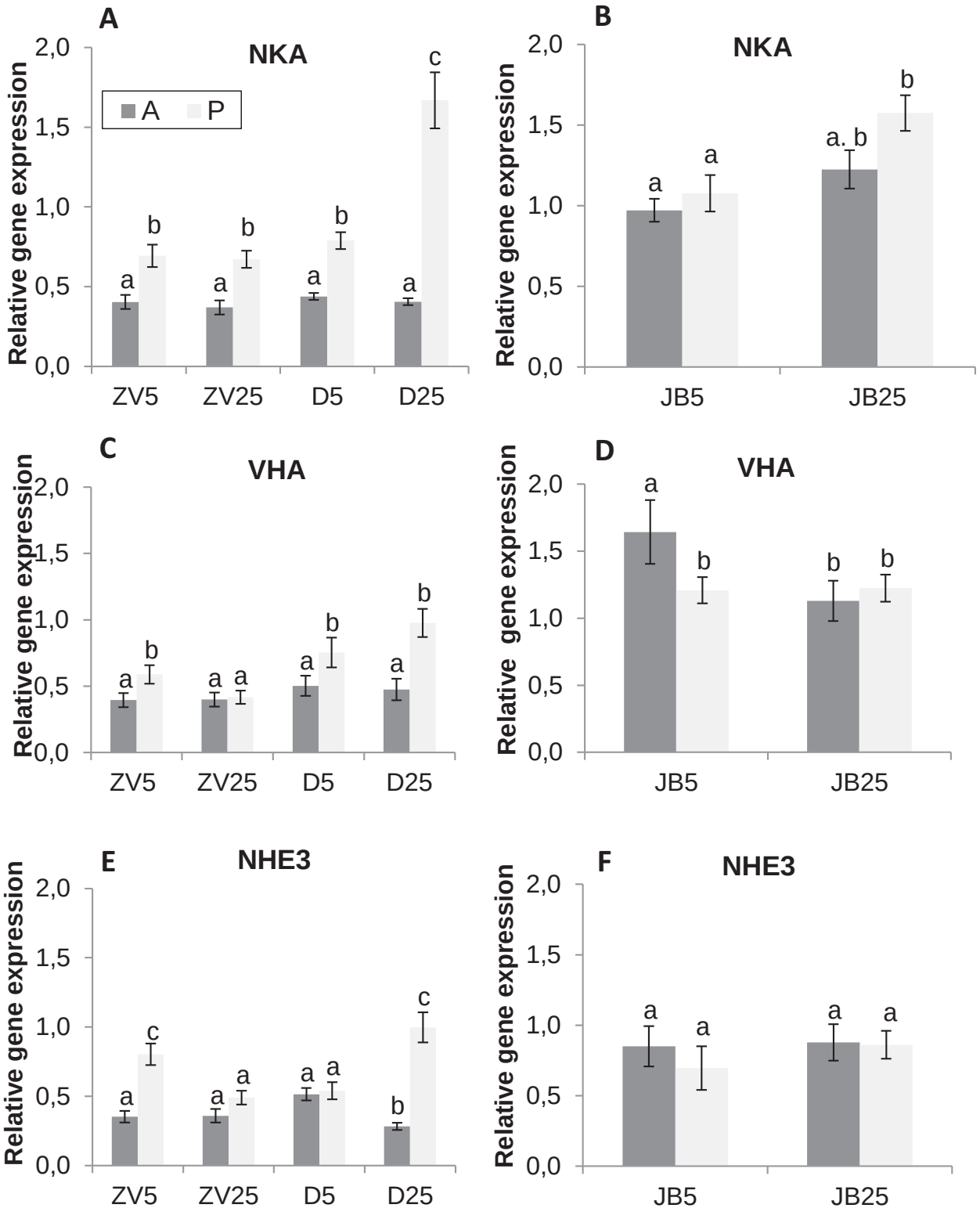
A
NKA
A
P
Relative gene expression
2,0
1,5
1,0
0,5
0,0
a
b
a
b
a
b
a
c
ZV5
ZV25
D5
D25
B
NKA
Relative gene expression
a
a
a. b
b
JB5
JB25
C
VHA
Relative gene expression
a
b
a
a
a
b
a
b
ZV5
ZV25
D5
D25
D
VHA
Relative gene expression
a
b
b
b
JB5
JB25
E
NHE3
Relative gene expression
a
c
a
a
a
a
b
c
ZV5
ZV25
D5
D25
F
NHE3
Relative gene expression
a
a
a
a
JB5
JB25

Fig. 2

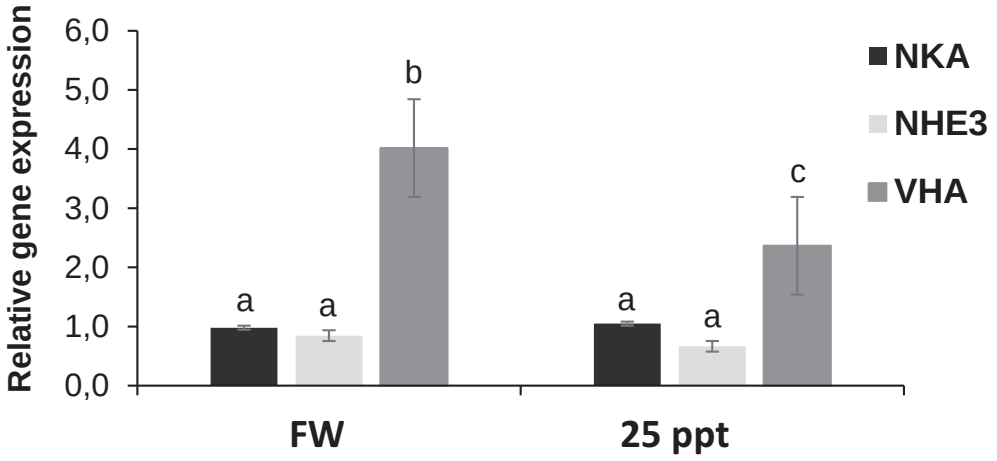
Relative gene expression
6,0
5,0
4,0
3,0
2,0
1,0
0,0
a
a
b
a
a
c
FW
25 ppt
NKA
NHE3
VHA

Table 1 :

Primer name	Nucleotide sequences (from 5' to 3')	Use	Expected size	Efficiency
EFd-F	GARTTYGARGCYGGTATCTC	CS	495 bp (Ituarte et al., in press)	-
EFd-R	GGWGGTATTGGWACWGTGCCC	CS		
NHE3d-F2	GGYTTCCACATGACKCCGAAG	CS	262 bp	-
NHE3d-R3	GGGCGTWCAGAGAGATTCCG	CS		
EF-F4	CAACCCAGCCATTGTACCCATC	qPCR	163 bp	1.938
EF-R4	GGGCTCGATGTTGTCCAGAGC	qPCR		
VHA-F1	TTCCTTCTACTCGACCGGCACG	qPCR	81 bp (Faleiros et al., 2010)	1.999
VHA-R1	TGCCAGGTAGACGTGGTTTCCC	qPCR		
NHE3-F4	GAAGGCGGCATCGGTGTCC	qPCR	121 bp	1.997
NHE3-R7	CGTGCCCAGGTGGTCGAAG	qPCR		
NKA-F3	TACACGCTCACCAAGACCTCCC	qPCR	102 bp (Faleiros et al., 2010)	1.946
NKA-R3	TGGCTTGCGGTGATGTTAAGGG	qPCR		

Table 2 :

Protein	**Species**	**Accession number**	**Query cover (%)**	**Amino acid homology (%)**
Elongation factor 1α (EF1α)	*Macrobrachium rosenbergii*	AGW23427.1	100	95
	Palaemon argentinus	AIW04742.1	98	91
	Penaeus monodon	AIS67294.1	100	85
	Scylla paramamosain	AFN08747.1	100	88
Sodium-hydrogen exchanger 3 (NHE3)	*Cherax destructor*	AJO70016.1	97	63
	Amyelois transitella	XP_013190821.1	97	63
	Cherax quadricarinatus	AIW68618.1	97	62
	Plutella xylostella	XP_011563892.1 XP_011563894.1	97	69