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Kinetic Analysis of Gill (Na^+,K^+)-ATPase Activity in Selected Ontogenetic Stages of the Amazon River Shrimp, *Macrobrachium amazonicum* (Decapoda, Palaemonidae): Interactions at ATP- and Cation-Binding Sites

Francisco Assis Leone · Douglas Chodi Masui · Thais Milena de Souza Bezerra · Daniela Pereira Garçon · Wagner Cotroni Valenti · Alessandra Silva Augusto · John Campbell McNamara

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Abstract We investigated modulation by ATP, Mg^{2+}, Na^+, K^+ and NH_4^+ and inhibition by ouabain of (Na^+,K^+)-ATPase activity in microsomal homogenates of whole zoeae I and decapodid III (formerly zoea IX) and whole-body and gill homogenates of juvenile and adult Amazon River shrimps, *Macrobrachium amazonicum*. (Na^+,K^+)-ATPase-specific activity was increased twofold in decapodid III compared to zoea I, juveniles and adults, suggesting an important role in this ontogenetic stage. The apparent affinity for ATP ($K_M = 0.09 \pm 0.01$ mmol L^{-1}) of the decapodid III (Na^+,K^+)-ATPase, about twofold greater than the other stages, further highlights this relevance. Modulation of (Na^+,K^+)-ATPase activity by K^+ also revealed a threefold greater affinity for K^+ ($K_{0.5} = 0.91 \pm 0.04$ mmol L^{-1}) in decapodid III than in other stages; NH_4^+ had no modulatory effect. The affinity for Na^+ ($K_{0.5} = 13.2 \pm 0.6$ mmol L^{-1}) of zoea I (Na^+,K^+)-ATPase was fourfold less than other stages. Modulation by Na^+, Mg^{2+} and NH_4^+ obeyed cooperative kinetics, while K^+ modulation exhibited Michaelis-Menten behavior. Rates of maximal Mg^{2+} stimulation of ouabain-insensitive ATPase activity differed in each ontogenetic stage, suggesting that Mg^{2+}-stimulated ATPases other than (Na^+,K^+)-ATPase are present. Ouabain inhibition suggests that, among the various ATPase activities present in the different stages, Na^+-ATPase may be involved in the ontogeny of osmoregulation in larval *M. amazonicum*. The NH_4^+-stimulated, ouabain-insensitive ATPase activity seen in zoea I and decapodid III may reflect a stage-specific means of ammonia excretion since functional gills are absent in the early larval stages.

Keywords (Na^+,K^+)-ATPase activity · Gill microsome · Cation-binding site · *Macrobrachium amazonicum* · Ontogenetic stage · Environmental salinity

F. A. Leone (✉) · D. C. Masui · T. M. de Souza Bezerra · D. P. Garçon
Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes, 3900, Ribeirão Prêto, SP 14040-901, Brazil
e-mail: fdaleone@ffclrp.usp.br

Present Address:
D. P. Garçon
Departamento de Biologia Molecular, Centro de Ciências Exatas e da Natureza, Universidade Federal da Paraíba, João Pessoa, PB 58059-900, Brazil

W. C. Valenti · A. S. Augusto
Campus Experimental do Litoral Paulista, Universidade Estadual Paulista Júlio de Mesquita Filho, São Vicente, SP 11330-900, Brazil

J. C. McNamara
Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Prêto, SP 14040-901, Brazil

Introduction

The success of a particular crustacean species in a given biotope depends on the adjustment of each ontogenetic stage to its specific surroundings. In aquatic environments, salt content constitutes the main factor with which organisms must contend (Charmantier 1998; Anger 2003). While some crustaceans spend their life cycles in waters where salinity varies little, others migrate between fresh- and brackish-water biotopes during development, exposing their successive ontogenetic stages to widely different salinity regimes (Charmantier 1998; Freire et al. 2003; Short 2004).

Crustaceans are predominantly marine organisms. Although many have become independent of seawater, completing their entire life cycles in freshwater, others still appear to be invading this medium, as suggested by their larval developmental sequence being dependent on brackish water and by their characteristic metabolic, osmotic and ion regulatory mechanisms (Sandifer et al. 1975; Read 1984; Moreira et al. 1986; McNamara et al. 1986; Freire et al. 2003; Faria et al. 2011). Marine crustaceans essentially osmoconform with their environment and only weakly regulate their hemolymph ionic concentrations. Brackish and freshwater habitats, however, represent challenging environments since hemolymph osmotic and ionic concentrations are held fairly constant at levels much higher than the surrounding medium, leading to passive ion loss and water gain. Crustaceans inhabiting these media have evolved mechanisms that regulate their hemolymph Na^+ and Cl^- concentrations, both by compensatory ion uptake and by diminishing passive ion loss across the gills and other body-surface epithelia (Onken et al. 1995; Péqueux 1995; Riestenpatt et al. 1996; Lucu and Towle 2003; Kirschner 2004; Freire et al. 2008; Faria et al. 2011; McNamara and Faria 2012).

All crustacean Na^+-absorbing epithelia appear to express (Na^+,K^+)-ATPase, an ion-transporting enzyme located in the basal membrane, together with basal K^+ and Cl^- channels. However, in strongly hyperosmoregulating crabs like *Eriocheir sinensis* and *Dilocarcinus pagei*, Na^+ channels, the V-type H^+ pump and Cl^-/HCO_3^- exchangers, located in the apical membrane, are also key components of salt uptake (Onken and Riestenpatt 1998; Onken and McNamara 2002; Weihrauch et al. 2004a). Salt-uptake models for freshwater palaemonid shrimps like *Macrobrachium amazonicum* propose that active Na^+ absorption ensues through Na^+ channels in the apical flange membranes of gill pillar cells in concert with the (Na^+,K^+)-ATPase located in the basal membrane of ion-transporting, septal cell ionocytes to which the pillar cells are structurally coupled (McNamara and Lima 1997; McNamara and Torres 1999; Belli et al. 2009; Faleiros et al. 2010). H^+ extrusion via the apical pillar cell V(H^+)-ATPase appears to drive Na^+ influx, leading to cellular hyperpolarization that facilitates basal Cl^- extrusion (Torres et al. 2003; Faleiros et al. 2010). Apical Cl^-/HCO_3^- exchangers, using HCO_3^- derived from CO_2 hydration by carbonic anhydrase, transport Cl^- into the pillar cell flanges, while Cl^- efflux to the septal cells proceeds through basal Cl^- channels. Together with active Na^+ transport to the hemolymph by the electrogenic (Na^+,K^+)-ATPase, K^+ recycling through basal membrane K^+ channels in the septal cells generates a negative electrical potential that drives Cl^- efflux to the hemolymph (for review, see Freire et al. 2008).

The (Na^+,K^+)-ATPase, found in the plasma membranes of all animal cells, underpins many homeostatic processes and is directly responsible for the asymmetrical, electrogenic countertransport of Na^+ and K^+ that results in strong ionic gradients across their membranes; i.e., the energy provided by ATP hydrolysis powers the countertransport of $3Na^+$ out of and $2K^+$ into the cytosol (Kaplan 2002; Jorgensen et al. 2003; Martin 2005; Sáez et al. 2009). Such gradients generate transmembrane electrical potential and drive transport processes like active transepithelial salt and passive water movement, Na^+/glucose/amino acid/nucleotide cotransport and cell volume regulation (Jorgensen and Pedersen 2001; Jorgensen et al. 2003; Martin 2005; Sáez et al. 2009). (Na^+,K^+)-ATPase is an oligomeric protein belonging to the P_{2C} subfamily of membrane-embedded P-type ATPases, whose hallmark is the formation of an acyl-phosphate intermediate during the catalytic cycle (Axelsen and Palmgren 1998; Jorgensen and Pedersen 2001; Kaplan 2002; Poulsen et al. 2010). The protein's X-ray crystal structure reveals a catalytic 110 kDa α-subunit and a 50 kDa β-subunit that constitute the active moiety of the enzyme, together with a regulatory FXYD protein (Morth et al. 2007; Geering 2008). Phosphorylation and dephosphorylation at the D376 residue in the α-subunit results in the transition between two main conformational changes: E_1, with a high affinity for intracellular Na^+, and E_2, characterized by high affinity for extracellular K^+ (reviewed by Jorgensen et al. 2003; Martin 2005; Morth et al. 2007; Sáez et al. 2009). Different tissue-specific α- and β-isoforms exhibit distinct transport and pharmacological properties (Blanco and Mercer 1998; Crambert et al. 2000; Blanco 2005).

Studies of crustacean ontogeny have dealt mainly with marine and estuarine decapods (Charmantier 1998), while osmoregulatory studies in freshwater Crustacea have focused mostly on adult crab, shrimp and crayfish species, mainly owing to their large size, which is convenient for in vivo and in vitro experiments (Péqueux 1995; Onken and McNamara 2002; Lucu and Towle 2003; Kirschner 2004; Freire et al. 2008). The larval stages have been neglected (Read 1984; Charmantier 1998; Haond et al. 1999; Khodabandeh et al. 2006) due to their small dimensions and reduced hemolymph volume (Charmantier 1998; Anger 2003; Augusto et al. 2007). The few studies that do correlate the salinity tolerance of early ontogenetic stages with their osmoregulatory capabilities have been hampered by a lack of information on the kinetic characteristics of the transporters involved in osmoregulation during larval development (Conte et al. 1977; Read 1984; Sun et al. 1991; Lee and Watts 1994; Escalante et al. 1995; Augusto et al. 2007; Ituarte et al. 2008).

The ontogeny of osmoregulation has been examined in *M. petersi* (Read 1984), and recently Charmantier and Anger (2010) explored ontogenetic osmoregulatory ability in two geographically isolated populations of

M. amazonicum from different Brazilian biomes. This species embraces both hololimnetic populations, in which the life cycle is entirely restricted to freshwater (Magalhães 1985; Collart and Rabelo 1996; Zanders and Rodriguez 1992), and diadromous populations, which are dependent on brackish water for larval development (McNamara et al. 1983, 1986; Charmantier 1998). *M. amazonicum* is widely distributed throughout neotropical South America, inhabiting inland and estuarine waters of the major hydrographic basins, such as the Amazon, Orinoco, São Francisco, Araguaia-Tocantins, Paraná and Paraguay watersheds, as well as coastal rivers in north and northeastern Brazil (Pettovello 1996; Ramos-Porto and Coelho 1998). Despite this ample geographical distribution and economic importance for fisheries (Maciel and Valenti 2009) and aquaculture (Moraes-Valenti and Valenti 2010), the larval biology of *M. amazonicum* is not well known (see Anger et al. 2009). The adult shrimp is a good hyperosmotic regulator, including excellent chloride regulatory capability, and has been used as a model organism for physiological studies of salinity tolerance and osmoregulatory mechanisms (McNamara et al. 1983; Zanders and Rodriguez 1992; Augusto et al. 2007; Santos et al. 2007; Faleiros et al. 2010). Larval growth patterns (Moreira et al. 1986), chemical composition (Anger et al. 2009) and osmoregulatory ability (Charmantier and Anger 2010) also have been investigated.

The life cycle of *M. amazonicum* consists of egg, larval, juvenile and adult stages; and the species has been well studied in its natural environment and under aquaculture and laboratory conditions (for review, see Maciel and Valenti 2009). In *M. amazonicum* from the Amazon delta, only newly hatched zoea I tolerate exposure to freshwater, maintaining a hemolymph osmolality of ≈300 mOsm/kg H_2O; larval instars II–IX and first-stage juveniles do not survive freshwater (Charmantier and Anger 2010). However, later juveniles and adults exhibit strong osmoregulatory capability in this medium compared to zoea I, a stage that also survives well at low salinities compared to instars II–IX, showing stronger osmoregulatory ability, which becomes maximal in juveniles and adults. In seawater (32 ‰ salinity), zoeae I–V and adults survive very well, in contrast to larval instars VI–IX, while late juveniles and adults hypo-osmoregulate slightly. Under strong hyperosmotic challenge (44 ‰ salinity), a few stage II–VII instars survive, showing hypo-osmoregulatory capability; however, zoea I, instars VIII and IX, juveniles and adults cannot tolerate this salinity (Charmantier and Anger 2010).

A kinetic characterization of (Na^+,K^+)-ATPase in such a diadromous species should allow insight into the development of osmoregulatory capability. Thus, in this study we investigated the modulation by ATP, Mg^{2+}, K^+, Na^+ and NH_4^+ and inhibition by ouabain of (Na^+,K^+)-ATPase from several ontogenetic stages of the freshwater shrimp *M. amazonicum*.

Materials and Methods

Material

All solutions were prepared using Millipore (Billerica, MA) MilliQ ultrapure apyrogenic water, and all reagents were of the highest purity commercially available. Imidazole, *N*-(2-hydroxyethyl) piperazine-N19-ethanesulfonic acid (HEPES), triethanolamine, ATP ditris salt, pyruvate kinase (PK), phosphoenolpyruvate (PEP), NAD^+, NADH, phosphoglycerate kinase (PGK), alamethicin, lactate dehydrogenase (LDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glyceraldehyde-3-phosphate (G3P), ouabain and 3-phosphoglyceraldehyde diethyl acetal were purchased from Sigma (St. Louis, MO). The protease inhibitor cocktail (5 mmol L^{-1} leupeptin, 5 mmol L^{-1} antipain, 1 mmol L^{-1} benzamidine, 1 mmol L^{-1} pepstatin A and 5 µmol L^{-1} phenyl-methane-sulfonyl-fluoride) was from Calbiochem (Darmstadt, Germany).

Reagents

G3P was prepared by hydrolysis of 3-phosphoglyceraldehyde diethyl acetal, barium salt with 150 µl HCl (d = 1.18 g/mL) in a boiling water bath for 2 min, after removal of the barium salt with Dowex $50H^+$ resin, as recommended by the manufacturer (see Sigma product information for G5376). Final pH was adjusted to 7.0 with 50 µl triethanolamine just before use. Ammonium-free suspensions were prepared by centrifuging 200 µl of 2.9 mol L^{-1} ammonium sulfate containing crystalline suspensions of LDH and PK at 20,000×*g* for 15 min, at 4 °C, in Eppendorf tubes. The pellet was resuspended in 300 µl of 50 mmol L^{-1} HEPES buffer (pH 7.5), transferred to a YM-30 Amicon (Billerica, MA) Microcon filter and centrifuged five times at 10,000×*g* in 300 µl of the same buffer until depletion of ammonium ions (tested with Nessler reagent). Finally, the pellet was resuspended to the original volume. For PGK and GAPDH, the suspension was treated as above with 50 mmol L^{-1} triethanolamine buffer, pH 7.5, containing 1 mmol L^{-1} dithiothreitol. When necessary, enzyme solutions were concentrated using YM-30 Amicon Microcon filters. Ammonium-depleted enzyme solutions were used within 2 days of preparation.

Shrimps

Amazon River shrimps, *M. amazonicum*, were produced at the Aquaculture Center, UNESP, Jaboticabal, São Paulo,

Brazil, from broodstock collected in freshwater at Furo das Marinhas near Santa Bárbara do Pará (1° 13.450′ S, 48° 17.632′ W), northeastern Pará State, Brazil, in 2001 (Araujo and Valenti 2007). The larval stages were identified according to Guest (1979); however, zoeae VII, VIII and IX are now termed decapodid I, II and III, respectively, according to the nomenclature proposed by Anger (2001).

Zoeae I (approximately 6,000 individuals/preparation, ≈60 μg wet mass) were obtained from hatching tanks (6 ‰ salinity) just after eclosion, guaranteeing that all individuals were in the same stage. The decapodid III stage was obtained from larviculture tanks (12 ‰ salinity), and individuals were separated under a stereomicroscope using morphological and behavioral characteristics. Groups of decapodid III (approximately 300 individuals/preparation, ≈650 μg wet mass) were held in aerated carboys containing 32 L water from the larviculture tanks. Juveniles (20 individuals/preparation, ≈700 μg wet gill mass) were collected from freshwater rearing tanks and held in carboys containing 32 L aerated freshwater. Adult male and non-ovigerous female shrimps (20 individuals/preparation, ≈6 g wet gill mass) were collected from freshwater ponds and maintained in carboys containing 32 L aerated pond water. These particular stages were chosen as they typify the different ontogenetic phases. Zoea I is a newly hatched, small, free-swimming larva that uses only internal yolk reserves as an energetic substrate. Decapodid III is the last larval stage; it requires brackish water for survival, its yolk supply has been long exhausted and exogenous feeding is necessary (Araujo and Valenti 2007). The juvenile represents the first benthonic freshwater stage, while adult shrimps are sexually mature.

The various salinities in which the different stages were reared represent those encountered by each ontogenetic stage in the natural environment. To avoid possible effects of the molting cycle, zoeae I were collected in the evening shortly after hatching. The decapodid III, juvenile and adult stages were used in intermolt, confirmed by stereoscopic microscopy. Individuals in the different stages were transported in their respective carboys to the laboratory and used immediately for microsomal preparation.

Preparation of Microsomal Fractions

For each homogenate prepared, shrimps were anesthetized by chilling on crushed ice immediately before dissection and homogenization. The gills of juvenile and adult shrimps were rapidly dissected, diced and homogenized in a Potter homogenizer in 20 mmol L^{-1} imidazole homogenization buffer, pH 6.8, containing 6 mmol L^{-1} EDTA, 250 mmol L^{-1} sucrose and a protease inhibitor cocktail (20 mL buffer/g wet tissue). For zoeae I and decapodid III, whole larvae were homogenized similarly; homogenates of whole juveniles and adults were prepared after removing the appendages and exoskeleton.

After centrifuging the crude homogenate at 10,000×*g* for 35 min at 4 °C, the supernatant was placed on crushed ice and the pellet was resuspended in an equal volume of homogenization buffer. After further centrifugation as above, the two supernatants were pooled and centrifuged at 100,000×*g* for 2 h at 4 °C. The resulting pellet containing the microsomal fraction was homogenized in 20 mmol L^{-1} imidazole buffer, pH 6.8, containing 250 mmol L^{-1} sucrose (15 mL buffer/g wet tissue). Finally, 0.5 mL aliquots were rapidly frozen in liquid nitrogen and stored at −20 °C. No appreciable loss of (Na^+,K^+)-ATPase activity was seen after 2 month storage of the microsomal enzyme prepared from either whole larvae or gill tissue. When required, the aliquots were thawed, placed on crushed ice and used immediately.

Measurement of ATP Hydrolysis

Total ATPase activity was routinely assayed at 25 °C using the PK/LDH linked system (Rudolph et al. 1979) in which the hydrolysis of ATP was coupled to the oxidation of NADH (Masui et al. 2008). The oxidation of NADH was monitored at 340 nm ($\varepsilon_{340\ nm,\ pH\ 7.5} = 6{,}200\ mol^{-1}\ L\ cm^{-1}$) in a Hitachi (Tokyo, Japan) U-3000 spectrophotometer equipped with thermostatted cell holders. Standard conditions were as follows: 50 mmol L^{-1} HEPES buffer (pH 7.5), 2 mmol L^{-1} ATP, containing 5 mmol L^{-1} $MgCl_2$, 20 mmol L^{-1} KCl, 0.14 mmol L^{-1} NADH, 2 mmol L^{-1} PEP, 82 μg PK (49 U) and 110 μg LDH (94 U), 50 mmol L^{-1} NaCl (for zoea I and juveniles) and 20 mmol L^{-1} NaCl (for decapodid III and adults), in a final volume of 1 mL. ATP hydrolysis was also estimated with 3 mmol L^{-1} ouabain to assess ouabain-insensitive activity. The difference in activity measured in the absence (total ATPase activity) or presence (ouabain-insensitive activity) of ouabain represents the (Na^+,K^+)-ATPase activity. Alternatively, ATPase activity was estimated using a GAPDH/PGK-linked system coupled to the reduction of NAD^+ at 340 nm (Masui et al. 2008). Standard conditions were 50 mmol L^{-1} triethanolamine buffer (pH 7.5), 2 mmol L^{-1} ATP, containing 5 mmol L^{-1} $MgCl_2$, 20 mmol L^{-1} KCl, 1 mmol L^{-1} NAD^+, 0.5 mmol L^{-1} sodium phosphate, 1 mmol L^{-1} G3P, 150 μg GAPDH (12 U), 20 μg PGK (9 U), 50 mmol L^{-1} NaCl (for zoea I and juveniles) and 20 mmol L^{-1} NaCl (for decapodid III and adults), in a final volume of 1 mL. The two coupling systems gave equivalent results, with a difference of $< 10\%$. ATP hydrolysis was also estimated at 25 °C after 10 min preincubation with alamethicin (1 mg/mg protein) to provide leaky and/or disrupted vesicles (Masui et al. 2008). Controls without added enzyme were included in each experiment to quantify the nonenzymatic hydrolysis of

substrate. Initial velocities were constant for at least 15 min provided that <5 % of the total NADH (or NAD^+) was oxidized (or reduced). The reaction rate for each modulator was estimated in duplicate using identical aliquots from the same preparation. Mean values were used to fit each corresponding saturation curve, which was repeated three times utilizing different microsomal homogenates ($n = 3$). One enzyme unit (U) is defined as the amount of enzyme that hydrolyzes 1.0 nmol of ATP per minute, at 25 °C; and (Na^+,K^+)-ATPase specific activity is given as U mg^{-1} total protein.

Protein Measurement

Protein concentration was estimated according to the Coomassie Blue G dye–binding assay of Read and Northcote (1981), using bovine serum albumin as the standard.

Estimation of Kinetic Parameters

The kinetic parameters V_M (maximum velocity), $K_{0.5}$ (apparent dissociation constant), K_M (Michaelis-Menten constant) and n_H (Hill coefficient) for ATP hydrolysis under the different assay conditions were calculated using SigrafW software (Leone et al. 2005a). The figures provide representative curves, each obtained from a single microsomal preparation. The kinetic parameters provided are calculated values, expressed as the mean ± SD, and represent the number of experiments performed with different preparations ($n = 3$). The apparent dissociation constant, K_I, of the enzyme-inhibitor complex was estimated as described by Marks and Seeds (1978). SigrafW software can be obtained from http://portal.ffclrp.usp.br/sites/fdaleone/downloads.

Results

(Na^+,K^+)-ATPase Activity in Whole Adult and Juvenile Shrimps

(Na^+,K^+)-ATPase activity assayed in the microsomal fraction of whole juveniles was 87.7 U mg^{-1}, ≈50 % less than in the corresponding gill microsomal preparation (176.2 U mg^{-1}). In whole adult shrimps, activity was 62.8 U mg^{-1}, representing ≈40 % of microsomal gill (Na^+,K^+)-ATPase activity (156.7 U mg^{-1}).

Modulation of (Na^+,K^+)-ATPase Activity by ATP

Figure 1 shows the effect of ATP concentration on (Na^+,K^+)-ATPase activity in microsomal fractions of *M. amazonicum* tissues from the four ontogenetic stages. Independent of stage, 70–85 % of total ATPase activity corresponds to (Na^+,K^+)-ATPase (insets to figures). Further, under saturating Mg^{2+}, Na^+ and K^+ concentrations, only a single ATP saturation curve, obeying Michaelis-Menten kinetics, was seen for the enzyme in the four stages (Table 1). Although the maximum (Na^+,K^+)-ATPase activities for zoea I and adult enzymes are similar, that for decapodid III is almost twofold greater (Table 1). The considerable ouabain-insensitive ATPase activity (54.0 ± 1.4, 104.5 ± 5.2, 49.3 ± 2.5 and 31.2 ± 1.8 U mg^{-1} for zoea I, decapodid III, juvenile and adult shrimps, respectively) also was stimulated over the same ATP concentration range, strongly suggesting Mg^{2+}-stimulated ATPase activities other than (Na^+,K^+)-ATPase. K_M values for the enzymes from the zoea I, decapodid III, juvenile and adult stages were fairly similar (Table 1). For ATP concentrations as low as 10^{-6} mol L^{-1}, a residual ATPase activity ranging 5–10 % of total ATPase activity was found for all stages (insets to figures).

Effect of Magnesium Ions

Figure 2 shows the modulation by Mg^{2+} of (Na^+,K^+)-ATPase activity in the four ontogenetic stages. A single saturation curve exhibiting positive cooperative effects ($n_H > 1.0$) was found over the range from 10^{-5} to 5×10^{-2} mol L^{-1}, independently of ontogenetic stage. The maximum stimulation rates for zoea I, juvenile and adult shrimps were similar; however, that for decapodid III was considerably greater (Table 1). $K_{0.5}$ values for Mg^{2+} stimulation of (Na^+,K^+)-ATPase activity varied very little (Table 1). Stimulation of ouabain-insensitive ATPase activity by Mg^{2+} varied considerably (see insets to figures), resulting in maximum stimulation rates of 51.6 ± 2.8 U mg^{-1} for zoea I, 104.6 ± 6.3 U mg^{-1} for decapodid III, 57.2 ± 2.9 U mg^{-1} for juvenile and 22.7 ± 0.8 U mg^{-1} for adult enzymes (insets to figures). A residual Mg^{2+}-stimulated ATPase activity of around 10 % of total ATPase activity for Mg^{2+} concentrations as low as 10^{-5} mol L^{-1} was detected in both zoea I and decapodid III but not in juveniles and adults. Interestingly, these Mg^{2+}-stimulated, ouabain-insensitive ATPase activities are similar to the ATP-stimulated, ouabain-insensitive ATPase activities (insets to figures), corroborating the likelihood of Mg^{2+}-stimulated ATPases other than (Na^+,K^+)-ATPase in the preparation. Independent of ontogenetic stage, stimulation by Mg^{2+} obeyed cooperative kinetics (Table 1), and Mg^{2+} concentrations >5 mmol L^{-1} markedly inhibited (Na^+,K^+)-ATPase activity (data not shown).

Effect of Sodium Ions

Under saturating ATP, Mg^{2+} and K^+ concentrations, stimulation of (Na^+,K^+)-ATPase activity by Na^+ resulted

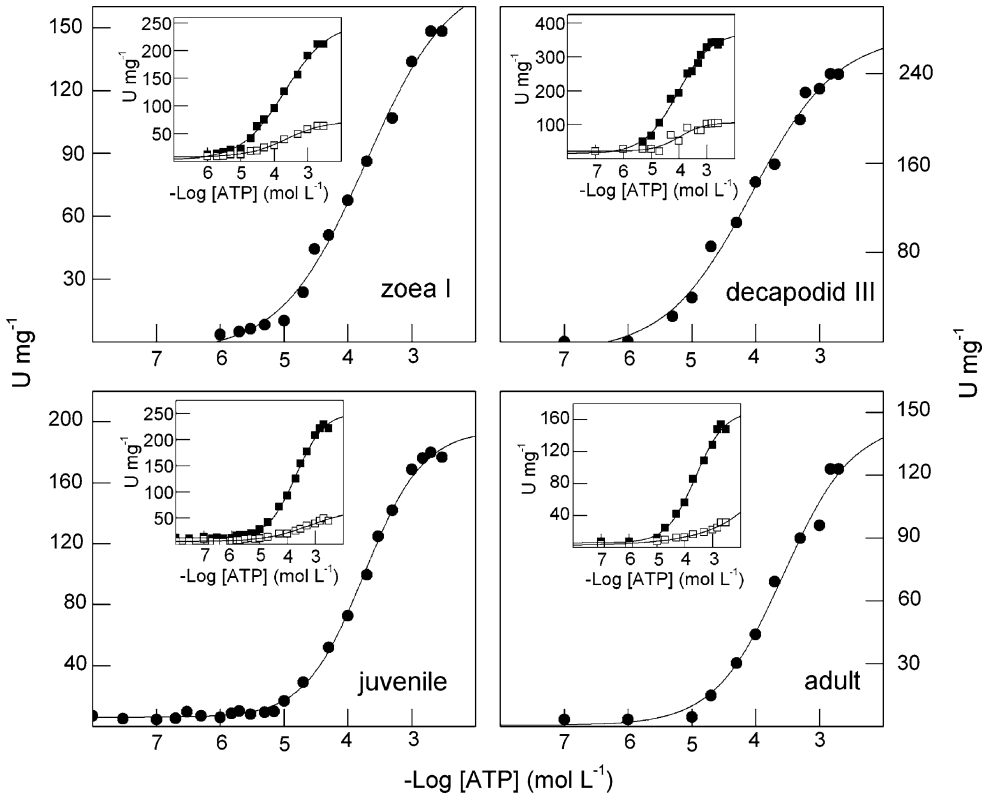

Fig. 1 Effect of ATP concentration on (Na^+,K^+)-ATPase activity in microsomal fractions from *M. amazonicum* at different ontogenetic stages. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} HEPES buffer, pH 7.5, containing 5.0 mmol L^{-1} $MgCl_2$, 20.0 mmol L^{-1} KCl, 0.14 mmol L^{-1} NADH, 2.0 mmol L^{-1} PEP, 82 μg PK (49 U), 110 μg LDH (94 U) and NaCl 50 mmol L^{-1} for zoea I and juvenile enzymes or 20 mmol L^{-1} for decapodid III and adult enzymes. Enzyme activity was assayed using 13.4, 7.2, 9.8 and 31.4 μg protein from homogenates of whole zoeae I and decapodid III, and gill homogenates of juvenile and adult shrimps, respectively. Experiments were performed using duplicate aliquots from three different homogenates; representative curves obtained from one homogenate are given. *Insets* effect of ATP concentration on ouabain-insensitive (*open square*) and total ATPase activities (*filled square*)

in single saturation curves, showing positive cooperativity (Fig. 3). Maximum rates for zoea I, decapodid III, juvenile and adult shrimps were similar to those for K^+ (Table 1). Except for zoea I, showing the highest $K_{0.5}$, the affinity constants of the enzymes in the other stages were about 4 mmol L^{-1} (Table 1). The ouabain-insensitive activity of zoea I was stimulated up to 15 % of total ATPase activity over the range 10^{-4} to 7×10^{-2} mol L^{-1} Na^+; however, stimulation by Na^+ was negligible in the other stages (insets to figures).

Effect of Potassium Ions

Figure 4 shows the effect of K^+ on (Na^+,K^+)-ATPase activity in the four different ontogenetic stages. Under saturating ATP, Mg^{2+} and Na^+ concentrations, enzyme activity is notably stimulated by K^+. Maximal stimulation rates were fairly similar for zoea I, juvenile and adult enzymes but about twofold less than for decapodid III (Table 1). Michaelis-Menten kinetics prevailed for K^+ stimulation of (Na^+,K^+)-ATPase in all stages (Table 1). The decapodid III enzyme exhibited the highest affinity for K^+ ($K_M = 0.91 \pm 0.04$ mmol L^{-1}). Except for zoea I, which showed maximal stimulation of ouabain-insensitive activity of 54.0 ± 1.3 U mg^{-1} (inset to figure), the decapodid III, juvenile and adult (insets to figure) enzymes were not stimulated by K^+, suggesting the absence of K^+-dependent ATPase activity. Considerable residual Mg^{2+}-ATPase activity, ranging 10–20 % of total ATPase activity, was found for K^+ concentrations as low as 10^{-5} mol L^{-1} (insets to figures).

Stimulation by Ammonium Ions

(Na^+,K^+)-ATPase activity in all four ontogenetic stages was stimulated by NH_4^+ under saturating ATP (2 mmol L^{-1}), Na^+ (50 mmol L^{-1} for zoea I and juvenile enzymes, 20 mmol L^{-1} for decapodid III and adult enzymes) and Mg^{2+} (5 mmol L^{-1}) concentrations, in the absence of K^+ (Fig. 5). Increasing NH_4^+ concentrations from 10^{-3} to 7×10^{-2} mol L^{-1} stimulated (Na^+,K^+)-ATPase activity to a maximum of ≈270 U mg^{-1} in zoea I

Table 1 Kinetic parameters for stimulation by ATP, Mg^{2+}, K^+, Na^+ or NH_4^+ and K_I values for ouabain inhibition of microsomal (Na^+,K^+)-ATPase activity in four ontogenetic stages (zoea I, decapodid III, juvenile and adult) of the Amazon River shrimp, *M. amazonicum*

Effector	V (U mg^{-1})				$K_{0.5}$ or K_M (mmol L^{-1})				n_H			
	Z I	D III	Juvenile	Adult	Z I	D III	Juvenile	Adult	Z I	DIII	Juvenile	Adult
ATP	140.4 ± 6.7	266.8 ± 12.6	194.4 ± 9.6	133.3 ± 6.4	0.19 ± 0.01	0.09 ± 0.01	0.18 ± 0.01	0.21 ± 0.01	0.8	0.7	1.0	1.0
Mg^{2+}	133.2 ± 6.2	245.0 ± 12.0	181.3 ± 7.7	139.4 ± 6.7	0.68 ± 0.03	0.56 ± 0.03	0.51 ± 0.02	1.03 ± 0.05	2.2	1.8	1.3	1.7
K^+	150.5 ± 7.3	247.0 ± 12.3	196.5 ± 10.1	137.1 ± 7.0	3.2 ± 0.16	0.91 ± 0.04	2.35 ± 0.12	2.02 ± 0.10	1.0	1.0	1.0	1.0
Na^+	144.4 ± 6.9	249.1 ± 11.9	186.8 ± 9.3	126.4 ± 6.3	13.2 ± 0.60	4.20 ± 0.20	4.06 ± 0.20	3.00 ± 0.15	1.5	2.2	1.3	2.2
NH_4^+	271.9 ± 11.8	273.8 ± 12.5	205.9 ± 9.4	194.2 ± 8.5	6.3 ± 2.1	3.12 ± 0.18	1.88 ± 0.08	4.76 ± 0.23	3.4	3.3	1.0	1.9
Ouabain inhibition												
	Z I				D III		Juvenile				Adult	
K_I (μmol L^{-1})	104.3 ± 5.3				92.0 ± 4.5		85.0 ± 4.1				126.4 ± 6.6	

Assays were performed in 50 mmol L^{-1} HEPES or triethanolamine buffer, pH 7.5, in a final volume of 1 mL. Microsomes were obtained by homogenizing whole zoeae I (Z I) and decapodid III (D III) or gill tissue (juveniles and adults). The effect of each modulator was evaluated under optimal concentrations of the others. Data are the mean ± SD from three different microsomal preparations

and decapodid III and to ≈200 U mg^{-1} in juveniles and adults (Table 1). Cooperative kinetics prevailed independently of developmental stage for stimulation by NH_4^+, although calculated $K_{0.5}$ values differed considerably (Table 1). Ouabain-insensitive ATPase activity was not stimulated by NH_4^+ in juveniles and adults, in contrast to zoea I and decapodid III, representing 15 and 45 %, respectively, of total ATPase activity (insets to Fig. 5).

Inhibition by Ouabain

Inhibition by 3 mmol L^{-1} ouabain of total ATPase activity in the four ontogenetic stages is shown in Fig. 6 with the corresponding K_I values in Table 1. In adult shrimps, ouabain inhibited 87 % of total ATPase activity (154.1 U mg^{-1}), resulting in ouabain-insensitive activity of 19.8 U mg^{-1}. For zoea I, the maximum ATPase activity of 180.1 U mg^{-1} decreased to 52.8 U mg^{-1} with ouabain. Total ATPase activities of both decapodid III and juveniles (344.0 and 231.1 U mg^{-1}, respectively) were inhibited by ouabain to about 50 U mg^{-1}.

Discussion

This is the first kinetic characterization of modulation by ATP, Mg^{2+}, Na^+, K^+ and NH_4^+ and inhibition by ouabain of a gill microsomal (Na^+,K^+)-ATPase in selected ontogenetic stages of the palemonid shrimp *M. amazonicum*. Our findings reveal considerable differences in modulation by Na^+ (zoea I), K^+ and ATP (decapodid III) of (Na^+,K^+)-ATPase activity, suggesting an important role in the osmoregulatory capability of the larval stages. While the similar K_M values estimated for ATP exclude the likelihood of different (Na^+,K^+)-ATPase isoforms, ouabain inhibition suggests that among the various ATPases expressed in the different ontogenetic stages, Na^+-ATPase may be involved in the ontogeny of osmoregulatory capability in larval *M. amazonicum*. Further, the NH_4^+-stimulated, ouabain-insensitive ATPase activity seen in zoea I and decapodid III may reflect a stage-specific means of ammonia excretion since functional gills are absent in the early larval stages.

Maximum specific activities estimated for tank-reared *M. amazonicum* zoea I, decapodid III and juvenile enzymes are considerably greater than in homogenates of whole *M. rosenbergii* larvae (Huong et al. 2004). Maximum activities lie between 3 and 100 U mg^{-1} in other shrimp species (Felder et al. 1986; Ituarte et al. 2008) and are approximately 50 U mg^{-1} in cephalothorax homogenates of larval *Homarus gammarus* (Thuet et al. 1988). Maximum rates of ATP hydrolysis for the gill enzyme in adult, pond-reared *M. amazonicum* are twofold less than in wild

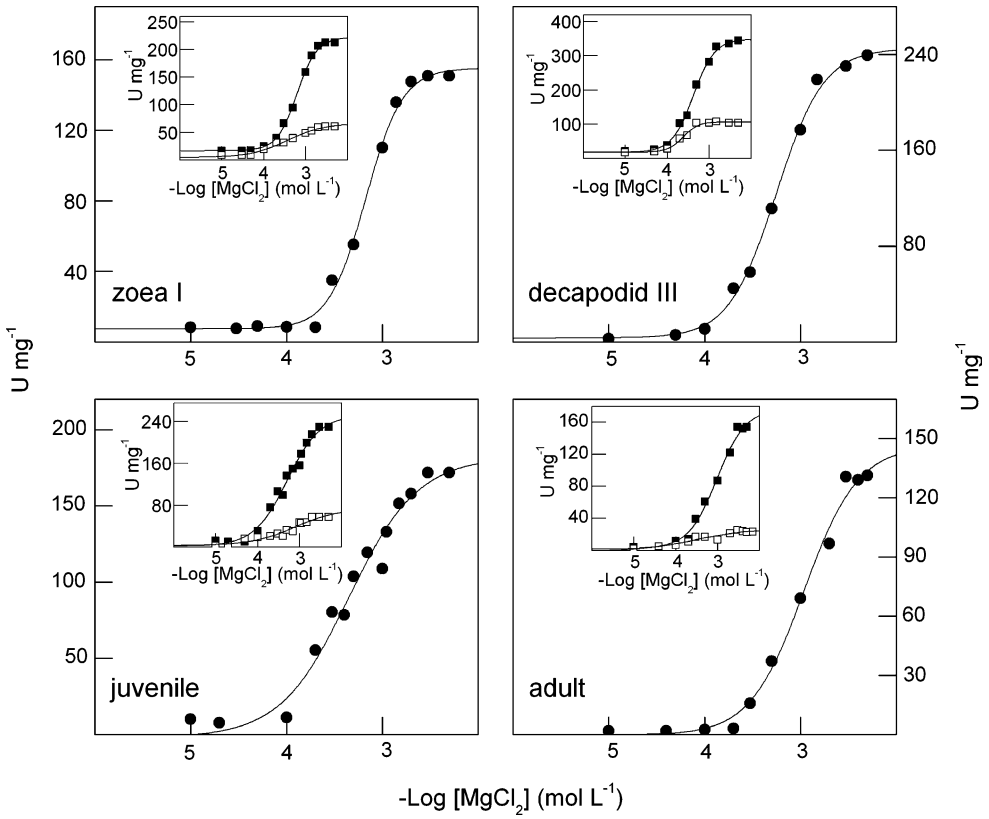

Fig. 2 Effect of Mg^{2+} concentration on (Na^+,K^+)-ATPase activity in microsomal fraction from *M. amazonicum* at different ontogenetic stages. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} HEPES buffer, pH 7.5, containing 2.0 mmol L^{-1} ATP, 20.0 mmol L^{-1} KCl, 0.14 mmol L^{-1} NADH, 2.0 mmol L^{-1} PEP, 82 µg PK (49 U), 110 µg LDH (94 U) and 50 mmol L^{-1} NaCl for zoea I and juvenile enzymes or 20 mmol L^{-1} NaCl for decapodid III and adult enzymes. Enzyme activity was assayed using 13.4, 7.2, 9.8 and 31.4 µg protein from homogenates of whole zoeae I and decapodid III, and gill homogenates of juvenile and adult shrimps, respectively. Experiments were performed using duplicate aliquots from three different homogenates; representative curves obtained from one homogenate are given. *Insets* effect of Mg^{2+}concentration on ouabain-insensitive (*open square*) and total ATPase activities (*filled square*)

M. amazonicum (Santos et al. 2007). Further, the single ATP stimulation curve seen in pond-reared adults also contrasts markedly with the biphasic stimulation by ATP of (Na^+,K^+)-ATPase activity seen in wild adult *M. amazonicum* (Santos et al. 2007). Whether this difference is a response to rearing conditions remains to be elucidated. Several *Macrobrachium* species show (Na^+,K^+)-ATPase activities ranging from 70 to 100 U mg^{-1} in gill homogenates (Moretti et al. 1991; Proverbio et al. 1991) and from 120 to 200 U mg^{-1} in microsomal fractions (Stern et al. 1984; Lima et al. 1997), including adult freshwater shrimp, *M. olfersi* (690 U mg^{-1}) (Furriel et al. 2000). While establishing the number of ATP binding sites on the gill enzyme in crustaceans is still controversial, K_M values for ATP hydrolysis are known for some species (Leone et al. 2005b; Lucu and Towle 2003) and may constitute a valuable tool to better compare enzyme modulation by its physiological substrate during ontogeny. The K_M for ATP binding sites on the gill (Na^+,K^+)-ATPase from adult, pond-reared *M. amazonicum* is comparable to wild adult *M. amazonicum* (Santos et al. 2007), *M. olfersi* (Furriel et al. 2000) and *M. rosenbergii* (Stern et al. 1984). However, the K_M for *M. amazonicum* decapodid III is about twofold less than the other stages, suggesting a greater affinity of the enzyme for its physiological substrate.

The crustacean (Na^+,K^+)-ATPase is a Mg^{2+}-dependent, Na^+ and K^+/NH_4^+-stimulated enzyme, Mg^{2+} being a cofactor required for enzyme phosphorylation and ATP hydrolysis either as free Mg^{2+} or as a Mg^{2+}-nucleotide complex (Robinson and Pratap 1991; Tentes and Stratakis 1991; Jorgensen et al. 2003; Leone et al. 2005b). Stimulation by Mg^{2+} was independent of ontogenetic stage in *M. amazonicum*, and $K_{0.5}$ values were similar to adult *M. olfersi* (Furriel et al. 2000) and wild adult *M. amazonicum* (Santos et al. 2007). A characteristic feature of crustacean (Na^+,K^+)-ATPase is inhibition by excess Mg^{2+} (Leone et al. 2005b) due to free Mg^{2+} binding to the E_2K enzyme form and to the decreased ATP affinity that occurs during the K^+-releasing step (Fontes et al. 1992). However, this is not the case for the ouabain-insensitive ATPase activity estimated here for the

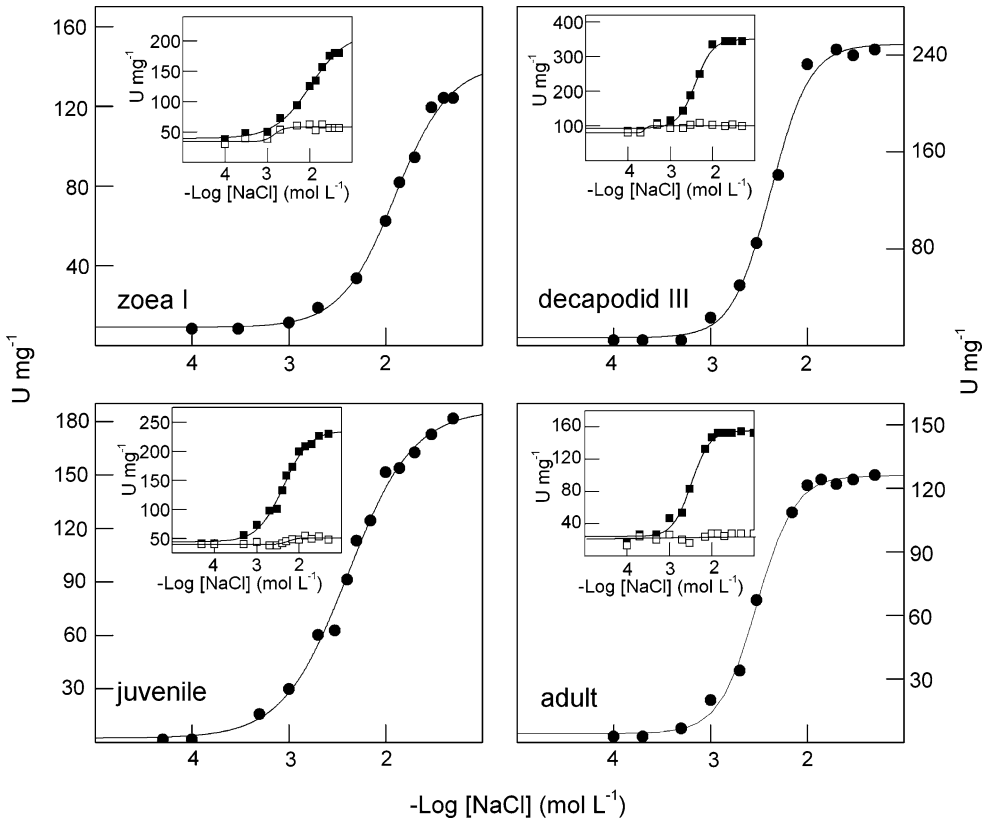

Fig. 3 Effect of Na^+ concentration on (Na^+,K^+)-ATPase activity in microsomal fractions from *M. amazonicum* at different ontogenetic stages. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} HEPES buffer, pH 7.5, containing 2.0 mmol L^{-1} ATP, 5.0 mmol L^{-1} $MgCl_2$, 20.0 mmol L^{-1} KCl, 0.14 mmol L^{-1} NADH, 2.0 mmol L^{-1} PEP, 82 µg PK (49 U), 110 µg LDH (94 U) and 50 mmol L^{-1} NaCl for zoea I and juvenile enzymes or 20 mmol L^{-1} NaCl for decapodid III and adult enzymes. Enzyme activity was assayed using 13.4, 7.2, 9.8 and 31.4 µg protein from homogenates of whole zoeae I and decapodid III, and gill homogenates of juvenile and adult shrimps, respectively. Experiments were performed using duplicate aliquots from three different homogenates; representative curves obtained from one homogenate are given. *Insets* effect of Na^+ concentration on ouabain-insensitive (*open square*) and total ATPase activities (*filled square*)

different ontogenetic stages, which remains virtually unchanged over a wide range of Mg^{2+} concentrations. The (Na^+,K^+)-ATPase and ouabain-insensitive ATPase activities estimated in the different stages of pond-reared *M. amazonicum* also reflect this typical behavior. The different rates of maximal stimulation of ouabain-insensitive ATPase activity by millimolar Mg^{2+} also suggest the presence of Mg^{2+}-stimulated ATPases other than (Na^+,K^+)-ATPase in the different ontogenetic stages of *M. amazonicum*. Whether these activities correspond to the same or to different Mg^{2+}-stimulated ATPases is unclear.

The kinetic characteristics of specific α-subunit (Na^+,K^+)-ATPase isoforms such as apparent affinity for Na^+ and K^+ when ATP is used as a substrate depend on the organism and tissue, membrane factors and posttranslational modifications (Therien et al. 1996; Sáez et al. 2009). The apparent affinity for Na^+ of the (Na^+,K^+)-ATPase from various species varies widely (4–25 mmol L^{-1}) among the Crustacea, although that for K^+ (0.5–2.5 mmol L^{-1}) is less so (for review, see Leone et al. 2005b). In *M. amazonicum*, (Na^+,K^+)-ATPase shows far lower Na^+ affinity in the earlier ontogenetic stages. Further, modulation by Na^+ obeys cooperative kinetics, in contrast to the Michaelis-Menten behavior seen for K^+ modulation, independently of ontogenetic stage. Except for decapodid III all stages show $K_{0.5}$ values of around 2.0 mmol L^{-1} for K^+ (see Table 1). The apparent affinity for K^+ of the enzyme from adult, pond-reared shrimps was about 2.5-fold less than in wild *M. amazonicum* (Santos et al. 2007) but similar to *M. olfersi* (Furriel et al. 2000). However, zoea I exhibited a considerably higher $K_{0.5}$ value, revealing low affinity for Na^+, possibly a consequence of protection against osmotic stress in the embryonic stage (Susanto and Charmantier 2001). Compared to juvenile and adult shrimps, the larval stages exhibit ATPase activities other than (Na^+,K^+)-ATPase. From the physiological standpoint, the 15 % stimulation of ouabain-insensitive ATPase activity, suggesting the presence of a Na^+-ATPase, warrants future investigation. Like the rat kidney cortex, this Na^+-ATPase activity may compensate for diminished Na^+

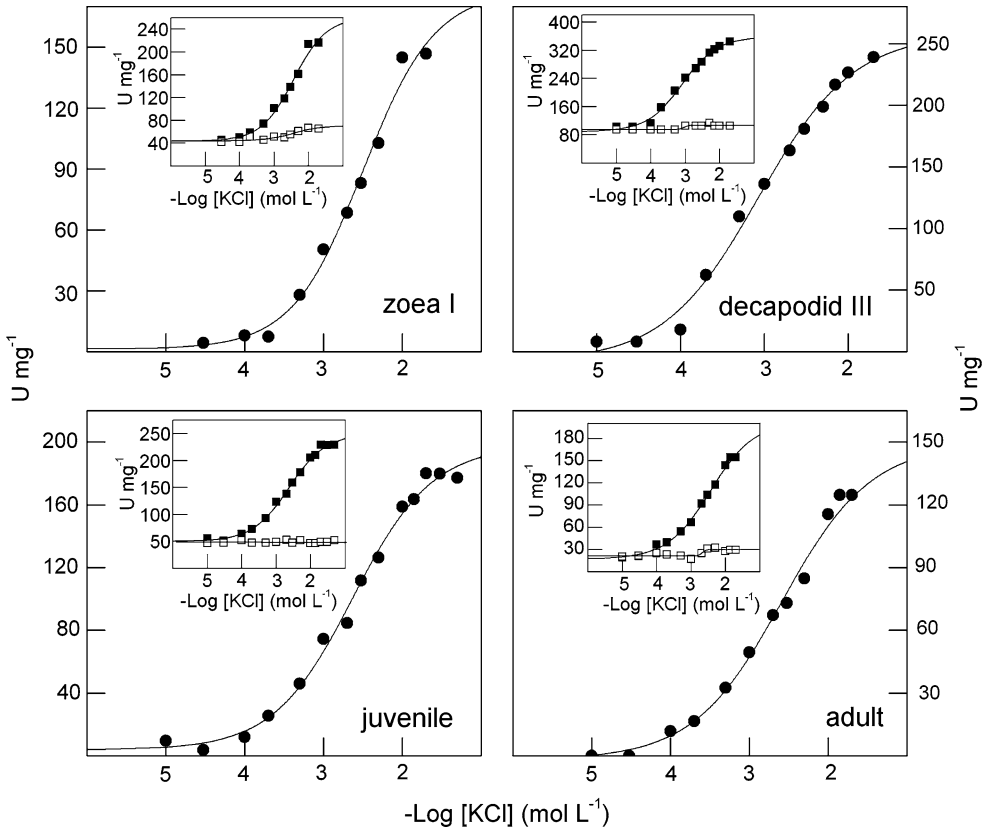

Fig. 4 Effect of K^+ concentration on (Na^+,K^+)-ATPase activity in microsomal fractions from *M. amazonicum* at different ontogenetic stages. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} HEPES buffer, pH 7.5, containing 2.0 mmol L^{-1} ATP, 5.0 mmol L^{-1} $MgCl_2$, 1.0 mmol L^{-1} NAD^+, 0.5 mmol L^{-1} sodium phosphate, 1.0 mmol L^{-1} G3P, 150 μg GAPDH (12 U), 20 μg PGK (9 U) and 50 mmol L^{-1} NaCl for zoea I and juvenile enzymes or 20 mmol L^{-1} NaCl for decapodid III and adult enzymes. Enzyme activity was assayed using 13.4, 7.2, 9.8 and 31.4 μg protein from homogenates of whole zoeae I and decapodid III, and gill homogenates of juvenile and adult shrimps, respectively. Experiments were performed using duplicate aliquots from three different homogenates; representative curves obtained from one homogenate are given. *Insets* Effect of K^+ concentration on ouabain-insensitive (*open square*) and total ATPase activities (*filled square*)

transport consequent to oscillation in (Na^+,K^+)-ATPase activity (Reyes et al. 2009).

The monophasic ouabain inhibition curves obtained suggest the absence of different (Na^+,K^+)-ATPase isoforms in the ontogenetic stages of pond-reared *M. amazonicum* as also seen in wild adult *M. amazonicum* (Santos et al. 2007) and *M. olfersi*, in which K_I is 2.5-fold less (Furriel et al. 2000). The comparable K_I values for ouabain inhibition of (Na^+,K^+)-ATPase in the early ontogenetic stages of *M. amazonicum*, considerably different from adult shrimps, may reflect the expression of different isoforms before larval metamorphosis. While different isoenzymes are expressed in crustacean gill tissues in response to salinity acclimation (Harris and Bayliss 1988; Genovese et al. 2004; Masui et al. 2005; Mendonça et al. 2007), no information is available on (Na^+,K^+)-ATPase isoform expression in the different ontogenetic stages. That newly hatched zoeae I of *Palaemonetes argentinus*, *M. petersi* and *M. amazonicum* hyperosmoregulate (Read 1984; Ituarte et al. 2008; Charmantier and Anger 2010), although functional gills are absent, suggests that the ion-transporting cells must be located elsewhere during the early stages, probably in the branchiostegite epithelium.

Owing to the absence of gills (zoea I) or their diminutive size (decapodid III), microsomal fractions of these stages were prepared using whole animals. While this procedure may occasion differences in enzyme-specific activity, the (Na^+,K^+)-ATPase activity of the decapodid III can be compared with that of zoea I and with the gill and whole-body preparations from juvenile and adult shrimps. Since we employed microsomal preparations rather than crude tissue homogenates, the activity increase in decapodid III cannot be attributed to a decrease in soluble proteins in the body fluids or associated with structural protein synthesis during metamorphosis. Further, any newly expressed structural proteins in the microsomal fraction from decapodid III would tend to decrease rather than increase (Na^+,K^+)-ATPase-specific activity. Thus, (Na^+,K^+)-ATPase activity in homogenates of whole adult and juvenile *M. amazonicum* is about one-third that of *P. argentinus*

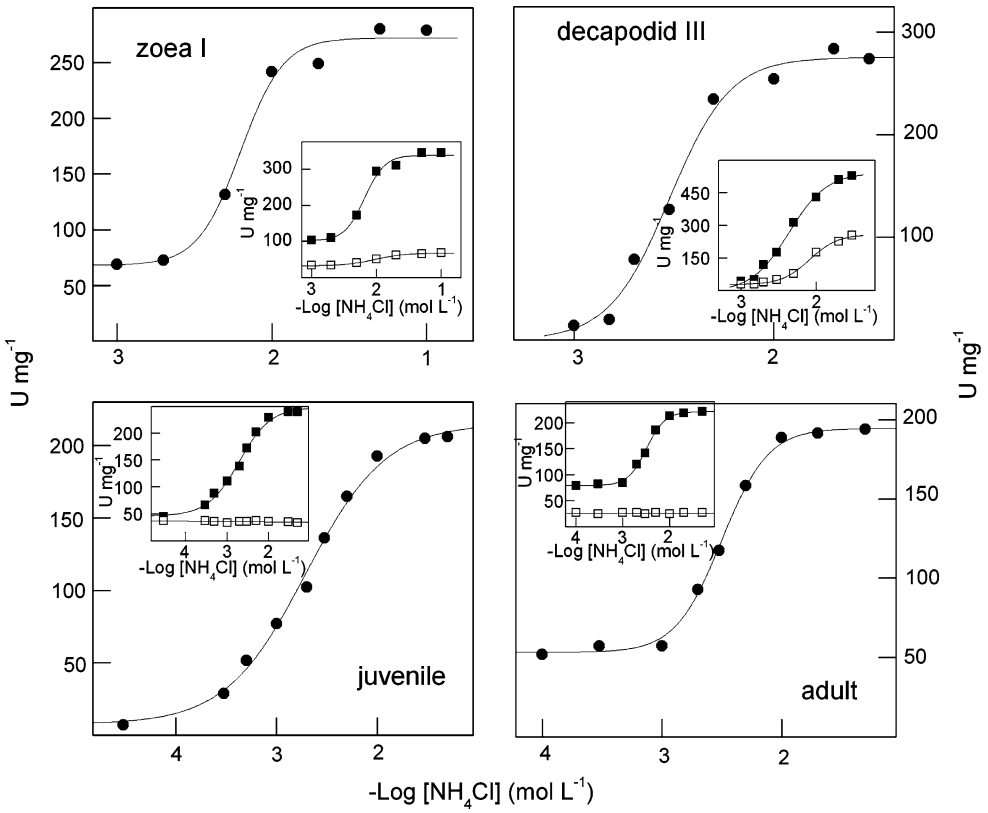

Fig. 5 Effect of NH_4^+ concentration on (Na^+,K^+)-ATPase activity in microsomal fractions from *M. amazonicum* at different ontogenetic stages. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} HEPES buffer, pH 7.5, containing 2.0 mmol L^{-1} ATP, 5.0 mmol L^{-1} $MgCl_2$, 1.0 mmol L^{-1} NAD^+, 0.5 mmol L^{-1} sodium phosphate, 1.0 mmol L^{-1} G3P, 150 μg GAPDH (12 U), 20 μg PGK (9 U) and 50 mmol L^{-1} NaCl for zoea I and juvenile enzymes or 20 mmol L^{-1} NaCl for decapodid III and adult enzymes. Enzyme activity was assayed using 13.4, 7.2, 9.8 and 31.4 μg protein from homogenates of whole zoeae I and decapodid III, and gill homogenates of juvenile and adult shrimps, respectively. Experiments were performed using duplicate aliquots from three different homogenates; representative curves obtained from one homogenate are given. *Insets* effect of NH_4^+ concentration on ouabain-insensitive (*open square*) and total ATPase activities (*filled square*)

gill tissue (Ituarte et al. 2008) and considerably lower than for zoea I and decapodid III. The few studies correlating the salinity tolerance of early ontogenetic stages with (Na^+,K^+)-ATPase activity have been undertaken mainly using crude tissue homogenates (Conte et al. 1977; Sun et al. 1991; Lee and Watts 1994; Escalante et al. 1995; Wilder et al. 2001; Huong et al. 2004; Ituarte et al. 2008) and not isolated microsomal fractions as employed here, and direct comparison is not possible.

The $K_{0.5}$ values for modulation by NH_4^+ of (Na^+,K^+)-ATPase activity in zoea I, decapodid III, juvenile and adult *M. amazonicum* are similar to those for *Callinectes danae* ($K_{0.5}$ = 4.61 ± 0.27 mmol L^{-1}, Masui et al. 2002), *Xiphopenaeus kroyeri* ($K_{0.5}$ = 3.7 ± 0.6 mmol L^{-1}, Leone et al. 2005b), *Clibanarius vittatus* ($K_{0.5}$ = 4.5 ± 0.2 mmol L^{-1}, Gonçalves et al. 2006), *Callinectes ornatus* ($K_{0.5}$ = 5.4 ± 0.2 mmol L^{-1}, Garçon et al. 2007), *M. amazonicum* ($K_{0.5}$ = 5.0 ± 0.2 mmol L^{-1}, Santos et al. 2007), *D. pagei* ($K_{0.5}$ = 1.92 ± 0.11 mmol L^{-1}, Furriel et al. 2010) and *M. olfersi* ($K_{0.5}$ = 8.4 ± 0.2 mmol L^{-1}, Furriel et al. 2004). NH_4^+ can replace K^+ in sustaining ATP hydrolysis in crustacean (Holliday 1985; Furriel et al. 2000; Masui et al. 2002; Gonçalves et al. 2006; Santos et al. 2007; Garçon et al. 2007; Furriel et al. 2010) and mollusk (Pagliarani et al. 2008) gill (Na^+,K^+)-ATPases, and NH_4^+ can substitute for K^+ as a counter ion in Na^+ transport in *Callinectes sapidus* (Towle and Holleland 1987). Like K^+, NH_4^+ can be actively transported by vertebrate (Na^+,K^+)-ATPase (Wall 1996). Synergistic stimulation by K^+ and NH_4^+ of crustacean (Na^+,K^+)-ATPase was first shown in *C. danae* (Masui et al. 2002, 2005) and now has been demonstrated in various crustaceans (Furriel et al. 2000; Gonçalves et al. 2006; Garçon et al. 2007, 2009; Santos et al. 2007; Furriel et al. 2010). While NH_4^+ excretion and active transport are not necessarily directly coupled in crabs (Weihrauch et al. 1999), exposure to elevated NH_3 may lead to substitution of K^+ by NH_4^+, decreasing intracellular K^+ (Towle and Holleland 1987). Further, acute exposure to NH_3 of *Neohelice* (=*Chasmagnathus*) *granulata* reveals hemolymph NH_3 to

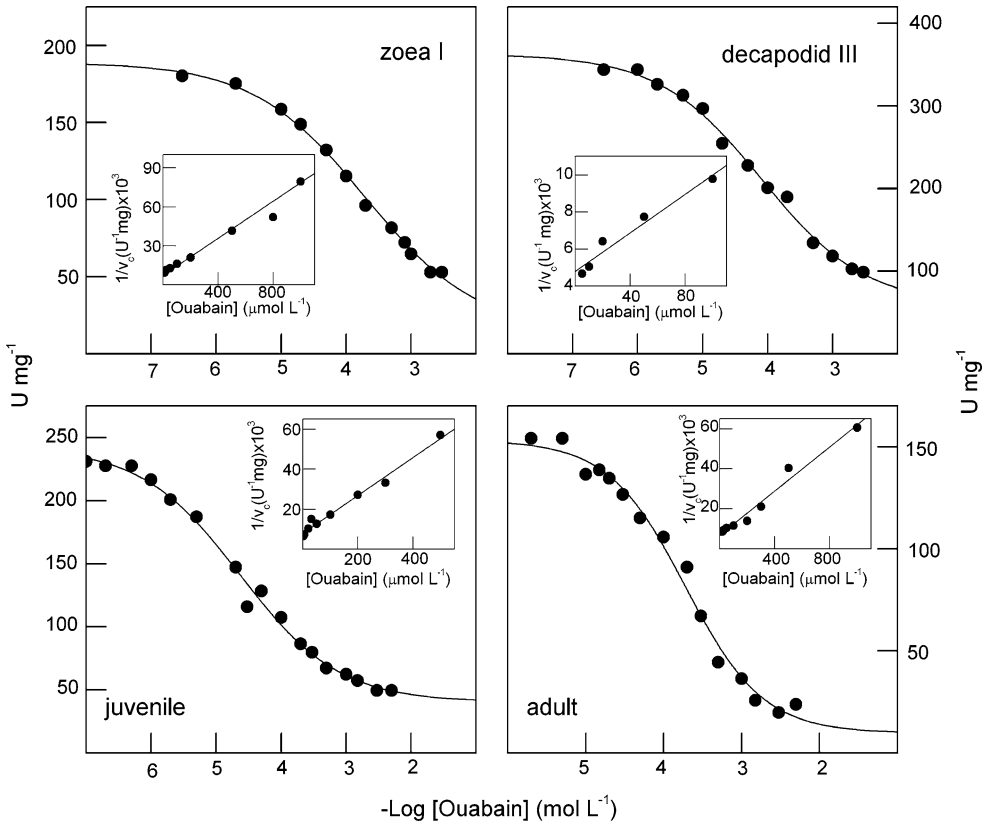

Fig. 6 Effect of ouabain on total ATPase activity in microsomal fractions from *M. amazonicum* at different ontogenetic stages. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} HEPES buffer, pH 7.5, containing 2.0 mmol L^{-1} ATP, 5.0 mmol L^{-1} $MgCl_2$, 20.0 mmol L^{-1} KCl, 0.14 mmol L^{-1} NADH, 2.0 mmol L^{-1} PEP, 82 μg PK (49 U), 110 μg LDH (94 U) and 50 mmol L^{-1} NaCl for zoea I and juvenile enzymes or 20 mmol L^{-1} NaCl for decapodid III and adult enzymes. Enzyme activity was assayed using 13.4, 7.2, 9.8 and 31.4 μg protein from homogenates of whole zoeae I, and decapodid III, and gill homogenates of juvenile and adult shrimps, respectively. Experiments were performed using duplicate aliquots from three different homogenates; representative curves obtained from one homogenate are given. *Insets* dixon plots for estimation of K_I, the enzyme-inhibitor complex dissociation constant, in which v_c is the reaction rate corresponding to (Na^+,K^+)-ATPase activity alone

be less than ambient (Rebelo et al. 1999). Consequently, and given that the NH_4^+ binding sites on the Na^+,K^+-ATPase molecule face the hemolymph, when hemolymph NH_3 exceeds physiological concentrations (100 μM), (Na^+,K^+)-ATPase may be stimulated, maintaining steady-state ammonia titers within physiological limits (Masui et al. 2002, 2005). In aquatic animals facing an inwardly directed ammonia gradient in their natural environment, the active component of an exocytotic ammonia excretion mechanism (Weihrauch et al. 2002) may provide protection for both the gill epithelial cells and the organism as a whole against passive NH_4^+ influx. The existence of such an excretion mechanism seems highly likely since, rather than diffusing freely across the cytoplasm, toxic ammonia would be trapped in intracellular vesicles, avoiding cytoplasmic damage (Weihrauch et al. 2002, 2004b).

Although these findings show that (Na^+,K^+)-ATPase activity in *M. amazonicum* is strongly modulated by Na^+, K^+, Mg^{2+} and NH_4^+ concentrations, further investigation is necessary to better comprehend modulation by ATP and ions of (Na^+,K^+)-ATPase activity during the ontogenesis of palemonid shrimps. We are undertaking a systematic investigation of Mg^{2+}-stimulated, ouabain-insensitive ATPase activities in addition to salinity acclimation studies to gather information on the different ATPases expressed during ontogeny. Such investigations should allow a better understanding of the marked, transitory increase in (Na^+,K^+)-ATPase activity in the decapodid III stage.

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