



# Activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase in a 'freshwater shrimp', *Palaemonetes argentinus* (Caridea, Palaemonidae): ontogenetic and salinity-induced changes

Romina B. Ituarte<sup>1,4,\*</sup>, Alejandra A. López Mañanes<sup>2,4</sup>, Eduardo D. Spivak<sup>1,4</sup>, Klaus Anger<sup>3</sup>

<sup>1</sup>Laboratorio Zoología Invertebrados, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Casilla de Correo 1245, 7600 Mar del Plata, Argentina

<sup>2</sup>Laboratorio Fisiología Bioquímica y Adaptativa, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3250, Mar del Plata B 7602 AYJ, Argentina

<sup>3</sup>Biologische Anstalt Helgoland, Stiftung Alfred-Wegener-Institut für Polar- und Meeresforschung, Meeresstation, 27498 Helgoland, Germany

<sup>4</sup>Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

**ABSTRACT:** Embryos, larvae, and adults of *Palaemonetes argentinus* tolerate a wide range of salinities (1 to 25‰). While osmoregulatory capacities have previously been demonstrated in all post-embryonic stages, little is known about the occurrence of osmoregulation during the embryonic phase. We examined ontogenetic and salinity-induced changes in the activity of a key enzyme involved in osmoregulation, Na<sup>+</sup>,K<sup>+</sup>-ATPase. Its activity was studied in: (1) eggs at an early (SI), an intermediate (SII), and a late stage of embryonic development (SIII); (2) in newly hatched larvae (Zoea-I, ZI); and in homogenates of (3) whole adults and (4) isolated gill tissue. All stages were directly exposed to 1, 15, or 25‰, and Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was chemically determined 24 h (embryos, larvae) or 48 h later (adults). Enzyme activity was detected in all developmental stages, being low in SI and SII, maximum in SIII, and intermediate in ZI and adults. Maximum salinity-induced activity changes prior to hatching (SIII) suggest that hyper-osmoregulatory functions are expressed by the end of the embryonic phase. The ontogenetic activity maximum at this stage, however, may also be related to the hatching process. Comparing different salinities, Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in SIII was always highest at 15‰, whereas the activity in gills was higher at both 15 and 25‰ than at 1‰. While gills are absent in the embryonic and early larval stages, ion-transporting cells must be located elsewhere during these early ontogenetic stages, probably in the brachiolegites.

**KEY WORDS:** Embryos · Salinity tolerance · Na<sup>+</sup>,K<sup>+</sup>-ATPase · Palaemonid shrimp · Gill

Resale or republication not permitted without written consent of the publisher

## INTRODUCTION

Euryhalinity is the ability of an aquatic organism to tolerate wide salinity variations without compromising life processes. It requires physiological, biochemical, morphological, and/or ecological adaptations, which may change during ontogeny. This trait is typically found in organisms living in estuaries, brackish coastal lagoons, mangrove swamps, and tide pools, where short-

term temporal as well as small-scale local variations in salinity occur.

In euryhaline species of decapod crustaceans, adaptive strategies and underlying physiological and biochemical mechanisms have mostly been studied in adult life-history stages (reviews in Péqueux 1995, Lucu & Towle 2003, Kirschner 2004). In the larval stages, by contrast, the physiological basis and metabolic implications of salinity tolerance have only re-

\*Email: ituarte@mdp.edu.ar

cently received increasing attention (Charmantier 1998, Agard 1999, Anger 2003), and the embryonic phase has, in this respect, remained even less researched (Charmantier & Charmantier-Daures 2001).

As in conspecific adults, larval euryhalinity is generally linked to the capability of osmoregulation (Read 1984, Charmantier 1998, Cieluch et al. 2007). Although experimental evidence has remained scarce, mainly due to technical problems of sampling and analyzing hemolymph from small crustacean embryos, the available information suggests that osmoregulatory capabilities may develop even earlier, i.e. at some stage during embryogenesis (Felder et al. 1986, Wilder et al. 2001, Taylor & Seneviratna 2005, Serrano et al. 2007). Even though similar physiological mechanisms have been found in larvae and adults of some species (Read 1984), numerous examples for dramatic ontogenetic changes have been observed in various others, mostly associated with ontogenetic migrations across different habitats (Charmantier 1998, Anger 2001). As a consequence, extrapolations of adult mechanisms to the early life-history stages may often result in wrong assumptions, so that separate studies of the ontogeny of species-specific traits remain necessary.

Osmotic and ionic regulation are possible through 2 different principal mechanisms (Péqueux 1995, Augusto et al. 2007): (1) intra-cellular isosmotic regulation, which is responsible for the maintenance of intra-cellular fluid composition and cell volume, and (2) anisosmotic extra-cellular regulation, which controls hemolymph osmolality, ionic composition, and extra-cellular liquid volume. Moreover, protection of the internal fluids against potentially detrimental passive osmotic changes may be achieved through reduced integumental permeability (Rainbow & Black 2001).

The enzyme  $\text{Na}^+, \text{K}^+$ -ATPase (the 'sodium pump'), which is the main focus of the present study, is fundamental to osmotic regulation in most eukaryotic cells (Balshaw et al. 2001, Jorgensen et al. 2003). While it has many additional functions, it also plays a key role in extra-cellular osmoregulation. In adult decapods exposed to hypo-osmotic media,  $\text{Na}^+, \text{K}^+$ -ATPase drives active ion absorption across transporting epithelia that are commonly located in the gills and antennal glands; in hyper-osmotic media, it mediates ion secretion in the reverse direction (Augusto et al. 2007, and references therein). The capability of hyper-osmoregulation in dilute media is considered to be a physiological key prerequisite for the invasion of brackish and freshwater environments (e.g. Freire et al. 2003). Although hypo-osmotic stress selects here for an early appearance of osmoregulatory functions, homeostatic ionic regulation processes have rarely been studied in larval and embryonic life-history stages of decapod crustaceans (Charmantier 1998, Charmantier & Charmantier-Daures 2001).

Among the Decapoda, palaemonid shrimps may be considered prime models for studies of physiological adaptations associated with limnic invasions by originally marine organisms (Freire et al. 2003). This caridean family represents a particularly diverse and ecologically important group, which can be found in marine, estuarine, as well as freshwater habitats (Bauer 2004). There is general consensus that this taxon evolved in the sea, before it invaded brackish coastal, estuarine, and eventually limnic environments (e.g. Freire et al. 2003, Augusto et al. 2007), and several genera may still be in a process of limnic radiation, showing convergent patterns of adaptation (e.g. Murphy & Austin 2005, Augusto et al. 2007). However, in this family as well, only little is known about the ontogeny of osmoregulation, especially during the embryonic phase (Wilder et al. 2001, Huong et al. 2004, Augusto et al. 2007).

Among the Palaemonidae, the so-called 'freshwater shrimp' *Palaemonetes argentinus* Nobili, 1901 should be a particularly good model for studies of physiological adaptations to brackish and freshwater conditions. This species occurs in limnic inland habitats such as lakes and streams, but also in brackish coastal lagoons connected to the sea, geographically ranging from central eastern Argentina to Uruguay and southern Brazil (Boschi et al. 1992, Spivak 1997). Its entire life cycle can be completed in freshwater or brackish conditions ranging from about 1 to 25‰ (Ituarte 2008). Preliminary laboratory experiments (Cieluch 2000, K. Anger unpubl. data), however, have shown that the larvae of this species prefer brackish conditions (5 to 15‰) for their development, regardless of whether the population originated from a land-locked limnic habitat or a brackish coastal lagoon. This suggests that *P. argentinus* has only recently invaded freshwater environments and may not have fully adapted to freshwater (Anger 2001). Consistent with their ecology, all postembryonic life-history stages of this species are strong hyper-osmoregulators in dilute media, but osmoconformers at high salinities (Charmantier & Anger 1999).

While the embryonic development of the nervous system of *Palaemonetes argentinus* has been studied in detail (Harzsch et al. 1997), no comparable data have become available for the embryogenesis of the ion-regulating system. When embryos (from a land-locked population) were cultured *in vitro* (i.e. isolated from potential maternal effects), they were able to survive and develop until hatching under a wide range of salinities (1 to 25‰), although the lowest and highest salinities caused osmotic swelling or shrinking, respectively (Ituarte et al. 2005). This suggests that at least a limited capability of osmoregulation may appear, for the first time in the ontogeny of this species, already

during the embryonic phase. This physiological function has been shown to be present at hatching and to gradually increase thereafter, throughout the larval and juvenile life-history stages (Charmantier & Anger 1999). In the embryos, however, euryhalinity could also be based on reduced permeability of the egg membrane (cf. Rainbow & Black 2001), while evidence for embryonic osmoregulation has been lacking.

In the present study, we quantified in *Palaemonetes argentinus* both ontogenetic and salinity-induced changes in the activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase, which may be considered an indirect indication of osmoregulation in early life-history stages. The enzyme activity was measured in 3 stages of embryonic development, in first-stage larvae, and in adult shrimps after short-term exposure to 3 different salinities (1, 15, 25‰). In addition, salinity effects on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity were determined in gills of adult shrimps, which are considered the main site of ionic and osmotic regulation (Freire et al. in press).

## MATERIALS AND METHODS

**Experimental procedure.** Ovigerous females, females with fully developed ovaries, and males of *Palaemonetes argentinus* were collected from Lake Chascomús (35° 36' S, 58° W), Province of Buenos Aires, Argentina. The salinity of Lake Chascomús may vary between oligohaline (0.5 to 5‰) and hypohaline conditions (<0.5‰), depending on rain, evaporation, and winds (Ituarte et al. 2005, 2007). During the present study, however, salinity varied only very little around 0.5‰ (conductivity ranging between 0.97 and 1.13 mS cm<sup>-1</sup>).

Shrimps were transported to the laboratory in water from the sampling site. Before the experiments, ovigerous females were maintained for at least 3 d in aquaria (30 × 30 × 50 cm) with dechlorinated tap water, oxygen supply, a temperature of 22 ± 2°C, an ambient photoperiod (~10 h light:14 h dark cycle), and TetraMin Pro Tropical Crisps provided as food. Non-ovigerous females with fully developed ovaries were kept in separate aquaria, together with males, in order to obtain newly fertilized eggs. The aquaria were examined twice daily.

Under the conditions described above, total embryonic development from egg-laying to hatching took from 26 to 27 d. Prior to experiments, 3 embryonic stages (reached after ca. 2, 12, and 24 d, respectively) were separated after inspection under a stereomicroscope: (1) newly produced embryos with little differentiation, 100% of egg volume occupied by yolk (early embryos, SI); (2) embryos with eyes visible as a reddish line, heartbeat already visible but often irregular, ca. 50 to 60% yolk (intermediate embryos, SII); and

(3) embryos with eyes fully developed, heartbeat regular, yolk largely depleted (late embryos, SIII). The same 3 major stages of embryonic development were identified and photographically documented by Harzsch et al. (1997; note, however, that the earliest stage shown in their paper was slightly more advanced than our initial stage SI). Additionally, entire larvae and adult shrimps (both males and females without eggs or developed ovaries) and isolated gill tissue from adult shrimps were used for experiments.

The experimental animals were directly transferred to salinities of 1, 15, and 25‰ (obtained by dilution of filtered seawater with dechlorinated tap water; Schleicher and Schuell filter paper 0859, pore size ca. 7 to 12 µm). Ovigerous females were kept for 24 h in each medium before embryos were carefully detached from the incubation chamber. Freshly hatched larvae (Zoea I, ZI) were obtained from females carrying late embryos. The acclimation time in larvae and adult shrimps was 24 and 48 h in each treatment, respectively (Charmantier & Anger 1999). Gills were removed with delicate tweezers from both the left and right sides of the branchial chamber of adult shrimps, after anesthetizing the shrimps by chilling on ice for at least 5 min. During the acclimation time, experimental animals were maintained without feeding.

**Measurement of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity.** Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was measured in 5 replicates (only 3 in isolated gills), with 40 to 50 mg of wet weight tissue per determination. Each replicate sample of embryos or larvae comprised pooled materials from 3 broods, while each replicate of adult shrimps was represented by a single individual. Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in gills was measured in pooled samples taken from 10 to 12 adults per each replicate. The preparation of samples for enzymatic determinations was consistently made at 0°C. All samples were rapidly mixed in homogenizing medium (0.25 M sucrose/0.25 mM EGTA-Tris, pH 7.4) and subsequently homogenized in a motor-driven, hand-operated Teflon-glass homogenizer. Glycerol (1.3% v/v) was added before Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was determined (López Mañanes et al. 2002).

Total Mg<sup>2+</sup>,Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was determined by measuring ATP hydrolysis in a reaction medium containing 100 mM NaCl, 30 mM KCl, 10 mM MgCl<sub>2</sub>, and 0.5 mM EGTA in 20 mM imidazol buffer (pH 7.4). Basal Mg<sup>2+</sup>-ATPase was determined in the same reaction medium, but without KCl and in the presence of 1 mM ouabain. Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was determined as the difference between both assays. An aliquot of the corresponding sample (5 µg of protein) was added to the reaction mixture and pre-incubated at 30°C for 5 min. The reaction was started by addition of ATP (final concentration 5 mM). Incubation was carried out at 30°C for 20 min. The reaction was stopped by addi-

tion of 2 ml of cooled Bonting reagent (560 mM sulfuric acid, 8.1 mM ammonium molybdate, and 176 mM ferrous sulfate). After 20 min at room temperature, the amount of released phosphate ( $P_i$ ) was determined by reading the absorbance at 700 nm of the reduced phosphomolibdate complex. In addition, protein was assayed following Bradford (1976), using bovine serum albumin ( $0.96 \text{ mg ml}^{-1}$ ) as a standard.

**Statistical analysis.** All values were expressed as arithmetic means  $\pm$  1 standard error (SE). Differences in  $\text{Na}^+, \text{K}^+$ -ATPase activity between life-history stages and salinity treatments were tested by 2-way ANOVA. The same method was used to test differences in basal activity ( $\text{Mg}^{2+}$ -ATPase) between life-history stages and salinity treatments. Effects of salinity on  $\text{Na}^+, \text{K}^+$ -ATPase activity in gill tissue, as well as effects on basal activity, were tested by 1-way ANOVA. All tests were performed after checks for normal distribution and equality of variance (Kolmogorov-Smirnov and Cochran tests, respectively; Underwood 1997). Data were transformed when necessary. When the ANOVA was significant, differences between treatments were tested *a posteriori* with planned comparisons (when there was a significant interaction) or Student-Newman-Keuls (SNK) tests (Underwood 1997).

## RESULTS

Activity of  $\text{Na}^+, \text{K}^+$ -ATPase was detected in all 3 salinity treatments and in all 5 life-history stages of *Palaemonetes argentinus* studied here. At each salinity level, it changed significantly across life-history stages (Fig. 1a, Table 1). At the lowest test salinity (1‰), enzyme activity was detected in newly laid eggs (SI). It subsequently doubled in SII embryos (reaching  $17.7 \pm 4.3$  vs.  $6.9 \pm 1.4 \text{ nmol } P_i \text{ mg}^{-1} \text{ protein min}^{-1}$ ); however, due to high variability this difference was not statistically significant. A significant increase occurred prior to hatching (SIII), reaching a maximum of  $142.1 \pm 9.5 \text{ nmol } P_i \text{ mg}^{-1} \text{ protein min}^{-1}$ . In post-embryonic stages (ZI larvae, adult shrimps), the activity of  $\text{Na}^+, \text{K}^+$ -ATPase was significantly lower than in SIII embryos ( $53.3 \pm 4.9$  and  $43.4 \pm 6.1 \text{ nmol } P_i \text{ mg}^{-1} \text{ protein min}^{-1}$ , respectively). At 15 and 25‰, ontogenetic patterns of change in activity were similar to those observed at 1‰ (Fig. 1a).

High ion concentrations (15 and 25‰) induced a significant increase in  $\text{Na}^+, \text{K}^+$ -ATPase activity, however, there was also a significant interaction between salinity and life-history stages (Table 1). The interaction was due to the fact that salinity significantly influenced the enzyme activity in SIII and post-embryonic stages, but not in the earlier embryonic stages (SI, SII; Fig. 1a).

Gill  $\text{Na}^+, \text{K}^+$ -ATPase activity was significantly affected by salinity ( $F_{2,6} = 25.86$ ,  $p < 0.01$ ), with activity levels

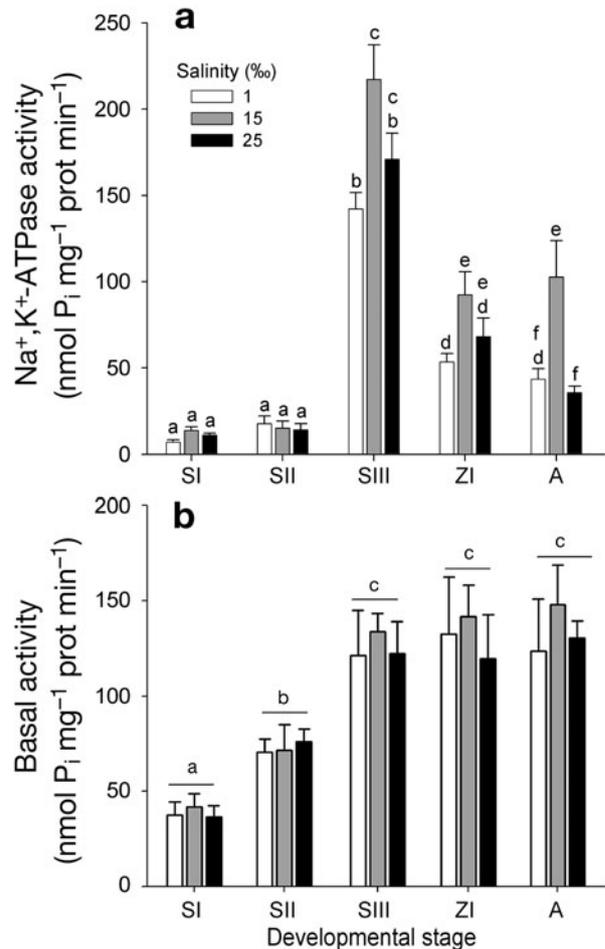


Fig. 1. *Palaemonetes argentinus*. (a) Ontogenetic and salinity-induced changes in  $\text{Na}^+, \text{K}^+$ -ATPase activity. Embryonic stages (SI, SII, SIII), first larva (ZI), and whole adults (A). Different letters above bars show significant differences among developmental stages at each level of salinity or among salinities for the same stage (planned comparisons,  $p < 0.05$ ). (b) Basal ( $\text{Mg}^{2+}$ -ATPase) activity. Different letters above bars show significant differences among developmental stages (Student-Newman-Keuls test,  $p < 0.05$ ); lines indicate non-significant differences among salinities

Table 1. *Palaemonetes argentinus*. 2-way ANOVA of  $\text{Na}^+, \text{K}^+$ -ATPase activity, in 5 ontogenetic stages (SI, SII, SIII, ZI, adults) and 3 salinity treatments (1, 15, 25‰). MS: mean squares; significant effects are in bold

Source of variation	df	MS	F	p
Stage	4	28.69	158.68	<b>&lt;0.0001</b>
Salinity	2	2.46	13.62	<b>&lt;0.0001</b>
Stage $\times$ Salinity	8	0.45	2.49	<b>0.02</b>
Error	60	0.18		

being higher at 15 and 25‰ than at 1‰ (Fig. 2a). In addition, high levels of  $\text{Na}^+, \text{K}^+$ -ATPase activity were observed in gill tissue compared to those in whole shrimps (Figs. 1a & 2a).

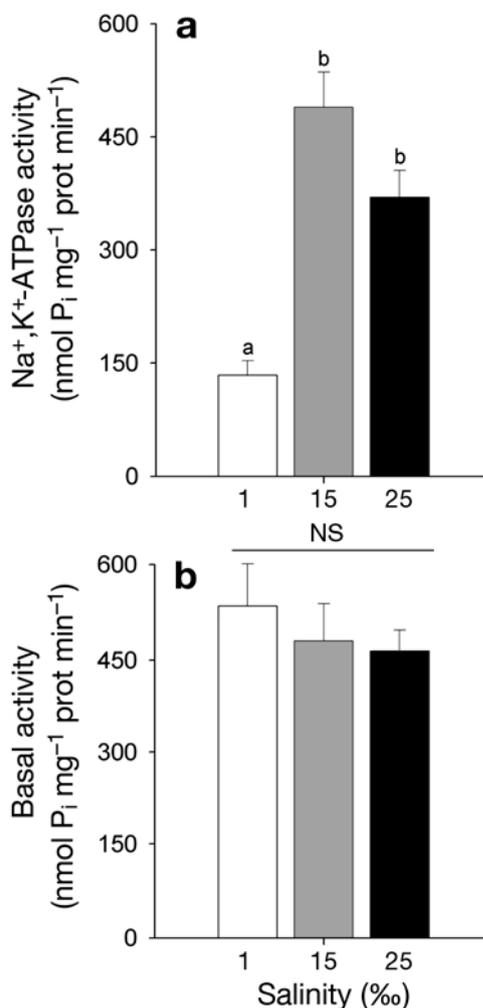


Fig. 2. *Palaemonetes argentinus*. (a) Salinity-induced changes on the Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in gill tissue. Different letters above bars show significant differences among salinities (Student-Newman-Keuls test,  $p < 0.05$ ). (b) Basal (Mg<sup>2+</sup>-ATPase) activity in gill tissue; no significant differences

Basal (Mg<sup>2+</sup>-ATPase) activity varied significantly among life-history stages ( $F_{4,60} = 33.77$ ,  $p < 0.0001$ ), but there was no significant effect of salinity treatments ( $F_{2,60} = 0.79$ ,  $p = 0.45$ ) or significant interaction between these factors ( $F_{8,60} = 0.21$ ,  $p = 0.99$ ). Activity increased significantly from SI to SIII, remaining on a similar level thereafter (no significant differences between SIII, ZI, adults; Fig. 1b). Likewise, the basal activity in gills was not affected by salinity ( $F_{2,6} = 0.39$ ,  $p = 0.69$ ) (Fig. 2b).

## DISCUSSION

In our experiments with *Palaemonetes argentinus*, a species of palaemonid shrimp that lives in both brackish and freshwater habitats, we showed that the activity of the ion-regulating key enzyme Na<sup>+</sup>,K<sup>+</sup>-ATPase

varies both ontogenetically and after variations in salinity. The latter response was similar in SIII embryos and in adult gill tissue, indicating that mechanisms, which are typical of euryhaline adults (i.e. active ion transport based on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity) may become functional in early life-history stages, most probably during the final phase of embryogenesis. This is consistent with the finding that all life-cycle stages co-occur in habitats, where low salt concentrations prevail. As an additional mechanism, a reduced ion permeability of the egg membranes may aid in embryonic osmo-protection.

An increasing activity of this key enzyme during the course of embryonic development has also been observed in several other species of decapod crustaceans. In embryos of *Palaemonetes argentinus*, the activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase increased up to 18-fold, depending on salinity conditions (Fig. 1a), and in those of a closely related species of freshwater shrimp, *Macrobrachium rosenbergii* de Man, 1879, it was almost 15-fold higher prior to hatching than in early embryos (Wilder et al. 2001). In an estuarine ghost shrimp, *Callinassa jamaicensis* var. *louisianensis* Schmitt, 1935, the activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase increased 12-fold during embryonic development (Felder et al. 1986), and Taylor & Seneviratna (2005) reported a 50-fold increase in embryos of an intertidal crab, *Hemigrapsus crenulatus* Milne Edwards, 1837.

Independent of their habitat (freshwater, brackish water, intertidal), all these species showed a high Na<sup>+</sup>,K<sup>+</sup>-ATPase activity close to hatching. While this pattern suggests that osmoregulatory structures become functional during late embryogenesis, subsequently decreasing Na<sup>+</sup>,K<sup>+</sup>-ATPase activities in larvae and adults (a similar result was reported for *Callinassa jamaicensis*; Felder et al. 1986) may be associated with other functions of this key enzyme (see Balshaw et al. 2001, Jorgensen et al. 2003). Maximum activity in late embryos (SIII) may be related to mechanisms of hatching, which involve numerous biochemical and ultrastructural reconstruction processes (Saigusa 1996). High enzyme activity should cause rapid Na<sup>+</sup> uptake and, as a consequence, increased osmotic pressure, followed by passive water influx, which may then lead to rupturing of the egg membrane and hatching.

Na<sup>+</sup>,K<sup>+</sup>-ATPase activity is concentrated in specialized ion-regulatory cells and tissues, which are mostly found in gills (Péqueux 1995, Charmantier 1998, Torres et al. 2007). In *Palaemonetes argentinus*, as in several other decapod taxa, however, the function of hyper-osmoregulation is already present at hatching, although functional gills are absent in the early larval stages (Felder et al. 1986, Charmantier 1998, Anger 2001). Hence, ion-transporting cells must be located elsewhere during these early ontogenetic stages. In

histological and electron-microscopical investigations, ion-transporting tissues have been detected in the dorsal organ of crab embryos (Seneviratna & Taylor 2006) as well as in larval structures associated to the branchial chamber (Felder et al. 1986, Cieluch et al. 2005, 2007). In larvae of *P. argentinus*, Cieluch (2000) found ultrastructural evidence for an occurrence of ion-transporting cells in the branchiostegites. The localization of ionocytes in embryos, however, remains to be evaluated in future studies.

In adult *Palaemonetes argentinus*, a suddenly increasing salt concentration induced in isolated gills an increase in the activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase, while it did not influence basal enzyme activity (Fig. 2). Moreover, higher levels of activity in gills than in whole shrimps show that Na<sup>+</sup>,K<sup>+</sup>-ATPase is more concentrated in these transporting organs than in other regions of the body. These observations corroborate a linkage between Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, gill tissue, and ion transport. In addition, however, Na<sup>+</sup>,K<sup>+</sup>-ATPase has also been located in 2 other epithelia of the branchial chamber of adult caridean shrimps, in the branchiostegites and epipodites (Martinez et al. 2005). Immunolocalization and molecular studies of the expression of mRNA coding for Na<sup>+</sup>,K<sup>+</sup>-ATPase should confirm our present biochemical evidence that gill tissue in *P. argentinus* also plays an essential function in ion transport.

While our findings may reflect consistent eco-physiological relationships between ontogeny, Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, hyper-osmoregulation in dilute media, and life under brackish and limnic conditions, the response to enhanced salinities is less clear. *Palaemonetes argentinus* is a strong hyper-osmoregulator in salinities from 1 to 17‰, but osmoconforming at levels >20‰ (Charmantier & Anger 1999). An increasing gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity at 15‰ is thus consistent with the ability to hyper-regulate at moderate to low salinities. High activity measured at 25‰, by contrast, where this species is an osmoconformer, should be associated with some other functions of this enzyme, e.g. in intracellular volume regulation. Enhanced Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in gills of *P. argentinus* exposed to high salinity (Fig. 2a) is contrary to the patterns observed in euryhaline crabs (e.g. Péqueux 1995, Lucu & Towle 2003, Kirschner 2004), but consistent with increasing levels of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in the gills of anadromous fish transferred to seawater (Deane & Woo 2004, Bystriansky et al. 2006). In the gills of another palaemonid freshwater shrimp, *Macrobrachium rosenbergii*, hyper-osmotic conditions did not significantly affect the activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase (Wilder et al. 2000). In the congener *M. olfersii* Wiegmann, 1836, in contrast, high salinity induced a reduction of the apical surface of ionocytes in gill tissue, so that ionic permeability and, probably, Na<sup>+</sup>,K<sup>+</sup>-ATPase

activity may have decreased (Lima et al. 1997, McNamara & Lima 1997). These inconsistent response patterns suggest that the tolerance of high osmotic pressures and related mechanisms of hypo-osmoregulation have not fully been understood and deserve further attention in future studies of comparative crustacean physiology.

In freshwater-tolerant species, lower isosmotic points are believed to reflect better osmotic adaptation (Augusto et al. 2007). The isosmotic point in adults of *Palaemonetes argentinus* (500 mOsm kg<sup>-1</sup> or 17‰; Charmantier & Anger 1999) is quite similar to that measured in fully limnic palaemonids, which complete their whole life cycle in freshwater, e.g. *Macrobrachium brasiliense*, *M. equidens*, and *M. potiuna* (521, 529, and 552 mOsm kg<sup>-1</sup>, respectively; Augusto et al. 2007 and references therein). However, *P. argentinus* from the limnic population of lake Chascomús shows a capability to also cope with suddenly increasing salinities, possibly based on increasing Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. Regulation of this key enzyme may thus allow this 'freshwater shrimp' to survive and reproduce in a wide range of salinities. This extremely euryhaline species may thus be a suitable invertebrate model for studies of physiological processes involved in salinity tolerance (e.g. Deane & Woo 2004). For instance, future studies should reveal if Na<sup>+</sup>,K<sup>+</sup>-ATPase activity is modulated through activation of pre-existing proteins and/or the synthesis of novel proteins, and if significant ontogenetic changes occur in major molecular and biochemical processes.

**Acknowledgements.** We thank S. Pinoni and C. del Valle for their assistance in the laboratory during the experiments. We also appreciate the constructive criticism from the editor and 3 reviewers, who helped to improve this manuscript. R.B.I. had a fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET). The present paper is based on work done by R.B.I. in partial fulfillment of the requirements for a PhD degree at the UNMDP and funded by grants from the University of Mar del Plata E15/149 and the Agencia Nacional de Promoción Científica y Tecnológica (PICT04 No. 21757).

#### LITERATURE CITED

- Agard JBR (1999) A four-dimensional response surface analysis of the ontogeny of physiological adaptation to salinity and temperature in larvae of the palaemonid shrimp *Macrobrachium rosenbergii* (de Man). *J Exp Mar Biol Ecol* 236:209–233
- Anger K (2001) The biology of decapod crustacean larvae. *Crustacean issues*, Vol 14. Balkema, Lisse
- Anger K (2003) Salinity as a key parameter in the larval biology of decapod crustaceans. *Invertebr Reprod Dev* 43: 29–45
- Augusto A, Greene LJ, Laure HJ, McNamara JC (2007) The ontogeny of isosmotic intracellular regulation in the diadromous, freshwater palaemonid shrimps, *Macro-*

- brachium amazonicum* and *M. olfersi* (Decapoda). *J Crustac Biol* 27:626–634
- Balshaw DM, Millette LA, Wallick ET (2001) Sodium pump function. In: Sperelakis N (ed) *Cell physiology source book: a molecular approach*. Academic Press, San Diego, CA, p 261–268
- Bauer RT (2004) Remarkable shrimps: adaptations and natural history of the carideans. University of Oklahoma Press, Norman
- Boschi EE, Fischbach CE, Iorio MI (1992) Catálogo ilustrado de los crustáceos estomatópodos y decápodos marinos de Argentina. *Frente Marítimo*, 10:7–94
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein-dye binding. *Anal Biochem* 72:248–254
- Bystriansky JS, Richards JG, Schulte PM, Ballantyne JS (2006) Reciprocal expression of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit isoforms  $\alpha$ 1a and  $\alpha$ 1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. *J Exp Biol* 209:1848–1858
- Charmantier G (1998) Ontogeny of osmoregulation in crustaceans: a review. *Invertebr Reprod Dev* 33:177–190
- Charmantier G, Anger K (1999) Ontogeny of osmoregulation in the palaemonid shrimp *Palaemonetes argentinus* (Crustacea: Decapoda). *Mar Ecol Prog Ser* 181:125–129
- Charmantier G, Charmantier-Daures M (2001) Ontogeny of osmoregulation in crustaceans: the embryonic phase. *Am Zool* 41:1078–1089
- Cieluch U (2000) Ultrastrukturelle Untersuchungen zum Einfluß von Salinität auf die Entwicklung von Transportepithelien bei *Palaemonetes argentinus* (Crustacea, Decapoda). MS dissertation, Tierärztliche Hochschule Hannover, Hannover
- Cieluch U, Charmantier G, Grousset E, Charmantier-Daures M, Anger K (2005) Osmoregulation, immunolocalization of Na<sup>+</sup>/K<sup>+</sup>-ATPase, and ultrastructure of branchial epithelia in the developing brown shrimp, *Crangon crangon* (Decapoda, Caridea). *Physiol Biochem Zool* 78:1017–1025
- Cieluch U, Anger K, Charmantier-Daures M, Charmantier G (2007) Osmoregulation and immunolocalization of Na<sup>+</sup>/K<sup>+</sup>-ATPase during the ontogeny of the mitten crab *Eriocheir sinensis* (Decapoda, Grapsoidae). *Mar Ecol Prog Ser* 329:169–178
- Deane EE, Woo NYS (2004) Differential gene expression associated with euryhalinity in sea bream (*Sparus sarba*). *Am J Physiol* 287:R1054–R1063
- Felder JM, Felder DL, Hand SC (1986) Ontogeny of osmoregulation in the estuarine ghost shrimp *Callinassa jamaicensis* var. *louisianensis* Schmitt (Decapoda, Thalassinidea). *J Exp Mar Biol Ecol* 99:91–105
- Freire CA, Cavassin F, Rodríguez EN, Torres AH, McNamara JC (2003) Adaptive patterns of osmotic and ionic regulation, and the invasion of fresh water by the palaemonid shrimps. *Comp Biochem Physiol* A 136:771–778
- Freire CA, Onken H, McNamara JC (in press) A structure–function analysis of ion transport in crustacean gills and excretory organs. *Comp Biochem Physiol* A
- Harzsch S, Anger K, Dawirs RR (1997) Immunocytochemical detection of acetylated  $\alpha$ -tubulin and *Drosophila* synapsin in the embryonic crustacean nervous system. *Int J Dev Biol* 41:477–484
- Huong DTT, Jayasankar V, Jasmani S, Saido-Sakanaka H, Wigginton AJ, Wilder MN (2004) Na/K-ATPase activity during larval development in the giant freshwater prawn *Macrobrachium rosenbergii* and the effects of salinity on survival rates. *Fish Sci* 70:518–520
- Ituarte RB (2008) Efectos de la salinidad sobre la reproducción y el desarrollo del ‘camarón de agua dulce’ *Palaemonetes argentinus*. PhD dissertation, Universidad Nacional de Mar del Plata, Mar del Plata
- Ituarte RB, Spivak ED, Anger K (2005) Effects of salinity on embryonic development of *Palaemonetes argentinus* (Crustacea: Decapoda: Palaemonidae) cultured *in vitro*. *Invertebr Reprod Dev* 47:213–223
- Ituarte RB, Spivak ED, Anger K (2007) Intraspecific variability in life-history traits of a ‘freshwater shrimp’, *Palaemonetes argentinus*. *Ann Limnol* 43:293–302
- Jorgensen PL, Håkansson KO, Karlsh SJD (2003) Structure and mechanism of Na,K-ATPase: functional sites and their interactions. *Annu Rev Physiol* 65:817–849
- Kirschner LB (2004) The mechanism of sodium chloride uptake in hyperregulating aquatic animals. *J Exp Biol* 207:1439–1452
- Lima GA, McNamara JC, Terra WR (1997) Regulation of hemolymph osmolytes and gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activities during acclimation to saline media in the freshwater shrimp *Macrobrachium olfersii* (Wiegmann, 1836) (Decapoda, Palaemonidae). *J Exp Mar Biol Ecol* 215:81–91
- López Mañanes AA, Meligeni CD, Goldemberg AL (2002) Response to environmental salinity of Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in individual gills of the euryhaline crab *Cyrtograpsus angulatus*. *J Exp Mar Biol Ecol* 274:75–85
- Lucu C, Towle W (2003) Na<sup>+</sup>+K<sup>+</sup>-ATPase in gills of aquatic Crustacea. *Comp Biochem Physiol* A 135:195–214
- Martinez AS, Charmantier G, Compère P, Charmantier-Daures M (2005) Branchial chamber tissues in two caridean shrimps: the epibenthic *Palaemon adspersus* and the deep-sea hydrothermal *Rimicaris exoculata*. *Tissue Cell* 37:153–165
- McNamara JC, Lima GA (1997) The route of ion and water movements across the gill epithelium of the freshwater shrimp *Macrobrachium olfersii* (Decapoda, Palaemonidae): evidence from ultrastructural changes induced by acclimation to saline media. *Biol Bull* 192:321–331
- Murphy NP, Austin CM (2005) Phylogenetic relationships of the globally distributed freshwater prawn genus *Macrobrachium* (Crustacea: Decapoda: Palaemonidae): biogeography, taxonomy and the convergent evolution of abbreviated larval development. *Zool Scr* 34:187–197
- Péqueux A (1995) Osmotic regulation in crustaceans. *J Crustac Biol* 15:1–60
- Rainbow PS, Black WH (2001) Effects of changes in salinity on the apparent water permeability of three crab species: *Carcinus maenas*, *Eriocheir sinensis* and *Necora puber*. *J Exp Mar Biol Ecol* 264:1–13
- Read GHL (1984) Intraspecific variation in the osmoregulatory capacity of larval, post-larval, juvenile and adult *Macrobrachium petersi* (Hilgendorf). *Comp Biochem Physiol* 78:501–506
- Saigusa M (1996) Two kinds of active factor in crab hatch water: ovigerous-hair stripping substance (OHSS) and a proteinase. *Biol Bull* 191:234–240
- Seneviratna D, Taylor HH (2006) Ontogeny of osmoregulation in embryos of intertidal crabs (*Hemigrapsus sexdentatus* and *H. crenulatus*, Grapsidae, Brachyura): putative involvement of the embryonic dorsal organ. *J Exp Biol* 209:1487–1501
- Serrano L, Towle DW, Charmantier G, Spanings-Pierrot C (2007) Expression of Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit mRNA during embryonic development of the crayfish *Astacus leptodactylus*. *Comp Biochem Physiol D Genomics Proteomics* 2:126–134
- Spivak ED (1997) Life history of a brackish-water popula-

- tion of *Palaemonetes argentinus* (Decapoda: Caridea) in Argentina. *Ann Limnol* 33:179–190
- Taylor HH, Seneviratna D (2005) Ontogeny of salinity tolerance and hyper-osmoregulation by embryos of the intertidal crabs *Hemigrapsus edwardsii* and *Hemigrapsus crenulatus* (Decapoda, Grapsidae): survival of acute hyposaline exposure. *Comp Biochem Physiol A* 140:495–505
- Torres G, Charmantier-Daures M, Chifflet S, Anger K (2007) Effects of long-term exposure to different salinities on the location and activity of Na<sup>+</sup>-K<sup>+</sup>-ATPase in the gills of juvenile mitten crab *Eriocheir sinensis*. *Comp Biochem Physiol A* 147:460–465
- Underwood A (1997) Experiments in ecology: their logical design and interpretation using analysis of variance. Cambridge University Press, Cambridge
- Wilder MN, Huong DTT, Atmomarsono M, Hien TTT, Phu T, Yang WJ (2000) Characterization of Na/K-ATPase in *Macrobrachium rosenbergii* and the effects of changing salinity on enzymatic activity. *Comp Biochem Physiol A* 125:377–388
- Wilder MN, Huong DTT, Okuno A, Atmomarsono M, Yang WJ (2001) Oubain-sensitive Na/K-ATPase activity increases during embryogenesis in the giant freshwater prawn *Macrobrachium rosenbergii*. *Fish Sci* 67:182–184

*Editorial responsibility: Victor Benno Meyer-Rochow, Bremen, Germany*

*Submitted: April 18, 2008; Accepted: July 29, 2008  
Proofs received from author(s): August 27, 2008*