

## Salinity Dependent Na<sup>+</sup>-K<sup>+</sup>ATPase Activity in Gills of the Euryhaline Crab *Chasmagnathus granulata*

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**Abstract.** The occurrence and response of Na<sup>+</sup>-K<sup>+</sup>ATPase specific activity to environmental salinity changes were studied in gill extracts of all of the gills of the euryhaline crab *Chasmagnathus granulata* from Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina). All of the gills exhibited a salinity dependent Na<sup>+</sup>-K<sup>+</sup>ATPase activity, although the pattern of response to environmental salinity was different among gills. As described in other euryhaline crabs highest Na<sup>+</sup>-K<sup>+</sup>ATPase specific activity was found in posterior gills (6 to 8), which, with exception of gill 6, increased upon acclimation to reduced salinity. However, a high increase of activity also occurred in anterior gills (1 to 5) in diluted media. Furthermore, both short and long term differential changes of Na<sup>+</sup>-K<sup>+</sup>ATPase activity occurred among the gills after the transfer of crabs to reduced salinity. The fact that variations of Na<sup>+</sup>-K<sup>+</sup>ATPase activity in the gills were concomitant with the transition from osmoconformity to ionoregulation suggests that this enzyme is a component of the branchial ionoregulatory mechanisms at the biochemical level in this crab.

**Key words:** *Chasmagnathus granulata* — Crustacean — Gill — Na<sup>+</sup>-K<sup>+</sup>ATPase — Osmoregulation

### Introduction

Decapod crustaceans that inhabit tide areas, coastal waters or estuaries, are exposed to frequent and abrupt changes in the environmental salinity. This requires biochemical, physiological, morphological and/or behavioural adaptations related with movements of water and ions between the animals and their medium (Kirschner 1991). The gills constitute the main site where processes of exchange and active

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transport of ions involved in the ionic and osmotic regulation of the corporal fluids are carried out (Towle 1993) In dilute media, to compensate for salt losses, hyperregulating crabs actively absorb NaCl from the external medium *via* the gills

In hyperregulating crabs, ion transporting capability appears to be mainly located in the posterior gills which contain discrete patches of mitochondria-rich epithelial cells exhibiting extensive basolateral membrane infoldings (Towle 1993, 1997) A generally accepted model of Na<sup>+</sup> uptake *via* posterior gills proposes that the Na<sup>+</sup>-K<sup>+</sup>ATPase located basolaterally plays a central role in establishing the electrochemical gradient of Na<sup>+</sup> and K<sup>+</sup> necessary for the activity of other transport systems, like an apical Na<sup>+</sup>-H<sup>+</sup> antiporter and co-transporters coupled with Na<sup>+</sup> (Siebers et al 1985, Lucu and Siebers 1986, Pequeux et al 1988, Burnett and Towle 1990, Lucu 1990, Towle 1990, Towle 1993, Onken 1996, Towle 1997, Onken and Riestenpatt 1998) Na<sup>+</sup>-K<sup>+</sup>ATPase is located to actively extrude Na<sup>+</sup> ions from the gill epithelial cell into the hemolymph, and the enhanced activity of the enzyme at low salinities would lead to a greater net accumulation of Na<sup>+</sup> ions in the hemolymph (Towle, 1997)

In this context, it is well-known that Na<sup>+</sup>-K<sup>+</sup>ATPase activity of posterior gills of several euryhaline crabs is higher than that in anterior gills, and increases after an abrupt change to reduced salinity, although the response pattern seems to be different depending on species (Siebers et al 1982, D'Orazio and Holliday 1985, Towle 1990, Towle 1993, Corotto and Holliday 1996, Towle 1997) However, to our knowledge, the response of Na<sup>+</sup>-K<sup>+</sup>ATPase activity to an abrupt salinity change in each individual gill pair of euryhaline crabs has not been studied Recently it has been suggested a functional difference in individual gills for Na<sup>+</sup> fluxes in the mangrove crab *Ucides cordatus*, in which active uptake of Na<sup>+</sup> in gill 5, and active extrusion in gill 6 were shown (Martinez et al 1998) Na<sup>+</sup>-K<sup>+</sup>ATPase activity of anterior gills of some euryhaline crabs, like *Callinectes sapidus* (Neufeld et al 1980), *Uca pugilator* (D'Orazio and Holliday 1985), *Uca pugnax* (Holliday 1985) and *Hemigrapsus nudus* (Corotto and Holliday 1996), also appears to increase upon acclimation to low salinity, and a subsidiary role for anterior gills in osmo-ionoregulatory process has been suggested (Corotto and Holliday 1996)

*Chasmagnathus granulata* is an euryhaline semiterrestrial crab which is found from Rio de Janeiro (Brazil) to Patagonia (Argentina) (Boschi 1964) In Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina) it inhabits areas with abrupt, frequent and highly variable changes in the environmental salinity Although *C. granulata* has been identified as a hyper- and hypo- osmoregulator crab (Mane Garzon et al 1974, Gnazzo et al 1978, Luquet et al 1998), its branchial ionoregulatory mechanisms at the biochemical level are still poorly understood

The aim of this work was to determine the distribution of Na<sup>+</sup>-K<sup>+</sup>ATPase activity among individual gills of *C. granulata* from Mar Chiquita coastal lagoon as well as to study the short- and long-term response of Na<sup>+</sup>-K<sup>+</sup>ATPase activity in all of the gills of *C. granulata* after an abrupt change to reduced salinity

## Materials and Methods

### *Chemicals*

$\text{Na}_2\text{ATP}$  (adenosine 5'triphosphate, vanadium free), Tris-(hydroxymethylamino-methane), EGTA (ethyleneglycol  $\text{N}'$ ,  $\text{N}'$ ,  $\text{N}'$ ,  $\text{N}'$ -tetraacetic acid), imidazol, bovine serum albumin and G-Strophanthin (ouabain) were obtained from Sigma (St Louis, MO, USA), sucrose, Coomassie Blue G250 and potassium phosphate were from Fluka Chemie AG (Buchs, Switzerland), 4-2-hydroxyethyl-1-piperazinethanesulfonic acid (HEPES) was from Boehringer (Mannheim, Germany), ethanol and phosphoric acid from Merck (Darmstadt, Germany). All chemicals used were of analytical grade. All solutions were prepared in distilled water.

### *Animal collection and maintenance*

Adult male *C. granulata* (carapace width > 30 mm) were collected from a single area of Mar Chiquita lagoon (Buenos Aires Province, Argentina) on September-October 1998. The animals were transported to the laboratory in lagoon water on the day of collection. The environmental salinity of the site varied between 33–35‰. Crabs were maintained in either 35‰ or 10‰ salinity for ten days *prior* to experiments. Aquaria contained 36 liters of water continuously aerated and filtered. A regime of 12 hours light/12 hours dark was applied and the temperature was kept at 20–24°C. The aquaria were shielded by a black screen to reduce disturbance. The animals were fed three times a week with commercial food (Tetra Bits, Tetrawerke, Germany) (0.07 g/individual) and fasted two days *prior* to experiments.

### *Preparation of enzyme gill extracts*

Crabs were cryoanesthetized by putting them on ice for 15–20 minutes. After removing the carapace the gills were immediately excised, taking care not to contaminate them with hepatopancreas tissue or gastric juice. Excised gills were mixed with homogenizing medium (0.25 mol/l sucrose, 0.5 mmol/l EGTA-Tris pH 7.4) (4 ml/g of gill tissue) and homogenized on ice with 20 strokes in a motor-driven hand-operated Teflon-glass homogenizer (Potter-Elvehjem, 1700 rpm). Gills 1, 2 and 3 were combined for assay because of their small size. Gills 4 to 8 were used separately. The homogenate was centrifuged at  $10\,000 \times g$  (Beckman, Microfuge B) for 30 seconds. The supernatant was separated into 200  $\mu\text{l}$  aliquots and stored at  $-20^\circ\text{C}$  until use. Glycerol (1.3% v/v) was added to samples before freezing. The freeze-thaw procedure did not alter  $\text{Na}^+$ - $\text{K}^+$ ATPase activity.

### *Assay of $\text{Na}^+$ - $\text{K}^+$ ATPase activity*

Total ( $\text{Mg}^{++}$ - $\text{Na}^+$ - $\text{K}^+$ )ATPase activity was determined by measuring ATP hydrolysis in a reaction medium containing 100 mmol/l NaCl, 30 mmol/l KCl, 10 mmol/l  $\text{MgCl}_2$ , 0.5 mmol/l EGTA in 20 mmol/l imidazol buffer (pH 7.4).  $\text{Mg}^{++}$ -ATPase activity was assayed in the same medium but without KCl and in the presence of 1 mmol/l ouabain.  $\text{Na}^+$ - $\text{K}^+$ ATPase activity was determined as the difference between both assays. An aliquot of gill extract was added to the reaction mixture.

and pre-incubated for 5 min at 30°C. The reaction was initiated by the addition of ATP (final concentration 5 mmol/l for anterior gills of individuals acclimated at both salinities and for posterior gills of crabs acclimatized to 35‰ salinity, and 10 mmol/l for posterior gills of individuals acclimatized at 10‰ salinity). These ATP concentrations were previously determined to be optimal for Na<sup>+</sup>-K<sup>+</sup>ATPase activity in anterior and posterior gills of *C. granulata* (del Prete et al. 1997, 1998). Incubation was carried out at 30°C for 15 min. The reaction was stopped by addition of 2 ml of cooled Bonting's reagent (560 mmol/l H<sub>2</sub>SO<sub>4</sub>, 8.1 mmol/l ammonium molybdate and 176 mmol/l FeSO<sub>4</sub>). After 20 min at room temperature the amount of released P<sub>i</sub> was determined by reading the absorbance at 700 nm of the reduced phosphomolybdate complex (Bonting 1970). Na<sup>+</sup>-K<sup>+</sup>ATPase activity was expressed as nmol P<sub>i</sub> min<sup>-1</sup> mg<sub>protein</sub><sup>-1</sup>.

#### *Measurement of hemolymph osmolality and ionic concentration*

Hemolymph was sampled from the infrabranchial sinus at the base of the chelipeds by means of a syringe and transferred to an iced centrifuge tube. After disrupting of clots by mechanical shaking, tubes were centrifuged for 20 min at 3500 rpm (IEC-Centra 7R) at 4°C. The supernatant was stored at -20°C until use. Media samples were stored similarly.

Na<sup>+</sup> and K<sup>+</sup> concentrations were determined by flame photometry (Radiometer, Copenhagen, FLM 3). Cl<sup>-</sup> concentration was measured by a coulometric method (Radiometer, CM 10) (ion selective) and osmolality with a microosmometer (Radiometer, Copenhagen, 3 MO).

#### *Protein analysis*

Protein was determined according to Bradford (1976). Bovine serum albumin was used as a standard.

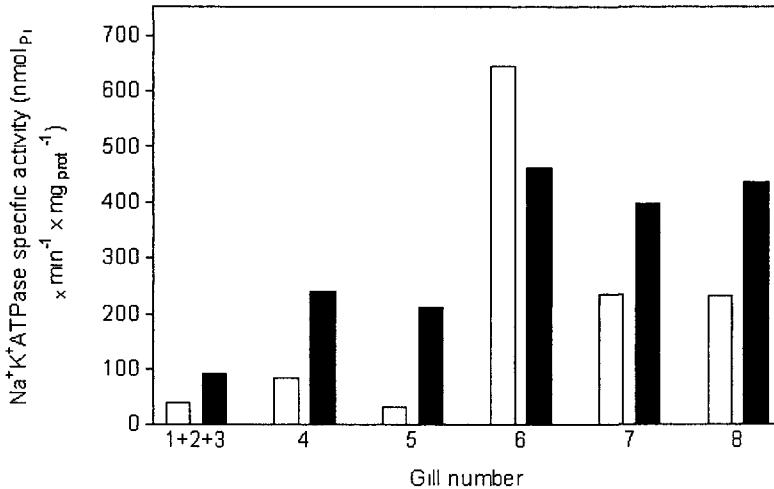
## **Results**

### *Na<sup>+</sup>-K<sup>+</sup>ATPase activity in gills of *C. granulata**

To determine the distribution of Na<sup>+</sup>-K<sup>+</sup>ATPase activity among gills of *C. granulata*, enzyme activity was determined in gills 1 + 2 + 3, and individual gills 4 to 8 of crabs maintained 10 days in either 35‰ or 10‰ salinity. Both in crabs acclimatized to 35‰ and 10‰ salinity, highest Na<sup>+</sup>-K<sup>+</sup>ATPase activity was found in posterior gills 6 to 8. With exception of gill 6, upon acclimatization to reduced salinity Na<sup>+</sup>-K<sup>+</sup>ATPase activity increased in all gills, although the enhancement of activity appeared to be higher in anterior gills 1 to 5 (Fig. 1).

### *Short- and long-term effects of salinity change on Na<sup>+</sup>-K<sup>+</sup>ATPase activity in gills of *C. granulata**

Crabs maintained 10 days at 35‰ salinity were abruptly transferred to 10‰ salinity and Na<sup>+</sup>-K<sup>+</sup>ATPase activity was measured before the transfer (time 0), 4 h, 24 h and 48 h (short term) and 37 days (long term) after the change.

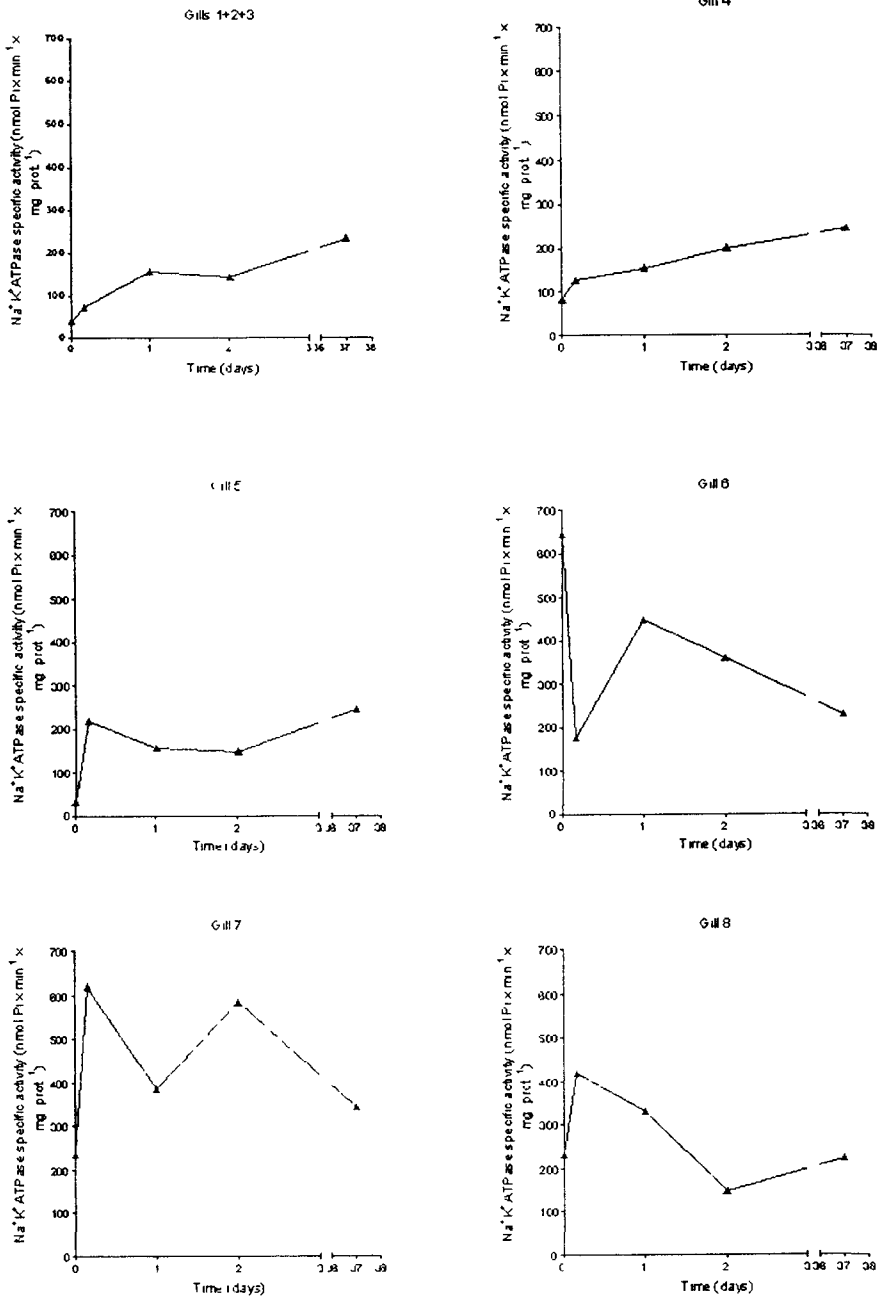


**Figure 1.** Distribution of  $\text{Na}^+/\text{K}^+$ -ATPase activity in gills of *Chasmagnathus granulata* acclimatized during 10 days to either 35‰ (□) or 10‰ (■) salinity. Each point represents the means from three assays from five crabs.

Anterior gills (1 to 5): after 4 h from the transfer an increase of  $\text{Na}^+/\text{K}^+$ ATPase specific activity occurred in gills 1 + 2 + 3 (79%) compared with the activity before the transfer. A further increase occurred 24 h after the salinity change (278%), remaining essentially constant till 48 hours after the transfer. In gill 4,  $\text{Na}^+/\text{K}^+$ ATPase activity also increased 4 h after the salinity change (50%) and continued to increase, being 138% higher after 48 h than the activity before the transfer. In gill 5, a high increase of activity occurred at 4 h (587%). At 24 h a decrease of  $\text{Na}^+/\text{K}^+$ ATPase activity occurred, but activity being still higher than before the transfer (391%). The activity maintained essentially constant up to 48 h after the salinity change (Fig 2).

Thirty seven days after the change to reduced salinity  $\text{Na}^+/\text{K}^+$ ATPase activity in gills 1 + 2 + 3, 4 and 5 was higher than the activity in the corresponding gills before the transfer (470%, 191%, 673%, respectively) (Fig 2).

Posterior gills (6–8): after 4 h from the salinity change a decrease of  $\text{Na}^+/\text{K}^+$ ATPase activity occurred in gill 6 (72%), whereas it increased at 24 h after the change, although until a value lower than that before the transfer (30%). Forty eight hours after the change,  $\text{Na}^+/\text{K}^+$ ATPase activity in gill 6 decreased again, being 55% of the activity before the salinity change. In gill 7 an increase of  $\text{Na}^+/\text{K}^+$ ATPase activity occurred at 4 h (163%), but it decreased by 24 hours, although activity being higher than before the transfer (63%). A further enhancement of  $\text{Na}^+/\text{K}^+$ ATPase activity occurred at 48 h reaching a similar value to that at 4 h after the transfer. In gill 8,  $\text{Na}^+/\text{K}^+$ ATPase activity also increased at 4 h (81%),



**Figure 2.** Effect of an abrupt salinity change from 35‰ to 10‰ salinity on Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in gills 1 + 2 + 3, 4, 5, 6, 7 and 8 of *Chasmagnathus granulata* Crabs acclimatized for 10 days at 35‰ salinity and transferred at zero time to 10‰. Each point represents the means from three assays from four to six crabs

but then it began to decrease reaching by 48 h a similar value to that before the transfer (Fig. 2).

After 37 days, Na<sup>+</sup>-K<sup>+</sup>ATPase activity in gill 6 was lower than the activity before the transfer (64%). In gill 7 Na<sup>+</sup>-K<sup>+</sup>ATPase activity was higher (46%), while in gill 8 it was similar to the activity at zero time (Fig. 2).

*Osmolality and ionic concentrations in hemolymph of C. granulata*

The osmolality and concentrations of ions mainly involved in ionoregulatory processes in osmoregulating decapod crustaceans (Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup>) were determined in hemolymph of *C. granulata* maintained for 10 days in either 35‰ or 10‰ salinity.

In 35‰ salinity the osmolality and hemolymphatic concentration of Na<sup>+</sup> were similar to those of the medium. K<sup>+</sup> concentration appeared to be slightly higher than that of external medium, whereas Cl<sup>-</sup> concentration was lower. In reduced salinity *C. granulata* exhibited hyperregulatory capacity with respect to hemolymphatic osmolality and Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> concentrations. The main osmolytes were Na<sup>+</sup> and Cl<sup>-</sup>, representing the sum of their concentrations more than 90% of hemolymph osmolality (Table 1).

**Table 1.** Osmolality and Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> concentrations in the external medium and in hemolymph of *C. granulata* kept 10 days at either 35‰ or 10‰ salinity

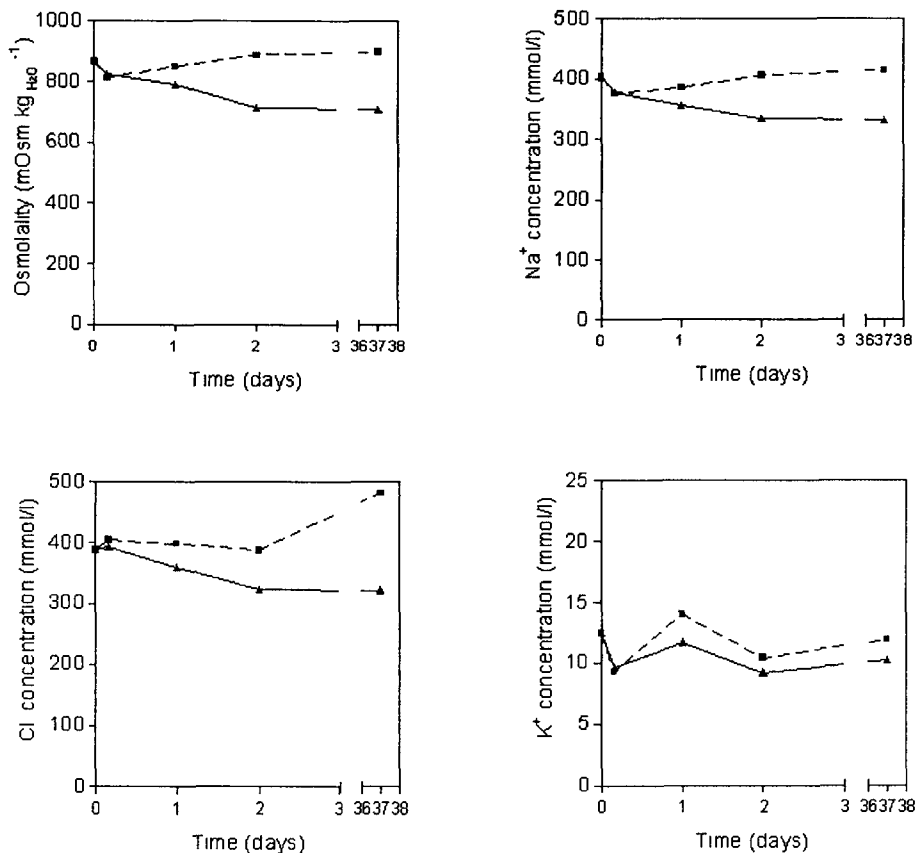
	10‰		35‰	
	Medium	Hemolymph	Medium	Hemolymph
Osmolality	316.5 ± 19.0	868.0	425 ± 62.0	759.0
Na <sup>+</sup>	41 ± 11.0	403.0	405 ± 31.0	356.0
K <sup>+</sup>	3.4 ± 0.0	12.5	9.15 ± 0.5	9.8
Cl <sup>-</sup>	164.5 ± 17.0	389.0	504.5 ± 37.0	415.0

Results of a single experiment using pooled hemolymph from 5 crabs are presented. Data for external medium are means ± S.E.M. of two assays

*Effect of an abrupt change of salinity on osmolality and ionic concentrations in hemolymph of C. granulata*

Crabs left for 10 days in 35‰ salinity were abruptly transferred to 10‰. Osmolality and concentrations of ions were determined before (time 0); and 4 h, 24 h, 48 h (short-term) and 37 days (long-term) after the transfer.

Upon transfer to reduced salinity, a slight decrease occurred in osmolality and Na<sup>+</sup> and Cl<sup>-</sup> concentrations, which appeared to stabilize 2 days after the change. Hemolymph osmolality and Na<sup>+</sup> and Cl<sup>-</sup> concentrations in transferred individuals were above those of the medium (Fig. 3). Thus, *C. granulata* appeared to exhibit a short-term hyperregulatory capacity after an abrupt change to reduced salinity.



**Figure 3.** Effect of an abrupt salinity change from 35‰ to 10‰ salinity on hemolymph osmolality and Na<sup>+</sup>, Cl<sup>-</sup> and K<sup>+</sup> concentrations of *C. granulata* Crabs acclimatized for 10 days at 35‰ salinity and transferred at zero time to 10‰ (full line) Crabs maintained at 35‰ salinity (dashed line) Each point represents the means from three assays from five crabs

*C. granulata* also exhibited hyperregulatory capacity for K<sup>+</sup>, although the response pattern was different to that of the osmolality, Na<sup>+</sup> and Cl<sup>-</sup>.

The main osmolytes in hemolymph of individuals transferred to reduced salinity were also Na<sup>+</sup> and Cl<sup>-</sup>, representing the sum of their concentrations more than the 90% of hemolymph osmolality.

## Discussion

Results described herein show that all of the gills of *C. granulata* from Mar Chiquita lagoon exhibited a Na<sup>+</sup>-K<sup>+</sup>ATPase activity sensitive to salinity (Figs. 1, 2).



Similarly to that described in other euryhaline crabs, highest Na<sup>+</sup>-K<sup>+</sup>ATPase specific activity was found in posterior gills, which was increased upon acclimatization of *C. granulata* to reduced salinity (Fig. 1), concomitant with the transition from osmoconformity to osmoregulation (Table 1, Fig. 3). This is in accordance with the main function attributed to posterior gills in other euryhaline crabs, i.e. ionoregulation (Siebers et al. 1985; Pequeux et al. 1988; Towle 1993, 1997; Pierrot et al. 1995). The role of anterior gills of euryhaline crabs in ionoregulation is still controversial, although a subsidiary role in this process has been suggested (Corotto and Holliday 1996). The fact that an increase of Na<sup>+</sup>-K<sup>+</sup>ATPase activity also occurred in anterior gills of *C. granulata* acclimatized to diluted media (Fig. 1), could suggest a role of these gills in acclimatization to reduced salinity in this crab.

Short- and long-term adaptive increases of Na<sup>+</sup>-K<sup>+</sup>ATPase specific activity has been shown to occur in posterior gills of several hyperregulating crabs after an abrupt change to low salinity media (Towle 1990, 1993). Trausch et al. (1989) suggested that both rapid and slower changes in Na<sup>+</sup>-K<sup>+</sup>ATPase activity in posterior gills occurred in the response of hyperregulating crabs to reduced environmental salinity. However, to our knowledge, both short and long term responses of Na<sup>+</sup>-K<sup>+</sup>ATPase activity in all of the individual gills of hyperregulating crabs has not been studied.

The results presented in this work show that the branchial Na<sup>+</sup>-K<sup>+</sup>ATPase activity in individual anterior and posterior gills of *C. granulata* from Mar Chiquita lagoon appeared to exhibit a different short and long term pattern of response upon an abrupt change to dilute media (Fig. 2). The enhanced Na<sup>+</sup>-K<sup>+</sup>ATPase activity in posterior gills 7 and 8 of *C. granulata* acclimatized to reduced salinity (Fig. 1) along with the increase of this activity in these gills after the change to dilute media (Fig. 2) were concomitant with hemolymph osmolality and Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> concentrations (Table 1, Fig. 3), suggesting that this enzyme could be a component of the branchial ionoregulatory mechanisms in posterior gills of this crab, probably accomplishing with the central role in the uptake of Na<sup>+</sup> from dilute media to hemolymph as proposed for other hyperregulating crabs (Towle 1997; Onken and Riestenpatt 1998).

In several semiterrestrial crabs it has been shown that the highly osmoregulatory branchiae increased in number in the species occupying higher habitats and that osmoregulatory function seemed to develop first in the posterior branchiae and then spread to the anterior branchiae (Takeda et al. 1996). Again, the short term increase in Na<sup>+</sup>-K<sup>+</sup>ATPase activity in anterior gills of *C. granulata* after an abrupt change from 35‰ to 10‰ salinity (Fig. 2) could suggest a role of anterior gills of *C. granulata* in processes of active ion uptake in this crab.

Short-term changes have been suggested to occur *via* regulation of pre-existing enzyme, by tissue Na<sup>+</sup> levels (Siebers et al. 1982; Harris and Santos 1993), and by biogenic amines and second messengers (Trausch et al. 1989; Kamemoto 1991; Morris and Edwards 1995; Mo et al. 1998). Whether this is the case for branchial Na<sup>+</sup>-K<sup>+</sup>ATPase activity in *C. granulata* remains to be investigated.

The fact that, conversely to expected,  $\text{Na}^+\text{-K}^+\text{ATPase}$  activity decreased in posterior gill 6 of *C. granulata* after change to diluted media could suggest a differential role of this gill in processes of ion transport. Functional differences in adjacent gills was suggested by Martinez et al. (1998) to occur in the hyper-hyporegulating crab *U. cordatus*, in which gill 5 was shown to be a site of  $\text{Na}^+$  uptake while gill 6 was shown to be a site of  $\text{Na}^+$  extrusion.

Long-term changes of branchial  $\text{Na}^+\text{-K}^+\text{ATPase}$  activity could be required for acclimatization to longer-term environmental conditions. Again, the increase of  $\text{Na}^+\text{-K}^+\text{ATPase}$  activity observed in anterior gills of *C. granulata* (Fig. 2) which may occur *via* synthesis of a new enzyme and cell proliferation (Neufeld et al. 1980; D'Orazio and Holliday 1985; Holliday 1985; Harris and Santos 1993; Corotto and Holliday 1996), suggests a role of these gills also in long-term acclimatization of this crab to reduced salinity. The fact that in posterior gills  $\text{Na}^+\text{-K}^+\text{ATPase}$  activity was nearly similar (gill 7 and 8) or lower (gill 6) than the activity before the transfer, could suggest either a deactivation or degradation of the enzyme to reach levels of  $\text{Na}^+\text{-K}^+\text{ATPase}$  activities sufficient to support the long-term ionoregulatory process.

In summary, the results of this work suggest a differential short- and long-term participation of  $\text{Na}^+\text{-K}^+\text{ATPase}$  activity in individuals gills of *C. granulata* from Mar Chiquita lagoon in ion transport process. Future studies should be focused on the mechanisms of regulation of this activity in both anterior and posterior gills, as well as on the identification of other ion transport mechanisms which could be also operating in gills of *C. granulata*.

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