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Response to environmental salinity of Na^+–K^+ATPase activity in individual gills of the euryhaline crab *Cyrtograpsus angulatus*

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Abstract

The occurrence, localization and response to environmental salinity changes of Na^+–K^+ATPase activity were studied in each of the individual gills 4–8 of the euryhaline crab *Cyrtograpsus angulatus* from Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina). Na^+–K^+ATPase activity appeared to be differentially sensitive to environmental salinity among gills. Upon an abrupt change to low salinity, a differential response of Na^+–K^+ATPase activity occurred in each individual gill which could suggest a differential role of this enzyme in ion transport process in the different gills of *C. angulatus*. With the exception of gill 8, a short-term increase of Na^+–K^+ATPase specific activity was observed in posterior gills, which is similar to adaptative variations of this activity described in other euryhaline crabs. However, and conversely to that described in other hyperregulating crabs, the highest increase of activity occurred in anterior gills 4 by 1 day after the change to dilute media which could suggest also a role for these gills in ion transport processes in *C. angulatus*. The fact that variations of Na^+–K^+ATPase activity in anterior and posterior gills were concomitant with the transition to hyperregulation indicate that this enzyme could be a component of the branchial ionoregulatory mechanisms at the biochemical level in this crab. The results suggest a differential participation of branchial Na^+–K^+ATPase activity in ionoregulatory mechanisms of *C. angulatus*. The possible existence of functional differences as well as distinct regulation mechanisms operating in individual gills is discussed.

Keywords: Branchial enzymes; Crabs; Crustaceans; *Cyrtograpsus angulatus*; Decapods; Gills Na^+–K^+ATPase; Osmoionoregulation

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1. Introduction

Crabs inhabiting coastal waters, tide areas or estuaries are exposed to frequent and abrupt changes in the environmental salinity which requires biochemical, physiological, morphological and/or behavioural strategies for controlling movements of water and ions between the individuals and their medium (Kirschner, 1991).

In euryhaline crabs, sodium and chloride constitutes the major contributors to hemolymph osmolality, the regulation of their fluxes and permeabilities being central to tolerate salinity gradients in these animals (Towle, 1993, 1997). In dilute media, hyper-regulating crabs absorb both sodium and chloride from the external medium via the gills thus regulating their concentrations in the hemolymph and compensating for salt losses. In species so far studied, the ability of active sodium and chloride uptake appears to be mainly located in posterior gills (Towle, 1993, 1997; Onken and Riestenpatt, 1998; Ahearn et al., 1999; Morris, 2001). Although differential characteristics in the apical absorption of sodium and chloride appear to occur in *Carcinus maenas* and in *Eriocheir sinensis*, which would reflect adaptation to brackish and fresh water, respectively (Onken and Putzenlechner, 1995; Onken and Riestenpatt, 1998), it is generally accepted that Na^+–K^+ATPase located basolaterally in posterior gills of hyperregulating crabs is a main component for physiological adaptations to reduced salinity. Na^+–K^+ATPase is assumed to play a central role establishing electrochemical gradients necessary for the activity of other transport systems like apicals Na^+/H^+ antiport and other cotransporter coupled with Na^+ (Siebers et al., 1985; Lucu and Siebers, 1986; Pequeux et al., 1988; Lucu, 1990; Towle, 1993, 1997; Onken, 1996; Onken and Riestenpatt 1998; Lucu and Flik, 1999). The active extrusion of sodium ions via the basolateral Na^+–K^+ATPase from the gill cell into the hemolymph and the enhanced activity of the enzyme at low salinities would lead to a greater net accumulation of Na^+ ions in the hemolymph (Towle, 1997). In relation to this, it has been clearly established that the adaptative increases of Na^+–K^+ATPase activity occurred in posterior gills both upon acclimation and after an abrupt change to reduced salinity, although the pattern of response seems to be different depending on species and to be correlated to the extent of hyperregulating ability (Siebers et al., 1982; D'Orazio and Holliday, 1985; Pequeux et al., 1988; Towle, 1990, 1993, 1997; Corotto and Holliday, 1996; Cooper and Morris, 1997).

On the other hand, the participation of anterior gills in the active absorption of ions from the external media appeared to be less clear although a subsidiary role for these gills in osmo-ionoregulatory process of *Hemigrapsus nudus* (Corotto and Holliday, 1996) and *C. maenas* (Lucu and Flik, 1999) has been suggested. However, to our knowledge, no studies have been carried out on the response of Na^+–K^+ATPase activity to an abrupt salinity change in each individual gill pairs of euryhaline crabs. Thus, available information about behaviour and pattern of response of Na^+–K^+ATPase activity to an abrupt salinity change is mainly concerned about the activity in one posterior gill or in pooled gills. Furthermore, it has been suggested a functional difference in individual gills of the mangrove crab *Ucides cordatus* for Na^+ fluxes, based on the fact that active uptake of Na^+ in gill 5, and active extrusion in gill 6 were shown (Martinez et al., 1998). This supports the importance of studying the response of Na^+–K^+ATPase activity and other branchial ion transport systems to salinity changes in the different individual gills.

Cyrtograpsus angulatus is a euryhaline crab which is found from Rio de Janeiro (Brazil) to Patagonia (Argentina) (Boschi, 1964) in habitats with all varying salinities from freshwater to seawater. In Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina), *C. angulatus* inhabits areas with abrupt, frequent, and highly variable changes in the environmental salinity ranging from 4‰ to 36‰, which indicates an extremely high degree of osmo-ionoregulatory capacity (Anger et al., 1994; Spivak et al., 1994). However, its ionoregulation ability and branchial ionoregulatory mechanisms at the biochemical level have been poorly investigated.

As part of our studies on ionoregulatory mechanisms in the gills of *C. angulatus* from Mar Chiquita lagoon, we have previously shown the occurrence of a Ca^{+}-ATPase and an anion-stimulated ATPase activity in gills plasma membrane fraction (Radin et al., 1995).

The aim of this work is to determine the occurrence and time course response to changes in environmental salinity of $Na^{+}-K^{+}$ATPase activity in individual gills of *C. angulatus* from Mar Chiquita lagoon.

2. Materials and methods

2.1. Chemicals

4-2(Hydroxyethyl)-1-piperazinethane sulphonic acid (Hepes) was obtained from Boehringer (Manheim, Germany); sucrose, Coomasie Blue G250 and potassium phosphate were from Fluka (Germany); Na_2ATP (adenosine 5′ triphosphate, vanadium-free), Tris-(hidroxymethylamino-methane) (Tris), ethyleneglicol *N*,*N*′,*N*′ -tetraacetic acid (EGTA), imidazole, bovine serum albumin and G-Strophantin (ouabain) were from Sigma (St. Louis, MO, USA); ethanol and phosphoric acid were from Merck (Darmstadt, Germany). All solutions were prepared in glass-distilled water.

2.2. Animal collection and maintenance

Crabs were caught from a single area of Mar Chiquita lagoon. Only adult male crabs with a carapace width greater than 2.5 cm were collected. Animals were transported to the laboratory in lagoon water on the day of collection. Crabs were maintained in natural seawater (35‰ salinity) for 2 weeks prior to use. The aquaria contained 36 l of water, continuously aerated and filtered. A regime of 12 h light/12 h dark was applied and the temperature was kept at 21±2 °C. Aquaria were shielded by a black plastic to reduce disturbance. Crabs were fed three times a week with commercial food (Tetrabits, Tetrawerke, Germany) (about 0.07 g per individual) but they were starved 48 h prior to experiments. Dilute seawater, when used, was obtained by dilution of natural seawater with distilled water.

2.3. Preparation of enzyme gill extracts

The crabs were cryoanesthesized by putting them on ice for about 30 min After removing the carapace, the gills were immediately excised, mixed with homogenizing

medium (0.25 M sucrose/0.5 mM EGTA–Tris, pH 7.4) (4 ml g^{-1} of gill tissue) and homogenized on ice with 20 strokes in a motor-driven hand-operated Teflon-glass homogenizer. Separate gills 4 to 8 were used. Gills 1, 2 and 3 were discarded due to their small size. The homogenate was centrifuged at 10,000×*g* (Beckman, Microfuge, B) for 30 s. The supernatant was separated into 50–100 μl aliquots and stored at −20 °C until use. Glycerol (1.3% v/v) was added to samples before freezing.

2.4. Assay of Na^+–K^+ ATPase activity

Total (Mg^{2+}–Na^+–K^+) ATPase activity was determined by measuring ATP hydrolysis in a reaction medium containing 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$, 0.5 mM EGTA in 20 mM imidazole buffer (pH 7.4). Mg^+–ATPase activity was assayed in the same medium but without KCl and in the presence of 1 mM ouabain. Na^+–K^+ATPase activity was determined as the difference between both assays. An aliquot of the corresponding sample (50–60 μg of protein) 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 mM). 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 mM sulphuric acid, 8.1 mM ammonium molybdate and 176 mM ferrous sulphate). After 20 min at room temperature, the amount of released Pi was determined by reading the absorbance at 700 nm of the reduced phosphomolybdate complex (Bonting, 1970).

The determination of enzyme activity was always performed with samples, which had been stored at −20 °C, without any previous thawing.

2.5. Measurement of hemolymph ionic concentration

Hemolymph (about 500 μl) was sampled from the infrabranchial sinus by mean of a syringe at the base of the cheliped, and transferred to an iced centrifuge tube. Serum was separated by centrifugation at 3500 rpm (IEC-Centra 7R) for 20 min at 4 °C. Na^+ and K^+ were determined by flame photometry (Radiometer Copenhagen, FLM 3). Cl^- was determined by a colorimetric method (Randox Commercial Kit) based on the formation of a blue Fe-2,4,6-tri-(2-pyridyl)-1,3,5-triazine-ferrous sulphate complex.

Ca^{2+} was determined by the o-CPC method (MercK commercial kit) and Mg^{2+} were determined by a colorimetric method (MercKotest commercial kit).

2.6. Protein analysis

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

2.7. Statistical analysis

Analysis of variance (ANOVA) was used to estimate the statistical significance of the differences and $P<0.05$ was considered to be significant.

3. Results

3.1. Localization and distribution of Na^+-K^+ ATPase activity in gills of C. angulatus

Na^+-K^+ATPase specific activity was determined in each of the gills pairs 4–8 of *C. angulatus* acclimated for 2 weeks to 35‰ salinity, salinity to which this crab is usually exposed in its natural environment. Highest Na^+-K^+ATPase activity was found in posterior gill 6 (437 ± 44 nmol Pi min^{-1} per mg $prot^{-1}$) which was significantly higher ($P<0.05$) (about 2-fold) than the activity in posterior gills 7 and 8 (Fig. 1). Furthermore, activity in gills 6–8 was significantly higher ($P<0.05$) than the activity in both anterior gills 4 and 5, although these gills exhibited a relatively high Na^+-K^+ATPase specific activity (about 60–85 nmol Pi min^{-1} per mg $prot^{-1}$).

3.2. Effect of an abrupt salinity change on Na^+-K^+ ATPase activity in gills of C. angulatus

Crabs acclimated for 2 weeks to 35‰ salinity were abruptly transferred to 6‰ salinity and Na^+-K^+ATPase activity was determined before (0 time) and at 1–3 days after the salinity change (Fig. 1). Both the amplitude of the salinity change as well as the experimental period were used simulating field conditions brought up by tidal variations usually faced by *C. angulatus* in Mar Chiquita lagoon.

After 1 day from the transfer of crabs to reduced salinity, Na^+-K^+ATPase activity increased considerably in anterior gill 4 (about 8-fold). Thereafter, it decreased by 3 days

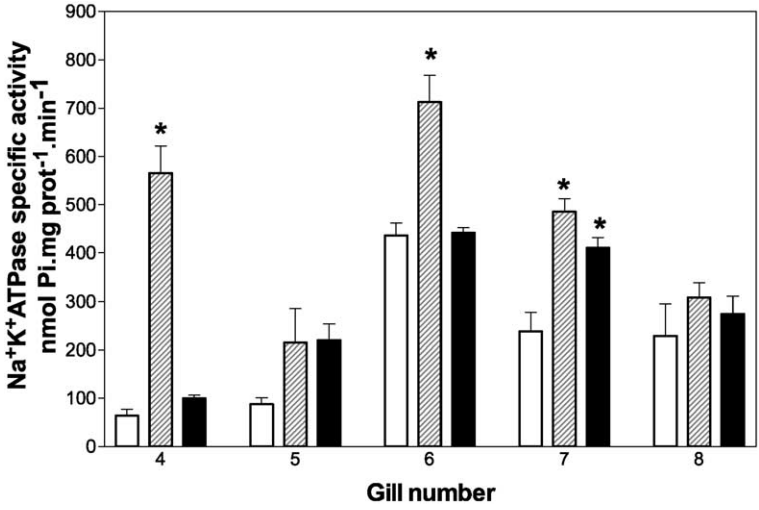

Fig. 1. Distribution and effect of an abrupt salinity change on Na^+-K^+ATPase activity in gill extracts of *C. angulatus*. Individuals acclimated for 2 weeks to 35‰ salinity were transferred (0 time) to 6‰ salinity. Data are the mean ± S.E. of three independent experiments using different preparations of gill extracts in which individual gills from three crabs were used. Open bars: individuals acclimated for 2 weeks to 35‰ salinity. Grey Bars: 1 day after the salinity change. Black Bars: 3 days after the salinity change. *Denotes significantly different from the activity before the transfer (0 time) ($P<0.05$).

to almost the value before the transfer. A slight increase of Na^+–K^+ATPase activity appeared to occur in gill 5 although the values reached were not significantly different to the activity before the transfer. Na^+–K^+ATPase activity in gills 6 and 7 increased at 1 day (by about 1.5- and 2.0-fold, respectively). By 3 days, activity decreased in gill 6—

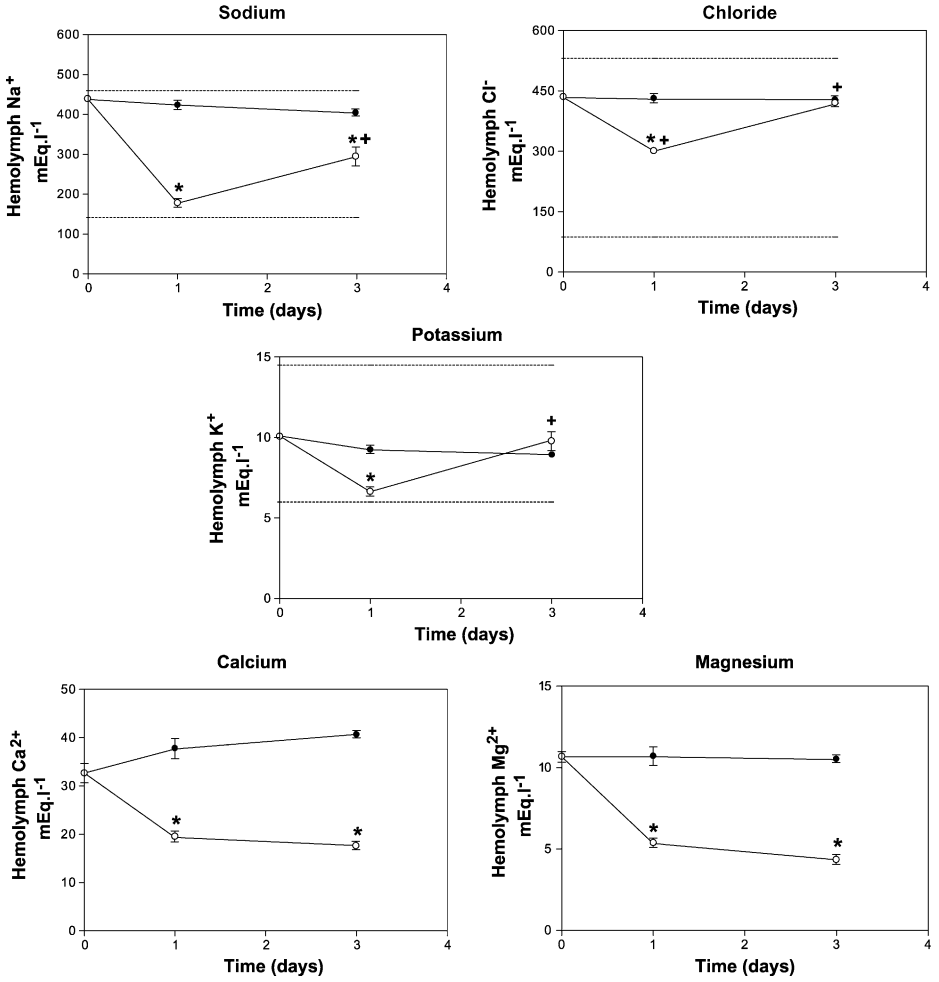

Fig. 2. Time course of changes in hemolymph ions concentrations of *C. angulatus* after an abrupt salinity change. Open circles: individuals acclimated for 2 weeks to 35‰ salinity and transferred to 6‰ salinity. Closed circles: individuals maintained in 35‰ salinity (not transferred crabs). Top and bottom dashed lines indicate ions concentration of the media of 35‰ and 6‰ salinity, respectively, except for Ca^{2+} and Mg^{2+} which concentrations in the external medium were not determined. Each point correspond to mean ± S.E. of three independent experiments in which pooled blood from three crabs were used. In some cases, deviation bars were smaller than symbols used. * Denotes significantly different from the values before the transfer (0 time) ($P<0.05$). $^+$Denotes significantly different from the values of the external medium (6‰ salinity) ($P<0.05$).

being no different to the activity before the transfer, whereas it remained essentially constant in gill 7. On the other hand, no significant changes of Na^+-K^+ATPase activity appeared to occur in gill 8 within the experimental period (Fig. 1).

In reduced salinity, the pattern of distribution of Na^+-K^+ATPase specific activity, appeared to be quite different compared to crabs acclimated to 35‰ salinity (Fig. 1). At 1 day from the transfer, no differences occurred between the activities in anterior gills 4 and posterior gills 6 and 7 and between anterior gills 5 and posterior gills 8. By 3 days, gills 6 and 7 exhibited the highest ($P<0.05$) Na^+-K^+ATPase activity, no occurring differences between the activity in gills 5 and 8, lowest activity being found in gill 4 ($P<0.05$).

3.3. Hemolymph ions concentration of C. angulatus

Na^+ and Cl^- concentrations in hemolymph of *C. angulatus* from Mar Chiquita lagoon acclimated to 35‰ salinity were not significantly different to those of external medium (Fig. 2). K^+ concentration appeared to be slightly below than that of the external medium. After transfer to reduced salinity, hemolymph Na^+ concentration decreased by 1 day but to values slightly above that of the external medium. By 3 days, Na^+ concentration increased reaching values highly above those of the external medium (above 2-fold) (Fig. 2). Hemolymph Cl^- concentration also decreased after 1 day from the transfer but to values significantly above to those of the external medium (about 3.5-fold). By 3 days, Cl^- concentration further increased reaching values similar to those before the transfer (Fig. 2). Hemolymph K^+ also decreased but after 3 days reached values similar to those before the transfer and significantly above that of the external medium. Thus, *C. angulatus* appeared to exhibit a short-term hyperregulatory capacity after an abrupt change to reduced salinity (Fig. 2). Ca^{2+} and Mg^{2+} concentrations also decreased but remained essentially constant and not reaching the values before the transfer within the experimental period.

4. Discussion

Results herein described show the occurrence of Na^+-K^+ATPase activity differentially sensitive to salinity in each individual gills 4 to 8 of the euryhaline crab *C. angulatus* from Mar Chiquita coastal lagoon. The increase of the activity in gills 6 and 7 of *C. angulatus* after an abrupt change to reduced salinity (Fig. 1) were concomitant with the transition from ionoconformity to hemolymph Na^+, K^+ and Cl^- hyperregulation (Fig. 2) suggesting that Na^+-K^+ATPase activity could be one of the components of the ionoregulatory mechanisms in posterior gills of *C. angulatus,* probably by directing the uptake of Na^+ from dilute media to hemolymph as proposed for posterior gills of other hyperregulating crabs (Towle, 1997; Onken and Riestenpatt, 1998; Onken et al., 2000). The fact that no changes occurred in gill 8 as long with the decrease in activity in gill 6 by 3 days when *C. angulatus* exhibited a strong hyperregulatory capability (Fig. 2) could suggest that levels of Na^+-K^+ATPase activity already existing in gill 8 and that one reached in gill 6 by 3 days are enough for the maintenance of short-term ionoregulatory

processes in this crab. In relation to this, the decrease in activity in posterior gill 6 is similar to that described for posterior gill 5 in the crayfish *Pacifasticus leniusculus* (Henry and Wheatly, 1988).

Short- and long-term adaptative increases of Na^+–K^+ATPase specific activity has been shown to occur in posterior gills of several hyperregulating crabs after an abrupt change to reduced salinity (Towle, 1990, 1993). It has been suggested that both rapid and slower changes in Na^+–K^+ATPase activity occur in posterior gills in the response to reduced environmental salinity (Trausch et al., 1989). Recently, we have shown a differential short- and long-term response of Na^+–K^+ATPase specific activity in all individual gills of *Chasmagnathus granulata*, the other dominant crab in Mar Chiquita lagoon, suggesting a differential participation of this enzyme in ion transport processes in the different gills (Schleich et al., 2001).

In most hyperregulating crabs, Na^+–K^+ATPase activity in posterior gills appeared to be more sensitive to changes in environmental salinity compared to the activity in anterior gills (Neufeld et al., 1980; Holliday, 1985; Corotto and Holliday, 1996). Furthermore, the role of anterior gills of euryhaline crabs in ionoregulation is still controversial, although a subsidiary role of these gills has been suggested (Corotto and Holliday, 1996). In *C. maenas*, there has been an observed increase in Na^+–K^+ATPase activity after an abrupt change to low salinity in pooled anterior gills. This increase, although significantly lower than that which occurred in posterior gills, was suggested to form part of the adaptative response to a hypoosmotic challenge (Lucu and Flik, 1999). Interestingly, in *C. angulatus*, the highest increase of Na^+–K^+ATPase activity upon abrupt change to reduced salinity occurred in anterior gill 4 by 1 day (Fig. 1), which could support the idea for a role of anterior gills in ion active uptake in this crab. Furthermore, the response of branchial Na^+–K^+ATPase activity of gill 4 of *C. angulatus* is in accordance with our previous results in *C. granulata* from Mar Chiquita lagoon, which showed a extremely high increase of this activity in anterior gills both upon acclimation and after an abrupt transfer to reduced salinity (Schleich et al., 2001). Nevertheless, since studies on branchial ion transport systems in most hyperregulating crabs so far studied have been mainly focused on posterior gills, further studies are required to clarify the role of Na^+–K^+ATPase activity as well as of other ion transport systems in anterior gills. Furthermore, the differential response of Na^+–K^+ATPase activity among individual gills of *C. angulatus* could be related to functional differences in each gill as it has been suggested for the hyper-hyporegulating crab *U. cordatus* in which active uptake of Na^+ in gills 5 and active extrusion of Na^+ in gills 6 were shown (Martinez et al., 1998).

The highest activity in posterior gills appeared to be a characteristic of euryhaline marine species which hyperregulate in dilute media. Na^+–K^+ATPase activity appeared to be heterogeneously distributed among gills of *C. angulatus* being higher in posterior gills 6 to 8 compared to the activity in anterior gills 4 and 5 (Fig. 1), which is in accordance with results shown in other several hyper-regulating crabs (Neufeld et al., 1980; D'Orazio and Holliday, 1985; Holliday, 1985; Harris and Santos, 1993; Corotto and Holliday, 1996). However, after an abrupt change to reduced salinity, the differential responses of the activity that occurred in the individual gills resulted in a quite different pattern of distribution of Na^+–K^+ATPase specific activity when hyperegulation in this crab was

started—also supporting the idea for a differential participation of individual gills in ionoregulatory process (Fig. 1).

The fact that Cl^- concentration in hemolymph of *C. angulatus* appeared to be regulated faster than that of Na^+ (Fig. 2) could suggest the participation of different ion transport membrane systems involved in chloride homeostasis of this crab. In relation to this, we have previously described the occurrence of an anion-stimulated ATPase in plasma membrane fraction isolated from gills of *C. angulatus* from Mar Chiquita lagoon acclimated to dilute media which could be involved in ionoregulation of this crab (Radin et al., 1995). Furthermore, in posterior gills of the Chinese crab *E. sinensis* acclimated to dilute media, the existence of two different routes for apical sodium and chloride absorption has been observed, in contrast to the posterior gills of *C. maenas*, which appear to absorb these ions in a coupled mode (Onken and Putzenlechner, 1995; Onken, 1999). *C. angulatus* appeared to exhibit a lower capability to regulate Ca^{2+} and Mg^{2+} after an abrupt change to reduced salinity although their concentrations were maintained essentially constant. Since no information is available, to the best of our knowledge, on Mg^{2+} branchial transport systems in hyperregulating crabs, and only fragmentary information exist on Ca^{2+} transport systems in *C. angulatus*, more studies are needed to clarify the regulation of the concentrations of these ions in hemolymph of this crab. In this relation, we have previously shown the occurrence of a Ca^{2+}-stimulated ATPase activity in gills of *C. angulatus*, which role in Ca^{2+} homeostasis that remains to be established in this crab.

In summary, our results show the existence of Na^+–K^+ATPase activity sensitive to salinity in the individual gills of *C. angulatus* and suggest a differential short-term participation of this activity among gills in the ion transport process.

Short-term changes of Na^+–K^+ATPase activity in posterior gills of some decapod crustaceans have been suggested to occur via regulation of pre-existing enzyme, by tissue Na^+ 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; Lucu and Flik, 1999; Morris, 2001). Furthermore, regulation by changes in the rate of serine exchange of phosphorylation–dephosphorylation cycle controlling the activity of different enzymes responsible for cellular responses in organs of euryhaline animals might be one mechanism which enables the rapid acclimatization of euryhaline crustaceans to different salinities (Zwingelstein et al., 1998).

Previous studies in our laboratory have shown a differential response to dopamine, dbAMPc and *myo*-inositol trisphosphate in gills 4, 5 and 6 of *C. granulata* from Mar Chiquita lagoon (Schleich et al., 1999). Whether the differential response of the activity in gills of *C. angulatus* is due to the existence of differential mechanisms of the regulation of Na^+–K^+ATPase operating in the individual gills remains to be investigated.

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