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Effects of long-term exposure to different salinities on the location and activity of Na^+–K^+-ATPase in the gills of juvenile mitten crab, *Eriocheir sinensis*

Gabriela Torres [a,b,*], Mireille Charmantier-Daures [c], Silvia Chifflet [d], Klaus Anger [a]

[a] *Biologische Anstalt Helgoland, Foundation Alfred Wegener Institute for Polar and Marine Research, 27498 Helgoland, Germany*
[b] *AG Zoosystematik und Morphologie, Carl von Ossietzky Universität Oldenburg, 26129 Oldenburg, Germany*
[c] *Equipe Adaptation Ecophysiologique et Ontogenèse, UMR 5119: ECOLAG, Université Montpellier II, 34095 Montpellier cedex 05, France*
[d] *Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, 11000 Montevideo, Uruguay*

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Abstract

The euryhalinity of mitten crab, *Eriocheir sinensis*, is based on osmoregulation, and thus on the activity of Na^+–K^+-ATPase. We studied location and activity of this enzyme in gills of juvenile crabs exposed to 5‰, 25‰, and 40‰ salinity. The posterior gills showed always a high number of immunopositive cells (IPC), staining with fluorescent antibody against Na^+–K^+-ATPase, covering at 5‰ the entire lamellae. At 25‰, they showed fewer IPC which occurred only at the bases of the lamellae. Enzyme activity was consistently higher in posterior than in anterior gills. Low salinity stimulated the activity only in posterior gills. Both histochemical and enzymatic results are consistent with previous ultrastructural observations showing that the epithelial cells of the posterior, but not the anterior gills exhibit typical traits of ionocytes. While an increase in Na^+–K^+-ATPase activity at a reduced salinity is consistent with a strong hyper-osmoregulatory capacity in juvenile crabs, a low activity at an enhanced salinity suggests a physiological response, directed towards a reduction of Na^+ uptake. The activity increase of ion-transporting enzymes is directly related to spatial changes in their distribution along the osmoregulatory tissue, i.e. an enhanced number of IPC scattered along the entire lamellae. In juveniles, this allows for successful development and growth at reduced salinities.

Keywords: *Eriocheir sinensis*; Gills; Na^+–K^+-ATPase activity; Na^+–K^+-ATPase immunolocalization; Osmotic stress

1. Introduction

The Chinese mitten crab, *Eriocheir sinensis* H. Milne-Edwards 1854 is a strongly euryhaline brachyuran. During its complex life cycle, the adults migrate downstream towards estuarine waters (Herborg et al., 2003), where they reach maturity, mate and release the planktonic larvae. Their following larval development, consisting of 5 zoeal stages and a megalopa (Kim and Hwang, 1995; Montú et al., 1996) takes place in estuarine and marine coastal waters. After metamorphosis, the juveniles start the upstream migration towards the limnic parental habitat (Herborg et al., 2003). This upstream migration involves an acclimation of the migrating juveniles to decreasing salinity, reaching eventually fresh water. The ability of *E. sinensis* to cope with the salinity variations occurring during its life cycle involves ontogenetic changes in the osmoregulatory capacity, i.e. from a moderately hyper-iso-regulating zoeal phase to a strongly euryhaline hyper-hypo-regulating first juvenile crab stage (Cieluch et al., 2007).

The increasing osmoregulatory capacity is indispensable to allow for upstream migration. It is achieved by maintaining an osmotic gradient through an active uptake of ions such as Na^+ and Cl^- across the posterior gills (Péqueux and Gilles, 1988). The absorption of Na^+ occurs through a coordinated action of apical Na^+ channels and basolateral Na^+–K^+-ATPase, while the

* Corresponding author. Biologische Anstalt Helgoland, Foundation Alfred-Wegener-Institute for Polar and Marine Research, 27498 Helgoland, Germany. Tel.: +49 4725 819 348; fax: +49 4725 819 369.
E-mail addresses: gtorres@awi-bremerhaven.de, gabriela-torres-@hotmail.com (G. Torres).

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uptake of Cl^- proceeds via an apical Cl^-/HCO_3^- antiporter and basolateral Cl^- channels (Onken et al., 1991) driven by the apical V-type H^+ pump (Putzenlechner et al., 1992; for a review see Towle, 1997). The study of developmental changes in histological characteristics and physiological capabilities showed a strong correlation between the ontogeny of osmoregulation and the expression of Na^+–K^+-ATPase in transport epithelia (Cieluch et al., 2007).

Adult *E. sinensis* possess eight lateral pairs of gills. The five anterior pairs are predominantly formed by thin epithelial cells, which are typical of gas exchange tissues (Péqueux, 1995). By contrast, ionocytes predominate in the three posterior gill pairs involved in the ion transport. They typically reveal abundant apical infoldings and basolateral invaginations with numerous mitochondria (Péqueux, 1995; Cieluch et al., 2007). The presence of numerous Na^+–K^+-ATPase molecules in the posterior gills was shown by immunofluorescence light microscopy (ILM), while immunopositive cells were undetectable in the anterior gills of the juvenile I and adult crabs (Cieluch et al., 2007).

The present study focuses on the involvement of Na^+–K^+-ATPase in the osmoregulation of juvenile *E. sinensis* during long-term exposure to different salinities, by localizing and measuring the activity of this enzyme in the gill tissues. The combination of histochemical and biochemical techniques should allow to explain how the regulation of Na^+ concentration occurs during hyper-osmoregulation. We expected (a) an increase in Na^+–K^+-ATPase activity during exposure to reduced salinities, as already documented for other crustacean species, and (b) that this effect is due to a modification in the abundance and distribution of Na^+–K^+-ATPase molecules along the posterior gills.

2. Materials and methods

2.1. Animals

Ovigerous female mitten crabs (*E. sinensis*, Crustacea: Decapoda, Brachyura) were collected near the island of Scharhörn in the mouth of River Elbe, northern Germany. They were transported to the Marine Biological Station Helgoland (Helgoland, Germany) and maintained in a circulating water system (25‰, 15 °C, and photoperiod 12:12 h). Freshly hatched larvae were mass-reared in 400-mL beakers (150 larvae L^{-1}) at 25‰ (optimal salinity; Anger, 1991), 18 °C, and a 12 h:12 h photoperiod. Water and food (*Artemia* sp. nauplii *ad libitum*) were changed in daily intervals. After metamorphosis to the juvenile I stage, the young crabs were divided in three groups and cultured for ca. 1 year at a reduced (5‰), an intermediate (25‰) and an enhanced (40‰) salinity under the same conditions of temperature, photoperiod, and food. We chose a salinity of 5‰ rather than freshwater as hypo-osmotic test condition, because the earliest juvenile crab stages are still relatively weak hyper-osmoregulators compared to the conspecific adults (Cieluch et al., 2007). Feeding and water change were performed every second day. Two months before the sampling of gills, the crabs were isolated to allow for control of the moulting cycle. Sampling took place during the intermoult stage.

2.2. Localization of Na^+–K^+-ATPase: immunofluorescence light microscopy

Crabs (ca. 15 mm carapace width) previously exposed to 5‰ and 25‰, respectively, were fixed for 48 h in Bouin's fixative. After rinsing in 70% ethanol and removal of the carapace, the posterior gills were dissected from the inner body wall. Subsequently, the gills were fully dehydrated in a graded ethanol series and embedded in Paraplast X-tra (Sigma). Sections (4 μm) were cut on a Leitz Wetzlar microtome, collected on poly-L-lysine-coated slides, and stored overnight at 38 °C. Sections were then pre-incubated for 10 min in 0.01% Tween20, 150 mM NaCl in 10 mM phosphate-buffered saline (PBS, pH 7.3) to permeabilize the tissues. In order to remove the fixatives' free-aldehyde groups, samples were treated for 5 min with 50 mM NH_4Cl in PBS. Sections were washed in PBS and incubated for 10 min with a blocking solution (BS) containing 1% bovine serum albumin (BSA) and 0.1% gelatine in 20 mM PBS to block non-specific binding sites. The primary antibody (monoclonal antibody IgGα5, raised against the avian α-subunit of the Na^+–K^+-ATPase) was diluted in PBS to 20 μg mL^{-1}, placed in small droplets of 100 μl on the sections, and then incubated for 2 h at room temperature in a moist chamber. Control sections were incubated in BS without primary antibody. To remove unbound antibodies the sections were then washed (3 × 5 min) in PBS, and subsequently incubated for 1 h with small droplets (100 μL) of the secondary antibody: fluoresceinisothiocyanate-labeled (FITC) goat anti-mouse IgG (Jackson Immunoresearch, West Baltimore, USA). After extensive washes in PBS (4 × 5 min), the sections were covered with a mounting medium and examined with a fluorescent microscope (Leitz Diaplan coupled to a Ploemopak 1-Lambda lamp) with an appropriate filter set (450 nm to 490 nm band-pass excitation filter).

2.3. Gill sampling for enzymatic determinations

Fifteen (five per each salinity) one-year-old crabs (carapace width 13.0–27.5 mm) were killed by short exposure to −20 °C. The gills were dissected, washed 2 s in pre-homogenization buffer (see Table 1), blotted dry, and frozen at −80 °C for later measurements of Na^+–K^+-ATPase activity and protein content.

2.4. Na^+–K^+-ATPase activity in gills tissues

The gills were homogenized in 300 μL of ice-cold homogenization buffer (see Table 1) by sonication (Branson Sonifier, Cell Disruptor B 15) with 5 strokes of 5 s, on ice. Na^+–K^+-ATPase activity was obtained as the difference between the inorganic phosphate (P_i) produced from the reactions in absence (total ATPase activity) and the presence of the Na^+–K^+-ATPase specific inhibitor ouabain (activity of "other" ATPase). The P_i concentration was determined using a modification of the

Table 1
Eriocheir sinensis

Solution	Reagents
Pre-homogenization buffer (PHB)	250.0 mM saccharose
	10.0 mM imidazol
	150.0 mM NaCl
Homogenization buffer (HB)	250.0 mM saccharose
	10.0 mM imidazol
Incubation buffer I (IB-I)	144.0 mM NaCl
	22.0 mM KCl
	3.3 mM $MgCl_2$
	4.4 mM NaN_3
	56.0 mM HEPES
	26.0 mM TRIS
	3.3 mM ATP
	1.1 mM ouabain
Incubation buffer II (IB-II)	144.0 mM NaCl
	22.0 mM KCl
	3.3 mM $MgCl_2$
	4.4 mM NaN_3
	56.0 mM HEPES
	26.0 mM TRIS
	3.3 mM ATP
Solution A	12% SDS
Solution B	6% ascorbic acid (in HCl 1 N)
Solution D	1% ammonium molybdate (in 12% SDS)
Solution E	2% sodium citrate
	2% sodium meta-arsenite
	2% acetic acid

Reagents for homogenization and incubation buffers, and solutions to determination of inorganic phosphate (modified from Chifflet et al., 1988).

method described by Chifflet et al. (1988). Reactions were initiated by the addition of 25 µL of the homogenate to 225 µL of incubation buffer I (IB-I; see Table 1) and to 225 µL of incubation buffer II (IB-II; see Table 1). After 15 min at 37 °C, the incubation was stopped by an addition of 250 µL solution A (see Table 1). To determine the P_i photometrically, 4 replicates of 75 µL from each of the above mentioned incubation mixtures (IB-I + solution A and IB-II + solution A) were placed in a 96-well microplate. Immediately thereafter, they were mixed with 75 µL of solution BD (1:1; see Table 1) and incubated for 5 min at room temperature. Subsequently, 115 µL of solution E (see Table 1) was added, and after 15 min at room temperature, absorbance was determined (Thermoelectron Multiskan® Spectrum spectralphotometer, wavelength: 850 nm). The calibration curve was obtained with appropriate dilutions of a K_2HPO_4 standard solution.

2.5. Protein determination

The protein content of the homogenate was determined using a modified method after Lowry et al. (1951) (kit: BioRad D_C Protein Assay). 25 µL of homogenate was mixed with 100 µL of ice-cold 20% trichloroacetic acid (TCA). After 10 min incubation at 4 °C, the samples were centrifuged at 10,000×*g* for 10 min at 4 °C; the supernatant was discarded. The remaining pellet was dissolved in 300 µL NaOH (1 M) and incubated with shaking at 1400 rpm for 30 min at 56 °C in a Thermomixer Eppendorf. After incubation, 4 replicates of 30 µL each of the dissolved sample were mixed with 20 µL of Reagent A and 300 µL of Reagent B (kit: BioRad D_C Protein Assay) in a 96-well microplate. The microplates were incubated for 15 min at room temperature in the dark and absorbance was measured (Thermoelectron Multiskan® Spectrum spectralphotometer, wavelength: 750 nm). The calibration curve was obtained by dilutions of bovine serum albumin (BSA, kit: BioRad D_C Protein Assay).

2.6. Statistical analysis

The data are expressed as mean values ± SD. The effect of exposure to different salinities on the activity of total ATPases and Na^+–K^+-ATPase in the different gills was tested with ANOVA following Zar (1996). Position (right vs. left), type (anterior vs. posterior) and gill number (3–8) were treated as repeated measures within-subject factors, while salinity (5, 25, 40‰) was a between-subject factor. When the ANOVA showed a significant effect, post hoc comparisons between mean values were performed using the Student–Newman–Keuls (SNK) test. Homogeneity of variance (Cochran test) and normality (normality plots) were checked. Statistical significance was accepted at $\alpha = 0.05$.

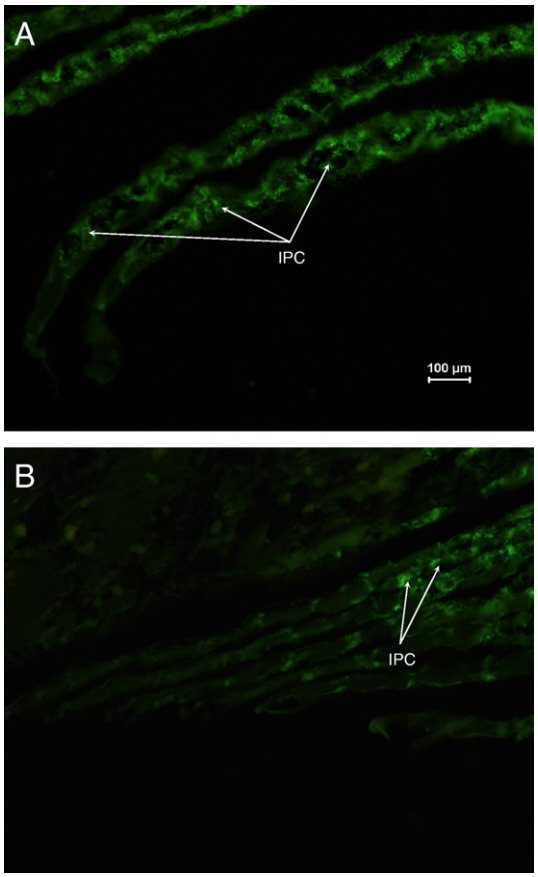

Fig. 1. *Eriocheir sinensis*. Immunolocalization of Na^+–K^+-ATPase in ionocytes of juvenile crabs (ca. 15 mm carapace width) after long-term exposure (one year) to 5‰ (A) or 25‰ (B). Equivalent sections of posterior gills show the apical side of the gill lamellae: more lamellae are shown in B, because they are ca. 1.5 times thinner than in A. IPC: immunopositive cells.

3. Results

3.1. Localization of Na^+–K^+-ATPase

Long-term exposure of *E. sinensis* to two different salinities (5‰, 25‰) induced changes in the location and number of ionocytes in the ion-transporting epithelia of the posterior gills. When animals were exposed to 5‰, these epithelia showed numerous immunopositive cells (IPC) scattered throughout the gill lamellae, indicating abundant Na^+–K^+-ATPase molecules (Fig. 1A). In gills of animals maintained at 25‰, by contrast, IPC were present in smaller amounts and located only at the base of the lamellae (Fig. 1B). Thus, a comparison between animals maintained at 5‰ and 25‰ showed not only a change in abundance of Na^+–K^+-ATPase molecules, but also in its distribution.

3.2. Na^+–K^+-ATPase activity in gill tissues

We show here only biochemical determinations from gill 3 to 8, because, the smallest anterior gills (1–2) did not reveal detectable enzymatic activities (neither total ATPase nor Na^+–K^+-ATPase).

ATPase activity (measured as P_i; given in nmol min^{-1} mg^{-1} protein) was significantly affected by salinity and gill type (anterior vs. posterior), but not by the lateral gill position (left vs. right; Table 2). The anterior gills (3–5) were not affected by salinity (Fig. 2A). In the posterior gills (6–8), by contrast, increasing salinities caused a significant decrease in enzyme activity (Fig. 2A). The anterior gills showed consistently low activities (245–367 nmol min^{-1} mg^{-1} protein), while the posterior gills revealed values of 485–933.

In the enzyme essay, the P_i produced through the activity of ATPase can be divided in two different fractions: (1) P_i produced by the activity of Na^+–K^+-ATPase alone and (2) P_i produced by "other" ATPases. In the anterior gills Na^+–K^+-ATPase activity represented only 30–60% of total ATPase activity, in the posterior gills, by contrast, this activity amounted to 70–73% of total ATPase activity.

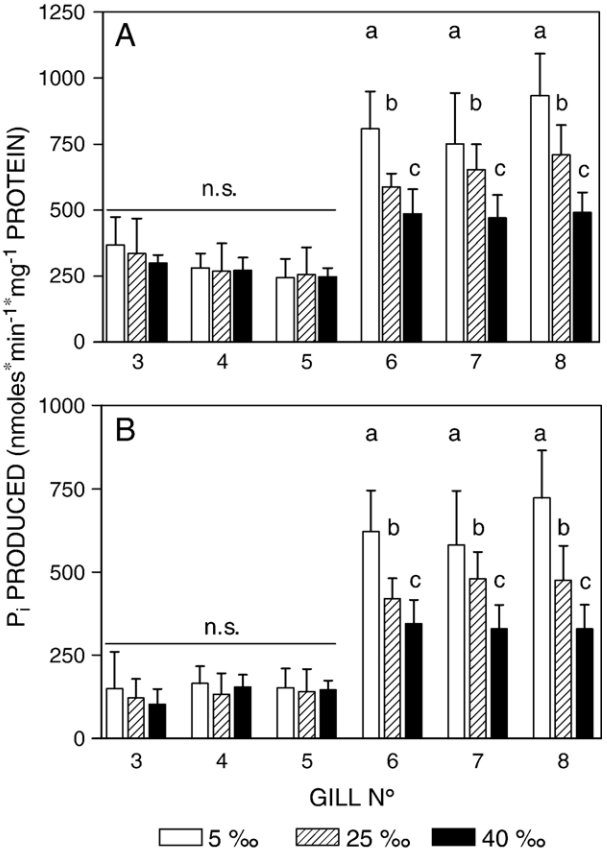

Fig. 2. *Eriocheir sinensis*. Activity of total ATPases (A) and Na^+–K^+-ATPase (B), expressed as amounts (in nmol) of inorganic phosphorous (P_i) produced per minute per milligram of protein. Different letters indicate significant differences between means; n.s., no significant difference.

Na^+–K^+-ATPase activity showed the same pattern of activity modification in response to different salinities as the total

Table 2
Eriocheir sinensis

	MSf	Dff	MSe	Dfe	*F*	*p*
Salinity (S)	523,075	2	54,000	12	9.69	**0.003**
Position (P)	15,860	1	5609	12	2.83	0.118
P*S	11,725	2	5609	12	2.09	0.166
Type (T)	6,022,160	1	32,251	12	186.73	<**0.001**
T*S	393,202	2	32,251	12	12.19	**0.001**
Gill no. (*N*)	22,389	2	8605	24	2.60	0.095
N*S	9318	4	8605	24	1.08	0.387
P*T	3074	1	3967	12	0.77	0.396
P*T*S	1328	2	3967	12	0.33	0.722
P*N	7660	2	2459	24	3.11	0.063
P*N*S	1797	4	2459	24	0.73	0.580
T*N	118,203	2	9001	24	13.13	<**0.001**
T*N*S	21,266	4	9001	24	2.36	0.082
P*T*N	16,214	2	5013	24	3.23	0.057
P*T*N*S	5159	4	5013	24	1.03	0.412

Four-way between- and within-subject ANOVA to evaluate the effect of salinity, gill position, type and number of total ATPase activity. Significant differences are in **bold**. Symbols: MSf: mean square of factors; Dff: degrees of freedom of factors; MSe: mean square of error; Dfe: degrees of freedom of error.

Table 3
Eriocheir sinensis

	MSf	Dff	MSe	Dfe	*F*	*p*
Salinity (S)	416,595	2	35,579	12	11.71	**0.002**
Position (P)	1088	1	3072	12	0.35	0.563
P*S	6424	2	3072	12	2.09	0.166
Type (T)	5,141,084	1	26,602	12	193.26	<**0.001**
T*S	310,537	2	26,602	12	11.67	**0.002**
Gill number (*N*)	17,813	2	6295	24	2.83	0.079
N*S	7788	4	6295	24	1.24	0.322
P*T	25	1	2676	12	0.01	0.925
P*T*S	5047	2	2676	12	1.89	0.194
P*N	5030	2	1380	24	3.65	**0.041**
P*N*S	1648	4	1380	24	1.19	0.339
T*N	9390	2	6842	24	1.37	0.273
T*N*S	15,834	4	6842	24	2.31	0.087
P*T*N	8829	2	3162	24	2.79	0.081
P*T*N*S	2423	4	3162	24	0.77	0.558

Four-way between- and within-subject ANOVA to evaluate the effect of salinity, gill position, type and number of Na^+–K^+-ATPase activity. Symbols as in Table 2.

ATPase activity (Table 3, Fig. 2B). Thus, the effect on total ATPase activity, i.e. an increase at decreasing salinity in the posterior but not in the anterior gills, could be attributed to an increase in Na^+–K^+-ATPase activity. In the former gill type, it increased on average from 329 to 723 nmol P_i min^{-1} mg^{-1} protein, while it remained low (122–166 nmol P_i produced min^{-1} mg^{-1} protein) in the latter.

4. Discussion

In previous studies of variation in Na^+–K^+-ATPase activities in crab gills exposed to salinity variation, long-term acclimation (through 1 year) has not been tested, but only exposures for several weeks (e.g. in the blue crab, *Callinectes sapidus*; Li et al., 2006).

Our study shows for the first time effects of salinity on the location of Na^+–K^+-ATPase molecules within ion-transporting gill tissues of early juvenile crabs. Also, the differential significance of individual gills, of the lateral position (left vs. right), and the type of gill (anterior vs. posterior) for Na^+–K^+-ATPase activity in relation to salinity is demonstrated. In *E. sinensis*, the early juvenile stages have previously been studied only with immunofluorescence light microscopy using specimens maintained at optimal salinities (Cieluch et al., 2007). Na^+–K^+-ATPase activities were studied in the posterior gills only (e.g. Cooper and Morris, 1997), or in pooled samples of anterior and posterior gills (e.g. Castilho et al., 2001) without testing for variability among the various gills. We found that, within either the posterior or anterior gills, Na^+–K^+-ATPase activity was independent of the body side or the individual gill number; while it differed substantially between anterior and posterior gills.

Our immunocytochemical study corroborates the importance of Na^+–K^+-ATPase in the posterior gills of brachyurans to cope with hypo-osmotic stress. In juveniles maintained at a low salinity (5‰), the gills showed an enhanced number of ionocytes, indicated by immunopositive cells (IPC) scattered throughout the entire lamellae. In specimens exposed to 25‰, by contrast, a lower number of IPC was detected, concentrated near the base of the lamellae. Besides, lamellae were thicker after exposure to 5‰ than to 25‰. In a previous study on early juvenile *E. sinensis* exposed to 25‰, the IPC were also located mainly on the base of the lamellae, but not on their apical side (Cieluch et al., 2007). This indicates that the number of ionocytes within the ion-transporting gill tissues increases in diluted media. The proliferation of ionocytes may provide the suitable machinery to face hypo-osmotic stress, allowing an enhanced expression of Na^+–K^+-ATPase, and thus an increase of the Na^+–K^+-ATPase activity.

The localization of ionocytes in our study is consistent with previous investigations on the European lobster, *Homarus gammarus* (Lignot et al., 1999; Lignot and Charmantier, 2001). When juveniles of this species were exposed to brackish seawater, they showed an increased number of widely scattered ionocytes in the epithelia of the epipodites and branchiostegites, which are in this species the ion-transporting epithelia involved in osmoregulation. In contrast, lobsters maintained in seawater possessed only low amounts of immunostained cells (Lignot et al., 1999; Lignot and Charmantier, 2001). The increased thickness of the lamellae in juvenile *E. sinensis* exposed to low salinity was consistent with previous studies of the posterior gills of *Carcinus maenas* and *Pachygrapus marmoratus* (Compère et al., 1989; Pierrot, 1994) and of the epithelium of epipodites and branchiostegites of *H. gammarus* (Haond et al., 1998). In conclusion, our study points to a common pattern in the relationship between variations in salinity and the distribution and abundance of ionocytes in ion-transporting tissues of decapod crustaceans.

The patterns of Na^+–K^+-ATPase activity found with biochemical techniques (present study) are also consistent with previous histological evidence (Péqueux, 1995). In crabs, all posterior gills showed both typical characteristics of ion transport tissues and higher enzyme activities than the anterior gills. The latter, by contrast, possessed typical features of respiratory tissues and changes in salinity did not influence their structure or abundance of IPC.

The increase in Na^+–K^+-ATPase activity in the posterior gills at 5‰ appears to be an adaptive physiological response, which enhances the function of hyper-osmoregulation. Lower salinities may either stimulate enzyme production, or uncover hidden active sites. Both mechanisms should cause an increase in the active transport of Na^+ ions. For *E. sinensis*, the immunocytochemical evidence suggests that *de novo* synthesis is the likely explanation for the increase in Na^+–K^+-ATPase activity. The same pattern was found not only in *E. sinensis* (Péqueux et al., 1984), but also in other hyper-regulating decapods (for a review see Lucu and Towle, 2003), e.g. *C. maenas* (Siebers et al., 1982, 1983), *H. gammarus* (Flik and Haond, 2000), *Chasmagnathus granulata* (Castilho et al., 2001). Inversely, the enzyme activity decreases during a transfer from freshwater to brackish water, e.g. in *Procambarus clarkii* (Sarver et al., 1994) or *Macrobrachium rosenbergii* (Wildera et al., 2000). In addition, osmoconformers, which are incapable of regulating their hemolymph concentration, do not show changes in the Na^+–K^+-ATPase activity during salinity variation, e.g. *Palinurus elephas* (Lucu et al., 2000).

The reduction of Na^+–K^+-ATPase activity after an extended exposure to 40‰ might be explained (a) as an adaptive response to reduce active Na^+ uptake, or (b) as a consequence of physiological disturbance due to high internal osmolality. *E. sinensis* is a weak hypo-osmoregulator at high salinities (Cieluch et al., 2007): in our experiments, the activity of ATPases other than Na^+–K^+-ATPase (data not shown) did not significantly decrease at 40‰. This suggests that a decrease occurred only in Na^+–K^+-ATPase activity. We may therefore conclude that this effect is an adaptive physiological response, which reduced the uptake of Na^+ ions.

The response of hypo-osmoregulators to changes in salinity varies among species. For instance, *C. granulata* shows in the posterior gills similar changes in Na^+–K^+-ATPase activity as *E. sinensis* (Castilho et al., 2001). However, it shows also a decreasing activity in the anterior gills. This suggests that, in this case, the hypo-osmoregulatory capacity may not be strong enough to prevent physiological stress. Other species do not

show any significant changes of enzyme activity when exposed to concentrated media (e.g. *C. maenas*: Hake and Teller, 1983; *Uca pugnax*: Holliday, 1985; *Uca pugilator*: Hake and Teller, 1983; D'Orazio and Holliday, 1985; *Sesarma reticulatum*: D'Orazio and Holliday, 1985). Others, by contrast, show under such conditions an increase in Na^+–K^+-ATPase activity (e.g. *Uca tangeri*: Drews, 1983; *Artemia salina*: Holliday et al., 1990). These inconsistent findings indicate that the mechanisms of hypo-osmoregulation in concentrated media require further comparative studies to enhance our understanding of responses to hyper-osmotic stress in decapod crustaceans.

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