



Osmolality and ionic status of hemolymph and branchial Na^+/K^+ -ATPase in adult mitten crab during seawater adaptation

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Abstract

Background: Invasive mitten crabs of the genus *Eriocheir*, are catadromous species. As in other decapod crustaceans, their hyperosmoregulation during and after migration into fresh water has been studied, but information about the physiology during seawater acclimation of adults is limited mostly to the dynamics of free amino acids. Therefore, the purpose of this study was to investigate the possible osmo/iono-regulation in adult mitten crabs (*E. japonica*) during adaptation to seawater.

Methods: Adult crabs collected in fresh water were transferred to 30-ppt seawater for 3 and 10 days, and the osmolality and ionic status of the hemolymph as well as gill Na^+/K^+ -ATPase, which has been implicated in ion transport in various crustaceans, were analyzed.

Results: Analysis of the hemolymph osmolality and Na^+ status indicated that adults were able to hypo-regulate these parameters in seawater. On the other hand, the free Ca^{2+} concentration was two-fold hyper-ionic to seawater in both fresh water and seawater, but relatively maintained compared with Na^+ and osmolality in seawater, while complexed calcium (total minus free calcium) increased after 3 days in the hemolymph. Examination of Na^+/K^+ -ATPase activity revealed that seawater acclimation decreased the activity in the most posterior gill (gill number 8), where immunoreactive Na^+/K^+ -ATPase was localized to the basolateral membrane region of gill epithelial cells in fresh water.

Conclusions: Adult mitten crabs can hypo-regulate the hemolymph concentrations of ions and their osmolality during seawater adaptation, partly by reducing the Na^+/K^+ -ATPase activity involved in ion absorption in fresh water and using the hemolymph complexed calcium as an internal reserve.

Keywords: Crustacean, osmoregulation, salinity, gills, Na^+/K^+ -ATPase

Introduction

Mitten crabs, genus *Eriocheir*, are notorious invasive animals and are considered to be the first (1912) documented case of a ballast water-transported species [1]. In addition, they are of considerable value for aquaculture in both local and international markets [2]. They are strongly euryhaline brachyurans and provide a unique model for studies on osmotic/ionic responses. During their life cycle, the early development [3,4] takes place in marine coastal waters and the metamorphosed juveniles migrate up rivers to limnic habitats [5]. Then, the adults migrate downstream to marine waters [5], where they reach maturity, mate, and release the planktonic larvae. These catadromous migrations involve their acclimation to changing salinities, reaching eventually fresh water or seawater. The ability of mitten crabs to cope with the salinity variations occurring during the life cycle involves changes in their osmoregulatory capacity, i.e., from an iso-hyper-regulating larval-juvenile phase to a hyper-hypo-regulating adult crab stage [5,6].

Osmoregulation of decapod crustaceans during hyposaline adaptation has been extensively studied. In crabs, the primary site of regulation is the posterior gills,

which have the highest specific activity of Na^+/K^+ -ATPase, the enzyme thought to provide the major driving force for salt uptake and be one of the central players in hyperosmotic and -ionic regulation. However the underlying cellular components of active ion uptake also include other transport proteins and transport-related enzymes, such as a Na^+/H^+ antiporter, a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter, V-ATPases, and carbonic anhydrases [7-16]. Indeed, the role of this Na^+/K^+ -ATPase in hyperosmoregulation during and after migration into rivers has been studied also in mitten crabs [17,18]. On the other hand, activation of this enzyme also occurs at high salinities in the posterior gills of some intertidal species [7,16,19-24]. However, information about the physiological regulation during seawater acclimation of the mitten crab adults is surprisingly limited to the dynamics of free amino acids and bioenergetics [25,26]. Furthermore, little attention has been given to Ca^{2+} regulation during adaptation of crustaceans to different salinities generally [23,27], while Ca^{2+} homeostasis in the molting cycle has been examined extensively [28,29]. For example, only the total calcium concentrations in the hemolymph [complexed, (protein bound) plus relevant

free (unbound) calcium] have been reported as the Ca^{2+} concentration in most of the studies on the responses to different salinities, while about 20% of the calcium in the hemolymph of intermolt crabs is protein bound, unlike other ions [9,30,31]. Recently, we have found that the free Ca^{2+} concentration in the hemolymph of the grapsid crab, *Gaetice depressus*, is well-maintained during salinity adaptations via use of complexed calcium in the hemolymph as an internal reserve [24]. In this study, therefore, we examined changes in the hemolymph osmotic and ionic concentrations and in the activity of branchial Na^+/K^+ -ATPase after transfer of adult mitten crab (*E. japonica*) from fresh water to seawater.

Methods

Collection and maintenance of animals

Adult male individuals of *E. japonica* weighing 40 to 60 g were collected in September from the fresh-water irrigation channel of the Yoshii River system located in Okayama Prefecture, Honshu, Japan [32,33]. Crabs were transferred to the Ushimado Marine Institute and held in fully aerated fresh water at a temperature of $23\pm 1^\circ\text{C}$. Animals were acclimated to laboratory conditions and fed a commercial diet *ad libitum* daily for one month prior to experimentation, but were not fed for 48 h prior to the start of the study. Crabs were sacrificed following anesthesia. All procedures were conducted in accordance with the Guidelines for Animal Experimentation established by Okayama University.

Experimental design

Each crab in freshwater was transferred to an individual 2-L aquarium with freshwater at a practical salinity of either 0 ppt (control) or 30-ppt natural seawater (448 mM Na^+ , 506 mM Cl^- , 9.7 mM Ca^{2+} , 9.7 mM K^+ , 994 mOsm kg^{-1}). The salinity was checked with a refractometer, and its osmolality later confirmed with a vapor pressure osmometer (Wescor Inc. 5500, Logan, UT, USA), an atomic absorption spectrophotometer (Hitachi Z5300; Tokyo, Japan), and an ion chromatograph (AV10, Shimadzu, Kyoto, Japan). The water in the tanks was replaced daily. Six crabs in each group were sampled on day 3 and on day 10. To assess the effects of salinity conditions on body mass, the crabs were weighed with a Shimadzu AB54 balance (accuracy: ± 1 mg) after the visible water had been removed with adsorbent tissue. Hemolymph (1 ml) was withdrawn from the arthroal membrane of the walking legs, immediately centrifuged (5 min at $6,000\times g$), and the supernate was analyzed. The eight gills were dissected out, blotted, and either placed in ice-cold SEI buffer (250 mM sucrose, 10 mM di-sodium EDTA, 50 mM imidazole, pH 7.3) and frozen immediately at -80°C ($n = 6$, [24]), or fixed for 48 h in Bouin's fixative ($n = 4$, [17]). Only specimens in intermolt stage C were retained for analysis [34-36].

Determination of osmolality and ionic concentrations

The osmolality was measured on 5 μL samples (diluted 1:1

with deionized water) using a vapor pressure osmometer [24]. Determinations of Na^+ and Ca^{2+} were made by the AV10 ion chromatograph using a cation-exchange column (IC-C3, Shimadzu, Kyoto, Japan). The lower detection limit in this study is ~ 5 μM . Standard curves for the anions and cations were prepared using commercial standard solutions (Shimadzu). Concentration was estimated from a regression line using Class-VP version 6.12 software (Shimadzu) [37,38]. In the case of calcium, total (free plus complexed) calcium was also analyzed since about 20% of the calcium in the hemolymph of intermolt crabs is a complexed moiety, unlike other ions [24,30,31]. A 5 μL aliquot of hemolymph was diluted 1:400 in deionized H_2O , and total calcium concentrations were determined by an atomic absorption spectrophotometer (Hitachi Z5300; Tokyo, Japan) [24].

Assay of Na^+/K^+ -ATPase enzyme activity

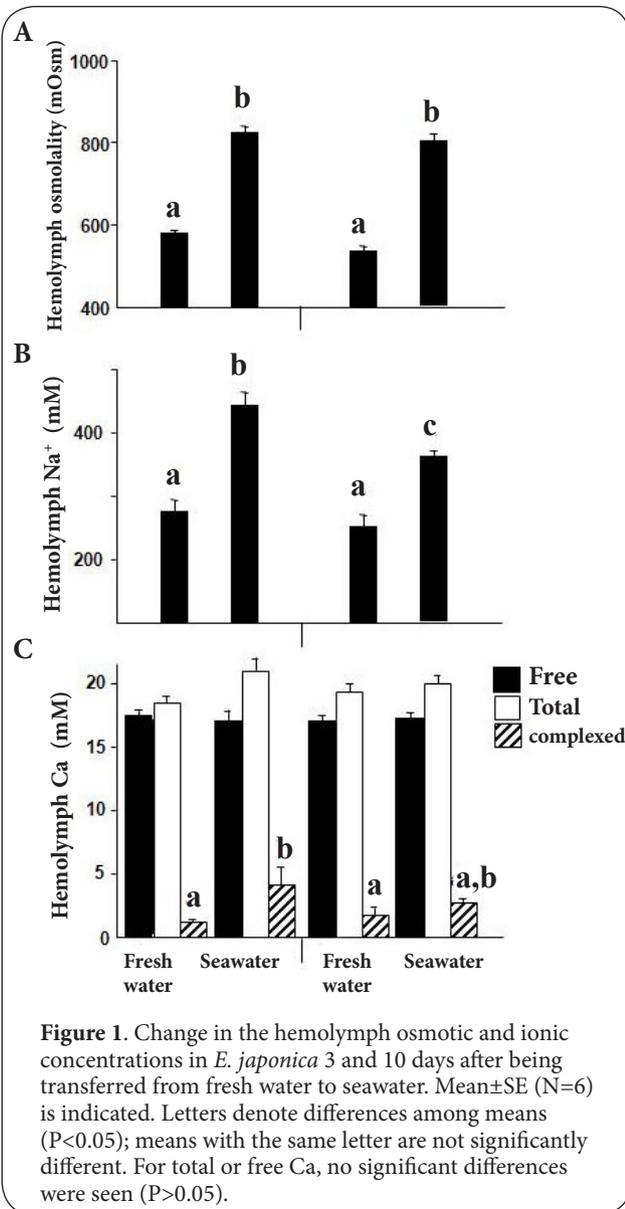
The Na^+/K^+ -ATPase activity was determined with a linked pyruvate kinase/lactate dehydrogenase-NADH assay [39], using a method validated for euryhaline crabs [24]. Gill tissue was homogenized in ice-cold 0.1%-deoxycholate SEI buffer (1:9 w/v) and centrifuged at $5,000\times g$. The resulting supernatant was diluted and assayed for Na^+/K^+ -ATPase activity. Each sample of gill homogenate was plated in quadruplicate assays of 10 μL ; two contained 2.8 mM ouabain and two did not. Fifty microliters of salt solution (50 mM imidazole, 189 mM NaCl , 10.5 mM MgCl_2 and 42 mM KCl) and 150 μL of assay mixture (50 mM imidazole, 2 mM phosphoenolpyruvate, 0.16 mM nicotinamide adenine dinucleotide, 0.5 mM adenosine triphosphate, 3.3 U/mL lactic dehydrogenase, and 3.6 U/mL pyruvate kinase) were added to each well. The kinetic assay was read at a wavelength of 340 nm at 24°C with a run time of 10 min and intervals of 10 s. The difference between the kinetic reading with and without ouabain is the Na^+/K^+ -ATPase activity and is expressed as micromoles ADP per mg protein per hour. Total protein in homogenates was measured using a BCA Protein Assay kit (Pierce Chemical Co., Rockford, Illinois, USA). Assays were run on a microplate reader (Multiskan Ascent, Thermo).

For further validation of this system, the standard conditions described above were employed, varying one factor while keeping all the other parameters constant. The ATPase activity inhibition was first examined by varying the ouabain concentration in the reaction mixture. Under standard conditions, the final concentrations of ouabain in the reaction mixture varied from 0, 0.5, 1.4, 2.8, and 5.0 mM in wells, and maximal inhibition was observed at 2.8 mM ouabain, and thus this concentration was fixed in the examination of other parameters in the remainder of the validation. Optimal conditions for actual analyses of the response to seawater transfer were set according to such results. In examinations of the effects of gill protein concentration on the enzymatic activity, it was seen that in a sample consisting of 1 mg protein/10 μL of

Table 1. Change in the wet body weight (%) of *E. japonica* after being transferred from fresh water to seawater.

	3 days	10 days
Fresh water (control)	-0.55 ± 0.23	-1.68 ± 0.93
Seawater	-0.58 ± 0.51	-1.08 ± 0.53

For each time period, no significant differences were seen ($P > 0.05$). Values are means ± SE (N = 6).



0.1%-deoxycholate SEI buffer diluted from 2- to 10-fold, activity decreased linearly in proportion to the protein quantity. Therefore, measurements were valid for samples diluted at least two-fold, but samples were usually diluted five-fold in this investigation.

Immunohistochemistry of Na^+/K^+ -ATPase

To label Na^+/K^+ -ATPase in the gills, we used a mouse monoclonal antibody IgGa5 against the avian α -subunit of the Na^+/K^+ -ATPase, as described before for the mitten crab [17]. Fixed tissue samples were dehydrated through graded alcohol concentrations and embedded in Paraplast. Sections were cut at 4 μm and attached to 3-aminopropyltriethoxysilane-coated slides. The slides were then immersed in 0.3% H_2O_2 in methanol at 20°C for 30 min to inactivate endogenous peroxidase activity. After being pre-incubated in 0.01% Tween20 in phosphate-buffered saline (PBS), the sections were placed in 5% normal goat serum in PBS at room temperature for 1 h to block non-specific binding. Sections were subsequently incubated for 2 h at room temperature with the primary antibody diluted to 20 $\mu\text{g}/\text{mL}$ in a solution containing 1% BSA (Sigma, Tokyo, Japan) and 0.1% gelatin in PBS. Sections were then washed 3 times in PBS, incubated with peroxidase-labeled goat anti-mouse secondary antibody (Sigma, Tokyo, Japan) diluted 1:70 in PBS containing 0.5% Triton X-100 and 1% BSA at room temperature for 1 h, and then developed for 5 min with DAB substrate solution (Roche, Tokyo, Japan). Controls omitting the Na^+/K^+ -ATPase primary antibody were included and yielded no immunoreactivity (data not shown).

Statistical analyses

Statistics were performed using Statview 4.11 (Abacus Concept). Since there was a significant interaction between the treatment (salinity condition) and time by two-way ANOVA, data for day 3 and day 10 were analyzed separately by the appropriate post-hoc test to determine the differences between the control (freshwater) and 30-ppt acclimated groups. All data were checked for normality and equal variances. Where assumptions of normality or equal variances were not satisfied, equivalent nonparametric tests were used.

Results

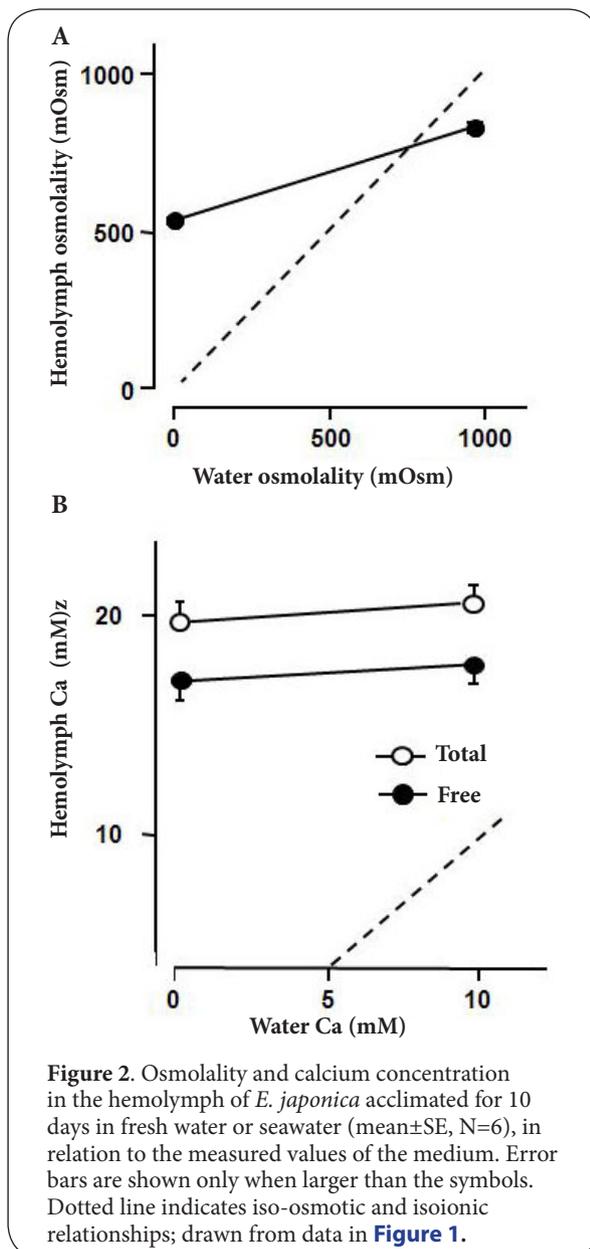
Body mass

Transfer of *E. japonica* from fresh water to seawater did not result in any significant change in body mass ($P > 0.05$, Table 1).

Hemolymph osmotic and ionic status

In crabs exposed to seawater, hemolymph osmolality increased from its freshwater level at 560 mOsm to its new acclimation level after 3 days which was about 200 mOsm hypoosmotic to the medium ($P < 0.001$, Figure 1A). Then, the level did not change significantly ($P > 0.05$) there after. The osmoregulatory performance of crabs acclimated for 10 days in fresh water or seawater is shown in (Figure 2A).

A virtually identical pattern was seen for the major hemolymph cation, Na^+ . The concentrations of this ion increased 3 days after transfer to seawater ($P < 0.001$). There after, it appeared to stabilize at new acclimated values, although the Na^+ levels decreased on day 10 after the transfer



compared to those on day 3 (**Figure 1B**).

The changes in Na^+ showed a relationship with changes in hemolymph osmolality, but the free Ca^{2+} concentrations appeared to be maintained at similar values in both fresh water and seawater (**Figure 1C**). The hemolymph Ca^{2+} was almost two-fold hyper-ionic to seawater. The complexed calcium (total minus free calcium) increased significantly ($P<0.05$) 3 days after transfer to seawater, but returned on day 10 to the fresh water levels. The calcium regulatory performance of crabs acclimated for 10 days in fresh water or seawater is shown in (**Figure 2B**).

Branchial Na^+/K^+ -ATPase

Branchial Na^+/K^+ -ATPase activity was unevenly distributed

among the eight gill pairs in crabs acclimated to either fresh water or seawater for 10 days (**Figure 3**, left). Activity tended to be high in the posterior (three) gills (G6 to G8). This distribution and the absolute values of the activity reported here were similar to those reported in the literature (see 'Discussion'). The Na^+/K^+ -ATPase activity in G8 decreased significantly after transfer from fresh water to seawater by day 10 ($P<0.05$). The Na^+/K^+ -ATPase activity in the other gills did not change during exposure to seawater ($P>0.05$). In the gills of the crabs exposed to seawater for 3 days, there was no significant difference in the Na^+/K^+ -ATPase activity ($P>0.05$, data not shown).

Immunohistochemical analysis of Na^+/K^+ -ATPase in sections of the G8 in the fresh-water crabs indicated its predominance in basolateral membrane regions of the epithelium and its absence from the apical region (**Figure 3**, right panel).

Discussion

The results of the transfer of the adult mitten crab to the higher salinity of seawater indicate that the crab is able to hypo-regulate the hemolymph concentrations of ions and its osmolality. Together with a previous report [26] on the increase in the free amino acid concentrations under hyperosmotic stress by mobilizing the amino-acid pool in muscle, the mitten crab appears to be able to efficiently adapt to seawater.

Changes in hemolymph Na^+ generally paralleled those in osmotic concentrations in response to seawater exposure, and it is likely that altered osmolality of the hemolymph under varying environmental conditions is based for the most part on altered levels of Na^+ and Cl^- [24,40]. On the other hand, an interesting finding of this study is the pattern of hemolymph calcium levels, particularly of complexed calcium. The levels of hemolymph free Ca^{2+} in crabs exposed to seawater were maintained at similar values to those in fresh water throughout the experiment, while complexed calcium increased temporally after 3 days. We speculate that hemolymph complexed calcium could serve as a surplus (unnecessary) calcium reservoir of decreased Ca^{2+} efflux by a decreased gradient for passive diffusion. These findings support a new control mechanism of hemolymph free Ca^{2+} by the complexed calcium as a reserve for free Ca^{2+} [24], and imply that hemolymph concentrations of both total and free calcium need to be analyzed. Calcium regulation in various environments has been studied in crustaceans, mostly with respect to the control of epithelial calcium transport [23,29,30,41]. At any rate, it appears that it is necessary to regulate free Ca^{2+} to a specific narrow range and that this control is separate from the osmoregulatory mechanisms.

One of the ion transporters that has received the most intensive studies in osmoregulating crustaceans is Na^+/K^+ -ATPase [7-15]. In addition to the Ca^{2+} channel, Ca^{2+} -ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger, the transportation of Ca^{2+} in the

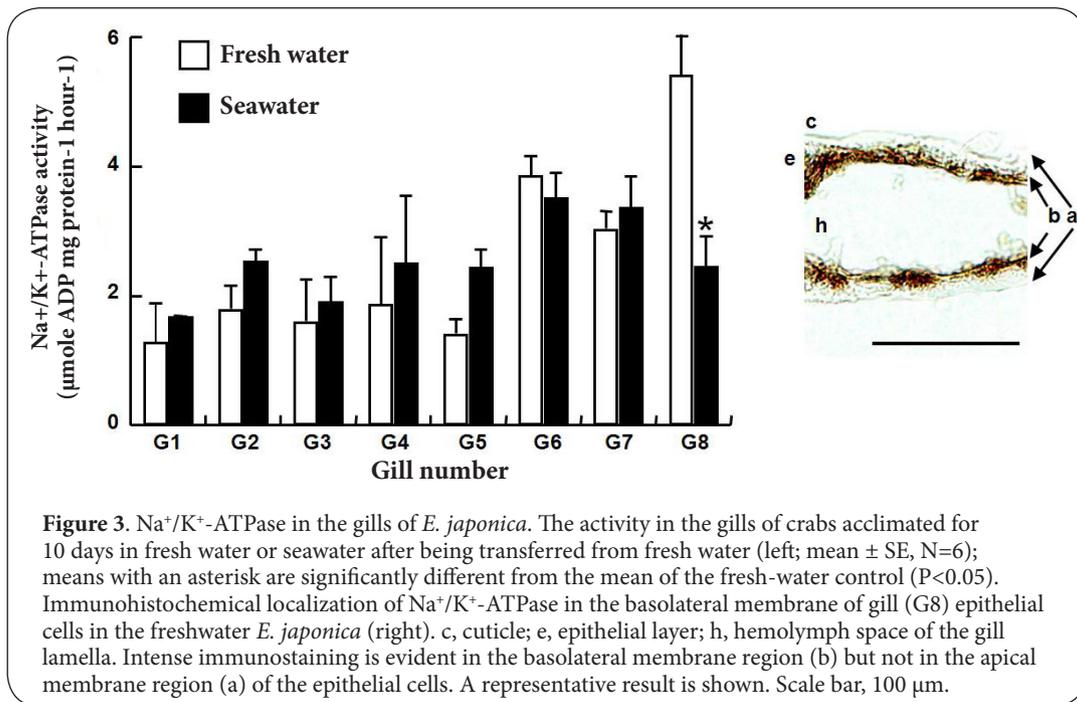


Figure 3. Na⁺/K⁺-ATPase in the gills of *E. japonica*. The activity in the gills of crabs acclimated for 10 days in fresh water or seawater after being transferred from fresh water (left; mean ± SE, N=6); means with an asterisk are significantly different from the mean of the fresh-water control (P<0.05). Immunohistochemical localization of Na⁺/K⁺-ATPase in the basolateral membrane of gill (G8) epithelial cells in the freshwater *E. japonica* (right). c, cuticle; e, epithelial layer; h, hemolymph space of the gill lamella. Intense immunostaining is evident in the basolateral membrane region (b) but not in the apical membrane region (a) of the epithelial cells. A representative result is shown. Scale bar, 100 μm.

hemolymph of crustaceans is also affected by the potential energy of the Na⁺ gradient, established by Na⁺/K⁺-ATPase activity [42]. In this study, we observed higher Na⁺/K⁺-ATPase activity in the posterior gills when compared with the anterior gills (see Figure 3), consistent with the molecular, biological, and physiological studies on various crab species, including juvenile mitten crab [7-9,15,17,23,24,27,43,44], which have designated the posterior gill epithelium, with its high Na⁺/K⁺-ATPase activity, as the principal site of osmoregulatory ion transport. These differences constitute the basis of the paradigm that anterior gills are structurally and functionally specialized for respiratory gas exchange, while the posterior gills have become specialized for active ion absorption counterbalancing passive losses in dilute media [23,27] as reflected in the significant decrease in the Na⁺/K⁺-ATPase activity of G8 after acclimation to seawater (Figure 3). Our immunohistochemical study identifying the Na⁺/K⁺-ATPase protein that is restricted to the basolateral membrane of the gill epithelial cells in the freshwater crab, is in agreement with the previously demonstrated localization of the Na⁺/K⁺-ATPase in the blue crab *Callinectes sapidus* [45], and also corroborates the importance of Na⁺/K⁺-ATPase in the posterior gills of brachyurans to cope with hypo-osmotic stress. Correspondingly, this enzyme activity inversely increases after transfer to low salinity in juvenile mitten crab [17]. The up-regulation of Na⁺/K⁺-ATPase activity even after a long-term (2-3 years) exposure to fresh water might reflect the history/origin of these mitten crabs [5].

The response of branchial Na⁺/K⁺-ATPase to high salinities varies among crustacean species [16]. For instance,

Chasmagnathus granulata shows in the posterior gills similar changes in Na⁺/K⁺-ATPase activity as those of mitten crabs [46]. Other species, including osmoconformers which are incapable of regulating their hemolymph concentration, do not show any significant changes of the enzyme activity when exposed to concentrated media (e.g., *Palinurus elephas* [47]). Others, by contrast, show under such conditions an increase in Na⁺/K⁺-ATPase activity (e.g., *Artemia salina* [48]). Recently, studies with the marble shore crab (*Pachygrapsus marmoratus*) and grapsid crab (*G. depressus*) have shown that the elevation in Na⁺/K⁺-ATPase levels was induced in only one of their gills following the transfer of crabs to high salinities but after being transferred to low salinities increased in the other/all gills. This suggests that the individual gills play distinct osmoregulatory roles in some intertidal crustaceans [24,49]. To enhance our understanding of responses to concentrated media in decapod crustaceans, future comparative investigations will examine the role of other transport proteins and transport-related enzymes in gills, including a Na⁺/H⁺ antiporter, carbonic anhydrase and Na⁺/K⁺/2Cl⁻ cotransporters [7,9-12,14,16,22,29,30].

In addition, following a salinity increase, the free amino acids are released primarily from the muscle into the blood, and the additional osmotic load at the blood level will control the water flow between the external medium. Thus, free amino acids are involved in osmoregulation in crustaceans and contribute to their osmoregulation capacity during ambient salinity changes [26,50]. It could be argued that although the mitten crab has an ion-regulatory capacity when it is in seawater on day 10, the osmotic pressure of the

hemolymph is still as high as on day 3. Therefore, it would be interesting to compare the respective roles of the Na⁺/K⁺-ATPase and key enzymes in osmolyte metabolism in the mitten crab (e.g., glutamate dehydrogenase expressed one of the highest degrees of adaptation to salinity in the muscle [26]).

Conclusions

Previous studies have shown that the adult catadromous mitten crab had increased free amino acids after ambient salinity increase to meet the demand of osmoregulation in hyperosmotic conditions. This paper indicates that this crab can also hypo-regulate the hemolymph concentrations of ions and osmolality values during seawater adaptation, by reducing Na⁺/K⁺-ATPase activity in the posterior gills which are involved in ion absorption in fresh water. Furthermore, the free Ca²⁺ concentration is well-maintained partly by hemolymph complexed calcium as an internal reserve.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	TS	SO	YN	WG	HT
Research concept and design	✓	--	--	--	--
Collection and/or assembly of data	✓	✓	✓	✓	✓
Data analysis and interpretation	✓	✓	✓	--	✓
Writing the article	✓	✓	✓	--	✓
Critical revision of the article	✓	--	--	--	--
Final approval of article	✓	✓	✓	✓	✓
Statistical analysis	✓	✓	✓	--	✓

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