

# Osmoregulation and Na,K-ATPase expression in osmoregulatory organs of *Scylla paramamosain*

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## Abstract

*Scylla paramamosain* is a euryhaline marine crab that can actively regulate its osmotic pressure of the hemolymph. We investigated the expression of Na,K-ATPase in the osmoregulatory organs, including anterior gills, posterior gills, antennal glands, and midgut. Our cDNA sequencing of the Na,K-ATPase  $\alpha$ -subunit revealed no isoforms in any of the four osmoregulatory organs studied. The full-length cDNA contains 3866 bp that encode a complete open reading frame of 1039 amino acids. The osmotic pressure of the hemolymph changed immediately after transference from 25 ppt to 5 and 45 ppt, and reached a new steady state within 3 days, showing the typical pattern of weak hyperosmoregulators. Expression of Na,K-ATPase  $\alpha$ -subunit mRNA level was determined for individuals acclimated in 5, 25, and 45 parts per thousand (ppt) for 7 days. Compared to the mRNA level of those from 25 ppt, the level in the posterior gills from 5 ppt was significantly higher. Also, the mRNA levels in the anterior gills, posterior gills and antennal glands from 45 ppt were significantly higher than that of the respective organs in 25 ppt. This increase in the 5 ppt treatment may be responsible for the observed increase of Na,K-ATPase activity on day 14 when the posterior gills had a significantly higher activity than those in the other two salinity treatments. No difference was found in the protein level across salinities. In conclusion, the posterior gills play the most important role in osmoregulation in dilute seawater and the increase in Na,K-ATPase activity on the 7th day may be due to its gene transcription and/or mRNA translation.

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## 1. Introduction

To keep the osmotic pressure of the hemolymph within a stable range, many brachyuran crabs living in intertidal regions and estuaries are hyperhypo-osmoregulators (Péqueux, 1995). The organs involved in crustacean osmoregulation include the gills, guts, and antennal glands (or excretory organs) (Mantel and Farmer, 1983). The gills are specialized for several functions, including gas exchange, osmoregulation, acid–base balance, and nitrogen excretion. The lamellar epithelium in the anterior gills is only about 2–4  $\mu\text{m}$  thick, much thinner than that in the posterior gills (10–20  $\mu\text{m}$ ) (Péqueux, 1995). The difference in diffusion distance implies that gas exchange takes place primarily in the anterior gills whereas the main

function of the posterior gills is osmoregulation (Mantel and Farmer, 1983; Péqueux, 1995).

The digestive tract of brachyurans, predominantly for digestion and absorption, can be divided into three regions including foregut, midgut and hindgut. The midgut is not lined with cuticle and the epithelial cells are in direct contact with the lumen. This makes the midgut more efficient in ion and water uptake (Icely and Nott, 1992). For example, in *Callinectes sapidus*, the rate of  $\text{Na}^+$  uptake is higher in the midgut than in the hindgut (Chu, 1986).

The antennal glands consist of the coelomosac, labyrinth, tubule and bladder, and their main function is urine production. By filtering the hemolymph, urine is produced and flows to the branchial chamber via the nephropore. Since some crustaceans can produce hypoosmotic urine (relative to the hemolymph), the antennal glands are also considered to participate in osmoregulation. (Mantel and Farmer, 1983; Morris, 2001).

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Na,K-ATPase provides the primary driving force for ion transport. The activity of Na,K-ATPase reflects the osmoregulation of crustaceans (reviewed by Lucu and Towle, 2003). Because of its role in regulation of osmotic pressure of the hemolymph, Na,K-ATPase activity is higher in the posterior gills than in the anterior gills of *Carcinus maenas* (Siebers et al., 1982; Henry et al., 2002), *C. sapidus* (Neufeld et al., 1980), *Uca vocans*, and *Uca lactea* (Lin et al., 2002). A discrepancy in activity of the enzyme among the anterior, the posterior gills and other osmoregulatory organs would suggest the relative importance of each organ in osmoregulation.

Previous studies focused primarily on either the gills or antennal glands alone (Piller et al., 1995; Lucu and Flik, 1999). Rarely have these osmoregulatory organs been investigated simultaneously. Although some studies examined several organs at the same time (Towle, 1981; Henry and Cameron, 1982; Siebers et al., 1982), these studies were not aimed at elucidating the relationship among the osmoregulatory organs in marine crabs. Towle (1981) investigated the enzyme activity of transport-related ATPases among the organs of *Birgus latro* and found the gills and the antennal glands had high Na,K-ATPase activity. He suggested that they might play a crucial role in the osmoregulation of terrestrial crabs, with the role of the gut varying between species. However, the inferences from that study were limited to terrestrial crabs. Whether they also apply to the euryhaline marine crabs need further investigation. Although Siebers et al. (1982) conducted an experiment on Na, K-ATPase activity in various organs of the euryhaline marine crab *C. maenas*, none of the organs examined (except the gills) were related to osmoregulation. An integrated study on how a euryhaline marine crab resolves osmoregulation upon salinity challenges is conducted in the present study.

The  $\alpha$ -subunit of Na,K-ATPase is a multi-transmembrane protein with a molecular weight of about 110 kDa. The catalytic site of the enzyme located on the  $\alpha$ -subunit is responsible for the transport property. The regulation of the enzyme can be categorized into short- and long-term regulation. Short-term regulation is accomplished via a change of enzyme kinetic behavior or translocation of the protein between the cell membrane and intracellular stores. Long-term regulation involves de novo synthesis of new enzymes or the degradation of pre-existing enzymes (Therien and Blostein, 2000). When *C. maenas* were transferred from seawater (SW) to diluted SW, they regulated their hemolymph osmolality by increasing the activity of pre-existing Na,K-ATPase in the short-run, and by synthesizing new enzymes for long-term regulation (Lucu and Flik, 1999).

Castilho et al. (2001) found that the anterior and posterior gills of *Chasmagnathus granulata* showed a different affinity to  $\text{Na}^+$ , suggesting different isoforms. A similar situation was found in *Uca minax* in that the anterior and posterior gills differed in their affinity to both  $\text{K}^+$  and ATP (Wanson et al., 1984). However, no difference in enzyme affinity to  $\text{Na}^+$  was found in the gills of *C. maenas* (Harris and Bayliss, 1988). There is no solid evidence for the presence of isoforms in crab gills. In the complete sequence analysis of the Na,K-ATPase  $\alpha$ -subunit conducted, no isoforms were found in the gills of *C.*

*sapidus* (Towle et al., 2001). In this study, we also cloned the complete sequence of the Na,K-ATPase  $\alpha$ -subunit of *Scylla paramamosain* to identify possible isoforms.

The mud crab *S. paramamosain* is distributed along the continental coast of the South China Sea and is an important species in aquaculture. The primary purpose of this study is to reveal the relative roles of the osmoregulatory organs via both mRNA and protein expression of the  $\alpha$ -subunit in Na,K-ATPase. The enzyme specific activity in Na,K-ATPase was also examined. This information was compared with two other marine species, *C. maenas* and *C. sapidus*.

## 2. Materials and methods

### 2.1. Animal maintenance and salinity transfer

Adult female mud crab *S. paramamosain* (Keenan et al., 1998) in stage C or early D of the intermoult cycle (Freeman et al., 1987) was purchased from Wu-chi fishing port, Taichung, Taiwan. The weights were  $150 \pm 25$  g. Except for those sacrificed immediately for gene cloning, all individuals were acclimated in aquaria with 25 ppt artificial seawater (Coralife) at  $28 \pm 1$  °C under a 14 h/10 h light/dark cycle for 1 week. They were fed a diet of fish (*Carassius auratus*) every 2 days until 3 days before sampling or the experiment. Crabs were then transferred to plastic containers with salinities of 5, 25 or 45 ppt and later sacrificed at 6 h, 12 h, 1 day, 3 days, 7 days, 14 days, and 28 days, respectively. Upon collection for sacrifice, a hemolymph sample of each individual was taken by puncturing the arthrodistal membrane at the base of the swimming pereopods with a 27-gauge needle. Its osmolality was measured with a vapor pressure osmometer (Wescor Model 5500) in 10  $\mu\text{l}$  aliquots. Prior to sacrifice, the crabs were anesthetized on ice for 5 to 10 min and were subsequently killed by rapid removal of the heart. The osmoregulatory organs in this study were gill 2 (the anterior gill), gill 6 (the posterior gill), the antennal glands, and the midgut. Any residue remaining in the tract was flushed with homogenizing buffer (25 mM Tris-HCl, 0.25 mM sucrose, 20 mM EDTA, 0.4% Na deoxycholate, 0.1 mM PMSF, pH 7.4) before use. The tissues subjected to RNA and cDNA preparation were immediately frozen with liquid nitrogen after sampling. Tissues subjected to protein preparation were immersed in homogenizing buffer on ice.

### 2.2. cDNA sequence cloning

Total RNA was obtained from individuals acclimated in 25 ppt following the Trizol reagent protocol (Life Technologies) under RNAase-free conditions and was stored at  $-20$  °C before use. The full-length cDNA was obtained and cloned with a commercial kit (SMART RACE cDNA Amplification Kit, Clontech).

### 2.3. Quantitative-PCR

The cDNA for quantitative PCR (Q-PCR) was reverse-transcribed from total RNA with oligo (dT)<sub>18</sub> primer and reverse

transcriptase III (Invitrogen). The Q-PCR reaction was performed with the SYBR Green master mix (ABI), and followed by the ABI PRISM 7000 sequence detection system (ABI). The primer pairs designed from the Na,K-ATPase  $\alpha$ -subunit and  $\beta$ -actin partial sequence were: NKA (104 bp fragment) forward: 5'-GACCTACCACGACCGCAAGATA-3', reverse: 5'-GGCGGGTCTTACAAATGATCAA-3' and ACT (103 bp fragment) forward: 5'-GAGAAGATCTGGCACCA-CACTTT-3', reverse: 5'-CGCGGTTGGCCTTGG-3'.

#### 2.4. Protein preparation

Unless otherwise stated, all the procedures were performed either on ice or at 4 °C. The organs were minced and homogenized in homogenizing buffer with 200: 1 protease inhibitor cocktail (3.31 mM antipain, 2.16 mM leupeptin, 63.86 mM benzamide, 100% aprotinin).

For protein preparations, the homogenates of the sampled organs were centrifuged at 1800 $\times g$  for 10 min in the first step to remove debris, chitinous material and cell nuclei. Parts of the supernatants were further centrifuged at 19,000 $\times g$  for 20 min and its supernatants were removed and tested for Na,K-ATPase activity assay. And other parts of supernatants from the first centrifuge were further centrifuged at 30,000 $\times g$  for 20 min. The pellets were resuspended in homogenizing buffer with protease inhibitor cocktail and stored at -70 °C prior to western blotting assay. Both centrifugates contained plasma membrane where the Na,K-ATPase located. For Western blot assay, this high speed centrifugation will give a better result with little background and non-specific binding of antibody. Protein concentration was determined spectrophotometrically at 595 nm with the reagent of Bio-Rad Protein Assay using bovine serum albumin as a standard.

#### 2.5. Na,K-ATPase activity assay

The method of Na,K-ATPase activity assay and subsequent calculation were slightly modified from Holliday (1985) and Lin et al. (2002). A pilot experiment for the optimal ionic conditions specifically for *S. paramamosain* was conducted prior to the study. In brief, an aliquot of the 10  $\mu$ l thawed protein extract was mixed with 400  $\mu$ l of inhibited reaction buffer (20 mM imidazole-HCl, 130 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM ouabain, pH 7.2) or non-inhibited reaction buffer (20 mM imidazole-HCl, 100 mM NaCl, 30 mM KCl, 10 mM MgCl<sub>2</sub>, pH 7.2). The reaction was initiated by adding 100  $\mu$ l Na<sub>2</sub>ATP to a final concentration of 1 mM and 30% trichloroacetic acid and centrifuged at 1800 $\times g$  for 10 min. The supernatant subsequently reacted with colorimetry solution (176 mM FeSO<sub>4</sub>, 560 mM H<sub>2</sub>SO<sub>4</sub>, 8.1 mM ammonium molybdate) and the liberated phosphate concentration was determined spectrophotometrically at 700 nm. Protein concentration was determined as described in Section 2.4. Enzyme specific activity (ESA) of Na,K-ATPase was calculated as the difference between phosphate (Pi) liberated by each homogenate in the two media and expressed as  $\mu$ mol PO<sub>4</sub><sup>2-</sup> per mg homogenate protein per hour.

#### 2.6. Western blotting

An aliquot of 35  $\mu$ g of proteins were fractionated by electrophoresis on 10% SDS-polyacrylamide gel and electroblotted onto PVDF membrane. The proteins were probed with anti-actin monoclonal antibody (Chemicon) overnight at 4 °C. Goat anti-mouse IgG conjugate peroxidase (Zymed) was used as a secondary antibody to visualize the epitope of actin. After acquiring the image, the membrane was incubated in stripping buffer (100 mM  $\beta$ -mercaptoethanol, 62.5 mM Tris-HCl, 2% SDS, pH 6.8) at 50 °C for 30 min. After washing, the membrane was subsequently reacted with Na,K-ATPase  $\alpha$ -subunit monoclonal antibody ( $\alpha$ 5, Developmental Studies Hybridoma Bank) and secondary antibody (goat anti-mouse IgG conjugate horseradish peroxidase, Zymed). The images obtained were

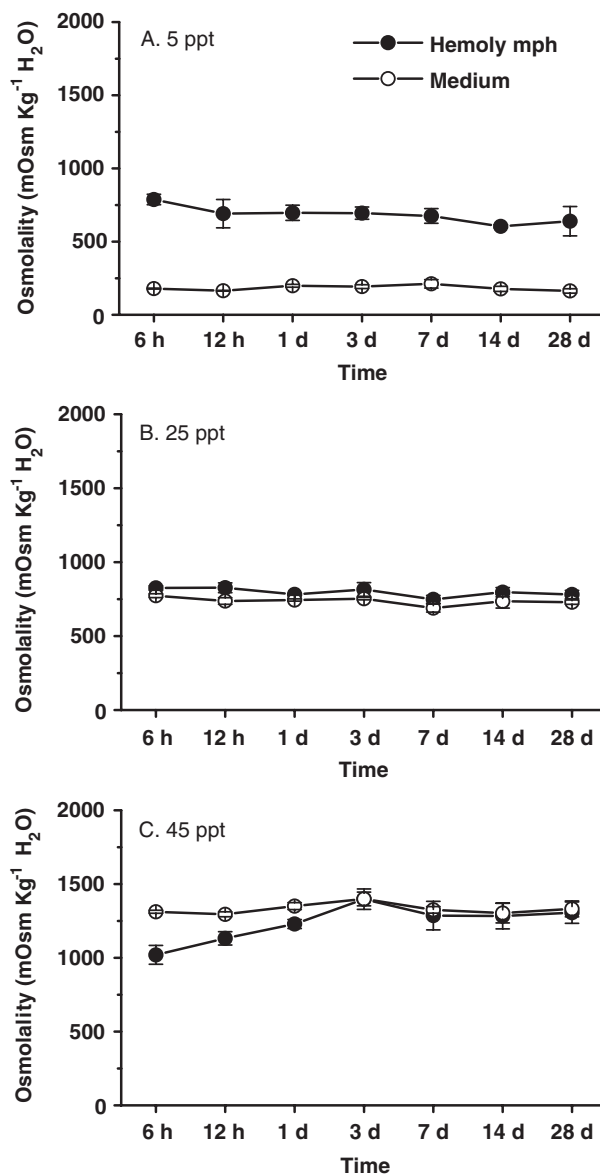


Fig. 1. Changes over time in hemolymph (filled circles) and acclimating medium (open circles) osmolalities for crabs transferred from 25 ppt to (A) 5 ppt, (B) 25 ppt, and (C) 45 ppt salinity. Values are mean  $\pm$  S.D.

analyzed with the software Image-Pro Plus 3.0. The protein expression was indicated as the relative intensity of Na,K-ATPase to beta-actin.

### 2.7. Statistical analysis

All the results from this study were expressed as mean values  $\pm$  S.D. For hemolymph osmolality analyses, significant differences among transfer time were determined by one-way ANOVA, and subsequent Tukey's pairwise comparison (Minitab 11). Significant differences between hemolymph and medium osmolalities were determined by paired *t*-test.

There were three parameters for representing the Na,K-ATPase expression, including enzyme specific activity,  $\alpha$ -subunit expression level (relative intensity) and mRNA level. When each of the parameters was measured from the four proposed osmoregulatory organs within the same individual, they were not independent (Tabachnick and Fidell, 2001). Therefore, a repeated-measures ANOVA was performed to determine significant differences among salinity treatments and among the organs. The grouping variables were salinities, including 5, 25 and 45 ppt. Within each organ, significant differences among medium salinities were analyzed with one-way ANOVA followed by Tukey's pairwise comparison. Significance was accepted as  $p < 0.05$ .

### 3. Results

The osmotic pressure of the hemolymph acclimated to 5, 25 and 45 ppt salinity is shown in Fig. 1. Six hours after transference to 25 ppt salinity, the osmotic pressure was  $825.5 \pm 15.2$  mosM  $\text{kg}^{-1}$   $\text{H}_2\text{O}$  ( $n=6$ ), higher than the medium (paired *t*-test,  $t=5.30$ ,  $p=0.003$ ,  $df=5$ ). Compared to the crabs in 25 ppt at 6 h, those placed in 5 ppt salinity decreased their hemolymph osmolality to  $788.0 \pm 35.5$  mosM  $\text{kg}^{-1}$   $\text{H}_2\text{O}$  ( $n=6$ ), and the value remained steady thereafter. On the other hand, the osmotic pressure of the hemolymph in crabs transferred to 45 ppt salinity increased to  $1019.8 \pm 64.0$  mosM  $\text{kg}^{-1}$   $\text{H}_2\text{O}$  ( $n=5$ ) at 6 h. Three days after transfer, the osmotic pressure was  $1397.3 \pm 69.1$  mosM  $\text{kg}^{-1}$   $\text{H}_2\text{O}$  ( $n=4$ ) and was not different from the medium ( $1399.0 \pm 46.6$  mosM  $\text{kg}^{-1}$   $\text{H}_2\text{O}$ , paired *t*-test,  $t=0.096$ ,  $p=0.930$ ,  $df=3$ ).

Since the osmotic pressure of the hemolymph of most crabs reached a stable state after 3 days (one-way ANOVA,  $F_{3, 20}=1.89$ ,  $p=0.164$ ), readings collected on and after day 3 were pooled to increase sample size for the following analysis. The osmotic pressure in 5 and 25 ppt salinity was significantly higher than the respective media while that of crabs in 45 ppt salinity was significantly lower (paired *t*-test, 5 ppt:  $t=32.59$ ,  $p=0.000$ ,  $df=19$ ; 25 ppt:  $t=8.47$ ,  $p=0.000$ ,  $df=24$ ; 45 ppt:  $t=2.16$ ,  $p=0.041$ ,  $df=23$ ). For the 45 ppt treatment, however, if the hemolymph osmolalities and their media are compared separately at each time point, no significant difference was found between them (paired *t*-test, 3 days:  $t=0.096$ ,  $df=3$ ; 7 days:  $t=0.997$ ,  $df=5$ ; 14 days:  $t=1.97$ ,  $df=6$ ; 28 days:  $t=1.93$ ,  $df=6$ ; all  $p > 0.05$ ).

Amplification and sequencing of Na,K-ATPase cDNA from *S. paramamosain* gills produced a full-length cDNA containing 3866 base pairs that encoded a complete open reading frame of 1039 amino acid (Fig. 2). The predicted molecular weight was 115.58 kDa. According to a hydrophobic assay, the amino acid sequence deduced from the cDNA contains 8 transmembrane domains. Comparing the amino acid sequence to other species, it had a similarity of 92% to blue crab (*C. sapidus*, AF327439), 78% to *Artemia franciscana* (X56650), and 73% to human (AB062885) Na,K-ATPase.

The product of PCR amplification from degenerate primers contained 759 base pairs and was used to design the primers for Q-PCR. The primitive mRNA concentration ratios of the Na,K-ATPase  $\alpha$ -subunit and actin were normalized with the minimal value, and are shown as normalized expression (Table 1). When *S. paramamosain* were subjected to three different salinities for 7 day, the four osmoregulatory organs were collected and their mRNA quantified. Both salinity of the medium and organ showed a significant effect on the expression of mRNA (repeated-measures ANOVA, salinity:  $F_{2,12}=44.48$ ,  $p < 0.0001$ ; organ:  $F_{3,36}=34.73$ ,  $p < 0.0001$ ; salinity  $\times$  organ:  $F_{6,36}=13.80$ ,  $p < 0.0001$ ). One-way ANOVAs showed the significant influences of salinity on Na,K-ATPase  $\alpha$ -subunit mRNA expression levels in gill 2, gill 6 and the antennal gland, but not in the gut (one-way ANOVA, gill 2:  $F_{2,12}=226.17$ ,  $p < 0.001$ ; gill 6:  $F_{2,12}=10.70$ ,  $p=0.002$ ; antennal gland:  $F_{2,12}=10.70$ ,  $p=0.002$ ; gut:  $F_{2,12}=2.49$ ,  $p=0.125$ ). The expression values were then divided by those of the corresponding organs in crabs acclimated to 25 ppt for 7 days. Relative to crabs in 25 ppt, the mean value of mRNA expression level in gill 2 increased about 1.8 times in 5 ppt, and 15.8 times in 45 ppt. In gill 6, it increased 6.5 times in 5 ppt and 4.9 times in 45 ppt. In the antennal gland, it increased about 5.2 times when crabs were transferred to 45ppt salinity but did not increase in 5 ppt salinity (Tukey's pairwise comparison,  $p > 0.05$ ).

At the protein level, we investigated the relative abundance of Na,K-ATPase  $\alpha$ -subunit in the osmoregulatory organs in a period of 28 days. The samples from different exposure times were loaded onto different gels and membranes and were, thus, confounded to the respective gel/membrane condition as well as actin standards. We chose a univariate repeated-measures ANOVA and focused on effects of salinity and organs at each sampling time. There was no significant difference in the protein expression level of the Na,K-ATPase  $\alpha$ -subunit (Table 2) due to salinity level at any time after transfer (repeated-measures ANOVA,  $p > 0.05$ ), but a significant difference was found among organs ( $p < 0.05$ ). The relative protein expression levels of the Na,K-ATPase  $\alpha$ -subunit in the posterior gill and the antennal gland are higher than those in the anterior gill and the gut.

Specific enzyme activity of Na,K-ATPase (Fig. 3) decreased slightly in gill 6 when the crabs were transferred to 45 ppt salinity for 6 h. A greater fluctuation was observed among those acclimated to different salinities for 12 h. Crabs in 5 ppt salinity for 12 h showed a higher enzyme activity in gill 2, gill 6 and the antennal gland than those in 25 or 45 ppt salinity. The change in

1  
 CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACGCGGGGTCGTGAGCGCGCCTCGAGGAGAGCGGACGTGCGCCCTGCCAGCAGTGTGCGAC  
 CACAAACACTC 120  
 121  
 CACACGCTGAAGAATGTGCTTGTAGCGAAAACCGTCACCAGTCCAAGAGGGCAACCAGAGCGAGTGAACGAGTGTACAGAGTGAACGTAGCCTAGTGGCGTGTACATA  
 AGTGTGTACATA 240  
 241  
 AGAGTGATCGGTGGTGTGCTGGTGAAGCCAGCGTCTGGCCGTTGTTTCGTGGATACCGTGTATCCTCTGCTGCCGTCTTCTGCTGAACAGGGCCGCCGACGCCCGC  
 CTACTCCAGCAA 360  
 361  
 CCATGGCTGACACGGGAAGAACAGATTTCGTACCGCCACGCTACGGATCGTAACGTCCCTGATGATAACCGAACTGTGAAGGGAGACCCCAAGTCAAAAAAGAAGAATG  
 TCAAGGCAAGA 480  
 1 M A D T G R T D S Y R H A T D R N V P D D N R T V K G D P K S K K K  
 N V K G K R 40  
 481  
 GGAAAGGGGAGAAGGAGAAGGATATGGACAACCTGAAACAGGAATTGGAACCTCGATGAACACAAGGTCCCATAGAGAAGTCTTTCAGCGCCTCTCTGTCAATCCTG  
 ACACAGGTTTGA 600  
 41 K G E K E K D M D N L K Q E L E L D E H K V P I E E L F Q R L S V N  
 P D T G L T 80  
 601  
 CACAGGCTGAGGCTCGCCGAGTTAGAAAAGGGACGGGCCCAACGCCCTCACCCCTCCCAAGCAAACCTCCAGAATGGGTCAAATTTTGAAGAACTTGTGGTGGAT  
 TCTCACTGCTGT 720  
 81 Q A E A R R R L E R D G P N A L T P P K Q T P E W V K F C K N L F G  
 G F S L L L 120  
 721  
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 TAATCATCACAG 840  
 121 W I G A I L C F I A Y S I E A A S E E E P N N D N L Y L G I V L T A  
V V I I T G 160  
 841  
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 TGCAGGCTGAGG 960  
 161 I F S Y Y Q E S K S S R I M E S F K N L V P Q Y A I V I R E G E K L  
 N V Q A E E 200  
 961  
 AACTATGCATAGGTGACATCATTGATGTTAAGTTTGGTGTATCGTATCCCTGCTGACATGCGAGTCAATGAAGCTCGAGGTTTAAAGTTCGACAACCTTCACTCACTG  
 GTGAGTCTGAGC 1080  
 201 L C I G D I I D V K F G D R I P A D M R V I E A R G F K V D N S S L  
 T G E S E P 240  
 1081  
 CACAGAGTCTGCTCTCCTGAATTACATCTGAGAATCCTCTTGAGACCAAGAACCTTGCCCTTCTTTCTACCAATGCTGTTGAAGGCACTTGCAAGGGTATTGTAATCA  
 ATATTGGTGACA 1200  
 215 Q S R S P E F T S E N P L E T K N L A F F S T N A V E G T C K G I V  
 I N I G D N 281  
 1201  
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 TCTTCTGGGTG 1320  
 282 T V M G R I A G L A S G L E T G E T P I A K E I S H F I H I I T G V  
A V F L G V 320  
 1321  
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 TCACTGTGTGTC 1440  
 321 T F F I I A F I L G Y H W L D A V V F L I G I I V A N V P E G L L A  
 T V T V C L 360  
 1441  
 TCACCCTCACTGCCAAGCGTATGGCTGCCAAGAAGTCTGGTCAAGAACCTGGAAGCTGTGGAGACCTGGGTTC AACCTTACCATCTGCTCTGACAAGACTGGCA  
 CCCTCACTCAGA 1560  
 361 T L T A K R M A A K N C L V K N L E A V E T L G S T S T I C S D K T  
 G T L T Q N 400  
 1561  
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 CTCTCTCCAGGA 1680  
 401 R M T V A H M W F D N T I I E A D T S E D Q S G C Q Y D K T S D G W  
 K A L S R I 440  
 1681  
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 TGAACCTGCTG 1800  
 441 A A L C N R A E F K T G Q E D V P I L K R E V N G D A S E A A L L K  
 C V E L A V 480  
 1801  
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 ACCTCCGCTACC 1920  
 481 G D V R G W R S R N K K V C E I P F N S T N K Y Q V S I H E T Q D K  
 N D L R Y L 520  
 1921  
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 ACGCTTACTTGG 2040  
 521 L V M K G A P E R I L E R C S T I F M N G E E K A L D E E M K E A F  
 N N A Y L E 560  
 2041  
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 TGCATGGTCTTC 2160  
 561 L G G L G E R V L G F C D Y V L P S D K Y P L G Y P F D A D A V N F  
 P V H G L R 600

2161  
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 ATCCCATCACTG 2280  
 601 F V G L M S M I D P P R A A V P D A V A K C R S A G I K V I M V T G  
 D H P I T A 640  
 2281  
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 CCAAGGCTGCTG 2400  
 641 K A I A K S V G I I S E G N E T V E D I A Q R L N I P I K E V D P R  
 E A K A A V 680  
 2401  
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 TCATGTGGAGG 2520  
 681 V H G S E L R D M T S E Q L D D V L I H H T E I V F A R T S P Q Q K  
 L I I V E G 720  
 2521  
 GCTGCCAGCAATGGGAGCCATTGTAGCTGTAAGTGGAGATGGTGTGAATGACTCCCTGCTCTCAAGAAGGCCGATATGGTGTGGCCATGGGTATCGCTGGATCCG  
 ATGTGCTAAGC 2640  
 721 C Q R M G A I V A V T G D G V N D S P A L K K A D I G V A M G I A G  
 S D V S K Q 760  
 2641  
 AGGCTGCCGACATGATTCTGTGGATGACAACCTTGTCTTATTGTCACTGGTGTGGAAGAGGGCAGGCTATTTTTGATAACCTGAAGAAATCCATTGCCTACACCC  
 TCACCTCCAACA 2760  
 761 A A D M I L L D D N F A S I V T G V E E G R L I F D N L K K S I A Y  
 T L T S N I 800  
 2761  
 TCCCTGAGATCTCCCCCTTCTGTGTTCTTCATGATTGCCTCCGTGCCTCTCCCTGCGGCACTGTTACCATCCTCTGCATGATCTGGTACTGACATGGTGCCTGCCA  
 TTTCCCTTGCC 2880  
 801 P E I S P F L F F M I A S V P L P L G T V T I L C I D L G T D M V P  
 A I S L A Y 840  
 2881 ATGAAGAAGCTGAGTCAGAT  
 ATTATGAAGCGCCAGCCCGCAATCCCTTACGGACAAGCTTGTGAACGAGAGGCTCATCTCCATGGCCTATGGTCAGATTGGCATGATCCAGGCTCTGG 3000  
 841 E E A E S D I M K R Q P R N P F T D K L V N E R L I S M A Y G O I G  
M I Q A L A 880  
 3001  
 CTGGATTCTTTGTGTAATTCGTCATCATGGCTGAGAATGGGTTCTGCCTCCCATTCTCTTTGGCATCCGTGAGCACTGGGACTCCAAGGCCATCAACGATCTGGAGG  
 ATTATTTAGGCC 3120  
 881 G F F V Y F V I M A E N G F L P P I L F G I R E H W D S K A I N D L  
 E D Y Y G Q 920  
 3121  
 AGGAATGGACCTACCACGACCGCAAGATACTTGTAGTACACCTGCCACACTGCGTTCCTTCGTGGCCATTGTGGTGGTGCAGTGGGCTGACTTGATCATTGTAGACCC  
 GCCGTAACCTCA 3240  
 921 E W T Y H D R K I L E Y T C H T A F F V A I V V V O W A D L I I C K  
 T R R N S I 960  
 3241  
 TCCTTACCAGGGCATGAAGAACATGGTGTCTTAACTTTGGGTTGTGTTTCGAGACCACACTGGCTGCCTTCTCTCTACACCCAGGCATGGACAAGGGTCTAAGGA  
 TGTATCCCCTCA 3360  
 961 L H Q G M K N M V L N F G L C F E T T L A A F L S Y T P G M D K G L  
 R M Y P L K 1000  
 3361  
 AATTCTACTGGTGGCTGCCAGCCCTCCCCTTCTCATTCTCATCTTCGTGTACGACGAGTGTGCCGGTTTCGTGCTGCGCAGGAACCTGGTGGCTGGGTTGAGATGG  
 AGACCTATTATT 3480  
 1001 F Y W W L P A L P F S F L I F V Y D E C R R F V L R R N P G G W V E  
 M E T Y Y \* 1040  
 3481  
 AAGGTTTGTAGTGAGCACAAGTTTACAAGAGCTCAAGAGcGTGGCCACCACCAGCAGCCTCCTCCCTCACAGCCAGCACTGTCATCACCATCAGTCTCCGTGATGTC  
 TGGGATTGGAA 3600  
 3601  
 CTGTTGTATTATAACCTTCAAAGAAATGCTATACTTTAATTAGAAACCAGAGAATATATAACAACCAAGTGTGGTGGCTCAGTAATGTGCTCTGTCAAATGATTC  
 ATTGCTTATTT 3720  
 3721  
 TATGTTACTTACTGGGATTTTAGTGTCTATTATGGTGTGTTCTAATTTCCCATGTTATATATAAGATTTGCAGTAAGTAACATTGAGATATTTGTACAGAATCCTG  
 GTGAAAGCATGT 3840  
 3841 ATTAAAAAAAAAAAAAAAAAAAAA  
 3866

Fig. 2. The cDNA and deduced amino acid sequence of Na,K-ATPase  $\alpha$ -subunit of *S. paramamosain*. The start codon is at site 363 from the 5'-end, and the stop codon is indicated by the asterisk. Eight predicted transmembrane domains are expressed as underlined amino acids.

enzyme activity among salinity treatments was the most obvious in gill 6. Gill 6 had a higher enzyme activity in 5 ppt salinity than in other salinities. This tendency started at 12 h and reached a stable state after 14 days. The enzyme activity was higher at 14 and 28 days than after transfer for 6 h (Tukey's pairwise comparison). However, in contrast to the 5 ppt salinity treatment that caused an increase in enzyme activity, 45 ppt salinity did not bring about such a clear increase or decrease in enzyme activity.

#### 4. Discussion

Although the osmotic pressure of the hemolymph is statistically lower than that of the medium at 45 ppt, it may not be biologically relevant. The osmotic pressure of the hemolymph changed immediately after transference from 25 ppt to 5 and 45 ppt, and reached a new steady state within 3 days, showing the typical pattern of weak hyperosmoregulators. Similar results have also been reported in *Scylla serrata*

Table 1  
Normalized expression of Na,K-ATPase  $\alpha$ -subunit mRNA levels, determined by Q-PCR, in *S. paramamosain* acclimated in three different salinities for 7 days

	Osmoregulatory organs			
	Gill 2	Gill 6	Antennal gland	Gut
5 ppt	14.57±10.39 <sup>a</sup>	151.10±49.12 <sup>a</sup>	12.73±4.65 <sup>a</sup>	3.29±1.73
25 ppt	8.09±2.81 <sup>a</sup>	23.13±11.33 <sup>b</sup>	10.46±3.89 <sup>a</sup>	2.22±0.65
45 ppt	127.63±12.39 <sup>b</sup>	112.90±49.43 <sup>a</sup>	54.26±33.13 <sup>b</sup>	7.52±6.42

The expression levels are indicated in mean±S.D. Different superscripted letters indicate significant differences among salinities based on Tukey's pairwise comparison ( $p < 0.05$ ).

(Davenport and Wong, 1987; Chen and Chia, 1997), and the shore crab *C. maenas* (Mantel and Farmer, 1983; Piller et al., 1995). All of these species act as a hyper-osmoregulator in diluted seawater and as an osmoconformer in seawater or concentrated seawater. The difference of osmolality between crustacean hemolymph and the medium is defined as osmoregulatory capacity (OC) (Charmantier, 1998). It is widely used as a biomarker to evaluate the degree of environmental stress on crustaceans, or as an indication of the presence of active osmoregulation in different developmental stages (Charmantier, 1998; Lignot et al., 2000). In *S. paramamosain*, a higher osmoregulatory capacity and a higher Na,K-ATPase activity were found in diluted SW. A similar relationship has been found in other crustaceans, including *C. maenas*, *C. sapidus*, *Callinectes similis*, *Hemigrapsus nudus* and *Homarus gammarus* (Siebers et al., 1982; Piller et al., 1995; Corotto and Holliday, 1996; Lucu and Devescovi, 1999).

It has been suggested that different isoforms may exist between the anterior and the posterior gills of *C. granulata* since different Na,K-ATPase affinity ( $K_m$ ) to  $\text{Na}^+$  was found between the gills (Castilho et al., 2001). However, gene cloning by Towle et al. (2001) produced no evidence for different Na,K-ATPase  $\alpha$ -subunit isoforms in *C. sapidus*. In the present study, no isoform was found among the four osmoregulatory organs of *S. paramamosain*. In our preliminary experiment (data not shown), the  $K_m$  values of Na,K-ATPase to  $\text{Na}^+$  and  $\text{K}^+$  were not significantly different between the anterior and posterior gills. Nevertheless, at least four  $\alpha$ -isoforms ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 4$ ) have been identified in mammalian cells (see review in Blanco and Mercer, 1998). Also, in *A. franciscana*, there are two isoforms of the Na,K-ATPase  $\alpha$ -subunit,  $\alpha 1$  and  $\alpha 2$  (Escalante et al., 1995). Based on phylogenetic analysis, the Na,K-ATPase  $\alpha$ -unit of *S. paramamosain* and *C. maenas* had a higher degree of similarity to  $\alpha 1$  than to  $\alpha 2$ . The function of  $\alpha 1$  in *A. franciscana* may be osmoregulatory-related, as suggested by its presence primarily in osmoregulatory organs, including the salt gland, antennal gland and midgut (Escalante et al., 1995). However, the isoforms are distributed differently among the organs in *Artemia salina* (Cortas et al., 1989). The functional differentiation of these isoforms in crustaceans deserves further investigation.

For crabs in 25 ppt salinity, the control group, the posterior gills (gill 6) and the gut had the highest and the lowest mRNA levels respectively among the organs studied. In diluted SW (5 ppt), a very significant mRNA level increase was found only in the posterior gills. A similar result can also be seen in *C.*

Table 2  
Univariate repeated-measures ANOVA of the effects of salinity on the relative protein levels of Na,K-ATPase  $\alpha$ -subunit in the osmoregulatory organs of *S. paramamosain*

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<b>6 h</b>				
Between subject effects				
Salinity (S)	2	54.136	1.54	0.2529
Error	12	35.044		
Within subject effects				
Organ (O)	3	217.280	6.54	0.0012
S*O	6	33.528	1.01	0.4347
Error	36	33.221		
<b>12 h</b>				
Between subject effects				
Salinity (S)	2	2.317	0.11	0.9000
Error	16	21.837		
Within subject effects				
Organ (O)	3	67.672	5.61	0.0022
S*O	6	12.349	1.02	0.4218
Error	48	12.068		
<b>1 day</b>				
Between subject effects				
Salinity (S)	2	1.398	1.96	0.1873
Error	11	0.714		
Within subject effects				
Organ (O)	3	2.317	12.47	<0.0001
S*O	6	0.171	0.92	0.4915
Error	33	0.186		
<b>3 days</b>				
Between subject effects				
Salinity (S)	2	1.865	2.83	0.0983
Error	12	0.659		
Within subject effects				
Organ (O)	3	4.334	6.32	0.0015
S*O	6	0.588	0.86	0.5345
Error	36	0.686		
<b>7 days</b>				
Between subject effects				
Salinity (S)	2	3.704	0.71	0.5106
Error	14	5.251		
Within subject effects				
Organ (O)	3	33.786	8.16	0.0002
S*O	6	3.844	0.93	0.4846
Error	42	4.140		
<b>14 days</b>				
Between subject effects				
Salinity (S)	2	9.828	1.91	0.1831
Error	15	5.159		
Within subject effects				
Organ (O)	3	17.559	7.70	0.0003
S*O	6	2.383	1.05	0.4093
Error	45	2.280		
<b>28 days</b>				
Between subject effects				
Salinity (S)	2	0.447	0.34	0.7151
Error	14	1.301		
Within subject effects				
Organ (O)	3	5.939	7.74	0.0003
S*O	6	0.475	0.62	0.7146
Error	42	0.768		

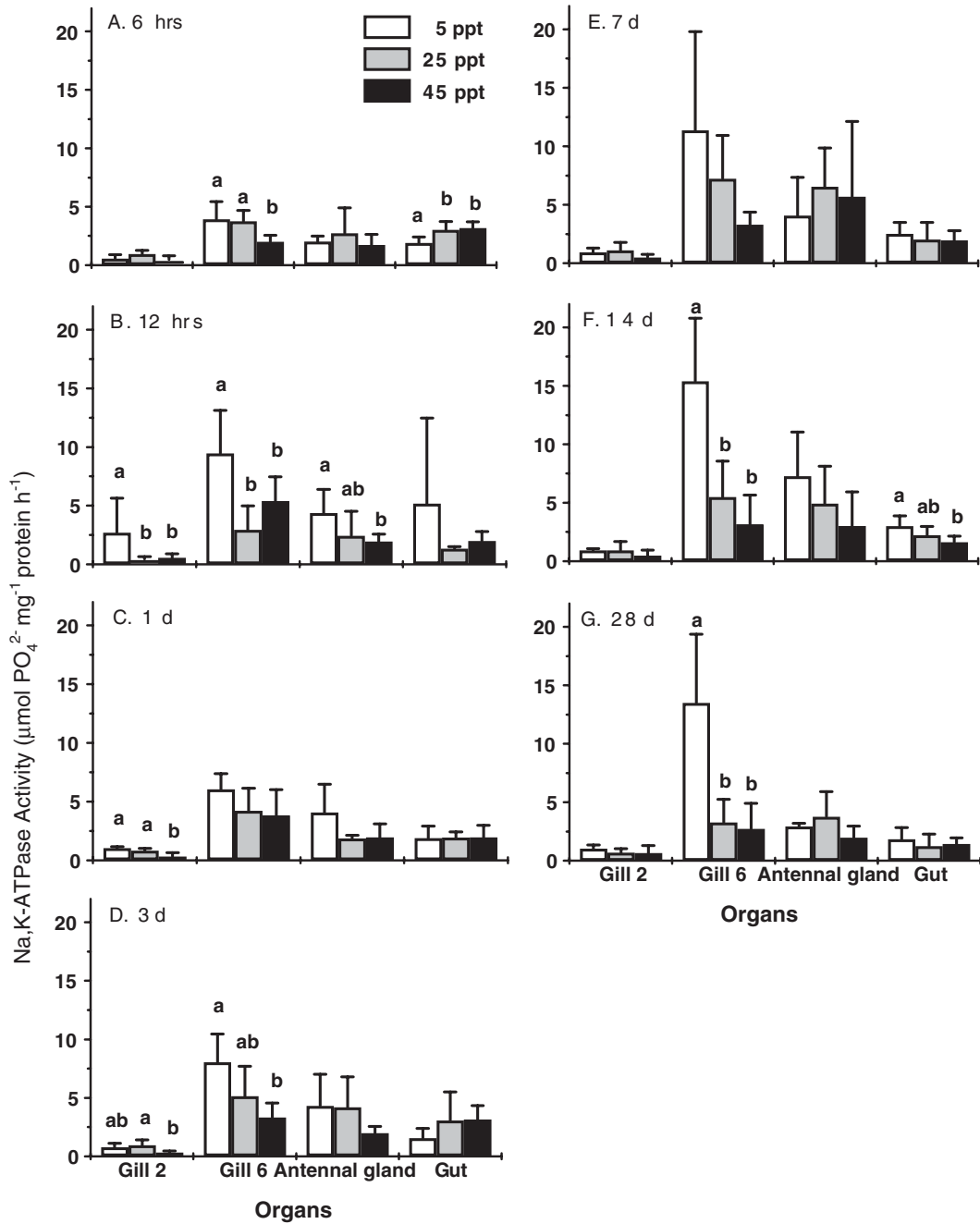


Fig. 3. Na,K-ATPase activity in osmoregulatory organs of *S. paramamosain* after the crabs were transferred to each salinity for (A) 6 h, (B) 12 h, (C) 1 day, (D) 3 days, (E) 7 days, (F) 14 days and (G) 28 days. The letters on the bars indicate significant differences among salinities of the organs investigated (Tukey's pairwise comparison,  $p < 0.05$ ).

*sapidus* by quantitative RT-PCR technique (Towle et al., 2001) in which the Na,K-ATPase  $\alpha$ -subunit mRNA was higher in the posterior than in the anterior gills. But there was no difference between the 5 and 35 ppt treatment and the authors suggested that it was probably due to the limitation of the technique. In the present study, the Q-PCR technique we used successfully revealed relative differences in expression of mRNA both among the organs and among salinities that was not possible using RT-PCR.

The antibody  $\alpha 5$  has a strong specificity for the Na,K-ATPase  $\alpha$ -subunit. It has been demonstrated to successfully

detect the crustacean  $\alpha$ -subunit in *C. maenas* (Lucu and Flik, 1999), *C. sapidus* (Towle et al., 2001) and *Macrobrachium olfersii* (Furriel et al., 2000). It also shows a strong specificity for the  $\alpha$ -subunit of *S. paramamosain* with a molecular weight of about 110 kDa. Not only the relative protein expression level of  $\alpha$ -subunit, but also Na,K-ATPase had a higher activity in the posterior gill and the antennal gland. However, because of inter-individual variation, neither salinity nor acclimation time showed a significant effect on the respective protein levels.

When crabs were transferred to a different salinity medium, their hemolymph osmolalities reached a new steady level after

3 days (45 ppt salinity). Na,K-ATPase activity in the posterior gill kept increasing after transfer to 5 ppt salinity and reached a significantly different level after 14 days. But protein level did not show such a significant difference among salinities as enzyme activity did. After 7 days the mRNA level of the posterior gill was already higher in 5 ppt salinity than in 25 ppt salinity. This suggested that the increase of mRNA may lead to an increase in Na,K-ATPase activity in diluted seawater after 14 days as a result of mRNA translation. In a study of *C. maenas* (Lucu and Flik, 1999), the authors suggested that Na,K-ATPase activity in crabs may be regulated by the pre-existing enzyme or the recruitment of silent enzyme in the short run (4 h), and by producing the new enzyme and/or by reducing the degradation rate in the long-term acclimation (3 weeks). In *C. sapidus*, only the enzyme activity, but not the mRNA or the protein level, showed a significant difference between salinity treatments (Piller et al., 1995; Towle et al., 2001). It is suggested that the activity might be regulated by a post-translational process. In *S. paramamosain*, the Na,K-ATPase activity in the posterior gill increased with time in diluted SW, but it did not increase as rapidly as osmotic pressure did. The discrepancy among these studies may be due to interspecific variation and the methods used for determining Na,K-ATPase activity.

On the other hand, the increase of mRNA did not lead to an increase of enzyme activity in concentrated SW. Crabs acclimated in 45 ppt salinity for 7 days increased the expression of mRNA in the anterior gill, the posterior gill and the antennal gland. But no similar increase was found in subsequent enzyme activity after 7 days. This discrepancy in mRNA and enzyme activity was reported recently in *Chasmagnathus granulatus*. When the hyper-hypo-regulating crab *C. granulatus* was acclimated in 45 ppt salinity, an increase in mRNA expression of Na,K-ATPase was found (Luquet et al., 2005) without a simultaneous increase in enzyme activity (Genovese et al., 2004). A higher degradation and recruitment rate of Na,K-ATPase may be responsible for this observation (Lucu and Flik, 1999; Therien and Blostein, 2000; Luquet et al., 2005). In concentrated SW, new Na,K-ATPase is synthesized continuously, but it may also degrade simultaneously. However, because Na,K-ATPase activity remained high after 7 days in *C. maenas* and 14 days in *S. paramamosain*, the role of Na,K-ATPase can not be excluded for long-term osmoregulation.

In other osmoregulatory organs, the guts and the antennal glands, the Na,K-ATPase activity did not change as drastically as in the posterior gills across salinities. Even though the gut changed Na,K-ATPase activity 6 h and 14 days after transfer to different salinities (Fig. 3), the degree and the tendency were not as clear as in the posterior gills. On the contrary, food and drinking water are the primary source of salt absorption for terrestrial crabs and the increase of Na,K-ATPase activity in the gut will enhance active salt uptake from freshwater. For example, Na,K-ATPase activity in the gut was higher when *B. latro* was provided with fresh drinking water than with seawater, but the differences between the treatments were not found in either gills or antennal gland even though their activities were much higher than the gut (Towle, 1981).

Except for highly terrestrial crustaceans, regulation in urine production rate might be one way that the antennal gland participates in osmoregulation. For *Penaeus monodon*, by regulating the urine production rate, its antennal glands managed to excrete isosmotic rather than hypoosmotic urine (Lin et al., 2000). A similar situation can also be observed in *C. maenas* and *Ucides cordatus* (Harris and Santos, 1993). By adjusting their urine production rates, *C. maenas* and *U. cordatus* spent less energy than by changing urine composition or reabsorption of ions from urine. Since the urine of these crustaceans are isosmotic or isoionic to their hemolymphs, the activities of osmoregulatory-related enzymes in the antennal gland, such as Na,K-ATPase and carbonic anhydrase, might remain constant no matter what the salinity is. Indeed, for *C. sapidus*, the carbonic anhydrase activity remained constant in the antennal gland although it fluctuated with salinity in the gills (Rathmayer and Siebers, 2001). This may explain why Na,K-ATPase activity in the antennal gland of *S. paramamosain* did not change with environmental salinity.

The relative weights of gill 2, gill 6, antennal glands and gut to the whole body weight are 0.07%, 0.52%, 0.14% and 0.01%, respectively. When higher weight is correlated to higher protein content or higher capacity to participate in osmoregulation, the relative importance of osmoregulation in these organs becomes apparent. When *S. paramamosain* was moved from 25 ppt to 5 ppt salinity for 14 days, the average Na,K-ATPase activity of gill 6, the antennal glands and gut increased by 9.9, 2.4 and 0.8  $\mu\text{mol Pi mg}^{-1}$  protein, respectively (no difference was found in gill 2). If we define the outcome value of the gut as 1x and multiply these activity values by their relative weights, the values of gill 6 and the antennal glands will be 455x and 29x, respectively. Based on this calculation of the relative importance of these organs, the ion transport of the posterior gills far exceeds that of other related organs.

Recently, there are some discussions on the possible contamination of other phosphatases and ATPases when ATP was used as the substrate for estimating Na,K-ATPase activities in gill homogenates (Furriel et al., 2001; Masui et al., 2003, 2005). Instead, these authors used a synthetic organic substrate, *p*-nitrophenylphosphate, to characterize gill Na,K-ATPase activity in the microsomal fraction. Although our method still serves as one the valid and popular methods, the concerns brought up by these researchers deserve further attentions in the future.

To sum up, the complete cDNA sequence of the Na,K-ATPase  $\alpha$  subunit was cloned and no isoform was found among the four osmoregulatory organs tested. This is the first time Q-PCR was used as a sensitive technology to successfully investigate the mRNA levels of these organs and their expression in different salinities. In diluted SW, the posterior gills still played the primary role in osmoregulation. The increase in Na,K-ATPase activity may result from increased gene transcription and/or mRNA translation. However, the crabs do not seem to undergo any active regulation in concentrated SW since they act almost as osmoconformers. These physiological regulations explain how *S. paramamosain*

can tolerate salinity fluctuations and live in a wide range of salinity in their habitats.

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