GILL Na^+, K^+-ATPase AS A FUNCTION OF SIZE AND SALINITY IN THE BLUE CRAB, *Callinectes sapidus* RATHBUN

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Approved by

Advisory Committee

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Chair

Accepted by

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Dean, Graduate School

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ABSTRACT

Some kinetic properties of gill Na^+,K^+-ATPase of the blue crab, *Callinectes sapidus*, and its relation to osmotic regulation in juvenile and adult crabs were analyzed. Results suggest the presence of some differences in transport mechanisms for juvenile and adult blue crabs to maintain hemolymph concentration in dilute media.

Adult and sub-adult crabs demonstrated fairly low levels of Na^+,K^+-ATPase activity in both anterior and posterior gills regardless of acclimation salinity. Juvenile blue crabs had much higher levels of Na^+,K^+-ATPase activity in both anterior and posterior gills relative to adults. Crabs acclimated to low salinity showed a significant increase in enzyme activity, both in anterior and posterior gills. The levels of enzyme activity in the anterior gills of juveniles showed a marked increase at low salinities. Enzyme activity in the posterior gills of juvenile crabs at 150 mOsm increased (35%) over sea water. However, there is a markedly higher specific activity increase (80%) in the anterior gills of smaller, juvenile crabs at this lower salinity. Sex had no effect on the enzyme activity relative to salinity.

Abrupt transfer of juvenile and adult crabs from 1000mOsm to 150mOsm resulted in a gradual change in the Na^+,K^+-ATPase activity that reached steady-state levels within 7-10 days after the transfer. The time course for the increase in activity was preceded by an increase in the expression of the Na^+,K^+-ATPase α-subunit mRNA.

The Na^+,K^+-ATPase of the posterior gills of both juvenile and sub-adult crabs showed an increased affinity for ATP at lower salinities, while that of the posterior gills of juvenile crabs had a higher affinity for Na^+ than that of sub-adults at low salinity.

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I would like to sincerely thank my committee members, Dr. Robert Roer, Dr. Thomas Shafer, and Dr. Stephen Kinsey for their invaluable guidance and suggestions, their help is crucial in the development of my research.

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Finally, I would like thanking my family for both the financial support and most importantly the moral support. Especially Dad and Mom for giving me the freedom to become my own person, while providing the love and guidance necessary for me to succeed at anything I choose. No matter where we go or what we encounter I know we will always be there for one other.

DEDICATION

This thesis is dedicated to my parents,

Yuanshan Li and Xisheng Wang,

who always wholeheartedly support me,

and

to my extraordinarily patient and understanding wife,

Bin Zhang.

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INTRODUCTION

As a group, crustaceans occupy a variety of habitats including the sea, brackish, fresh and saline water, and land. Their capacity for regulating the osmotic concentration of body fluids over such a salinity range is equally diversified. However, few species can match the capacity for hyperosmoregulation exhibited by *Callinectes sapidus* (Pequeux, 1995). From an academic, economic, and ecological standpoint, the blue crab is an important and interesting species. The blue crab is an estuarine-dependent species whose life history involves a complex cycle of planktonic, nektonic, and benthic stages which occur throughout the estuarine-nearshore marine environment in a variety of habitats along the Atlantic and Gulf coasts. The blue crab is one of the more abundant estuarine macro-invertebrates and supports valuable commercial and recreational fisheries in the above areas. Many researchers have used the blue crab as an experimental animal because of its ready availability, economic value, hardiness, and complex life cycle. The blue crab plays a crucial role in the estuarine food web, providing prey for many species and in turn a voracious predator on other species.

Blue crabs can actively transport salts into their body fluids, and keep the ion concentration and osmotic pressure of their blood higher than that of a more dilute external medium, and are called hyperosmoregulators. Because the osmotic pressure of their blood is higher than that of the medium, blood water activity is lower and water moves into the crab by osmosis. Most studies show that the crab deals with this osmotic water load by increasing its rate of urine production. However, crabs can not excrete urine that differs in

concentration from the blood because, unlike freshwater crustaceans and vertebrates, they cannot reabsorb salts from the urine. This, in turn, means that the crab loses a lot of salt in its urine when it is in dilute waters at the head of an estuary. Sodium loss via urine may be up to 12% of the total sodium loss (Charmantier, 1991). If the crab is to be able to keep its blood from becoming diluted, this lost salt must be replaced by increased inward salt pumping by the gills.

Hyperosmoregulating crabs use their gills to actively pump salts into their blood as necessary to osmoregulate. Their gills have patches of special epithelial cells known as "ionocytes" and the ionocytes are most densely located in the posterior gills; the anterior gills have few or no ionocytes. The ionocyte has structural features typical of animal ion transport epithelia: large numbers of basal in-foldings, many mitochondria to make the ATP which powers the sodium pump and "tight junctions" between cells at the apical surface. In crustacean gills, large numbers of apical in-foldings are present just under the exoskeleton (cuticle). These apical in-foldings increase in size when the crab is actively transporting ions into its blood against large concentration gradients.

Life Cycle

Like many estuarine and marine invertebrates, the blue crab has a complex life cycle. It undergoes migrations at several stages. For instance, juvenile blue crabs move from mesohaline to oligohaline areas of estuaries in the Cape Fear River. The observation that the female blue crabs migrate from fresh and brackish waters to sea water show that the zoeal stages require full strength seawater, as megalops survive to first crabs at low salinity. Juveniles represent an important bottleneck in the life history of blue crabs. A

significant proportion of juveniles rely on mesohaline to oligohaline areas of Cape Fear River to provide protection from predation until they reach a critical size (30-50mm carapace width). However the osmoregulatory demands associated with using lower salinity habitats may have physiological and energetic costs.

In this habitat, the blue crab confronts a highly variable environment that can include rapid changes in salinity. The ability of blue crab to cope with salinity changes is essential for the establishment of a population in this habitat. Estuarine crabs acclimated to 100% sea water undergo an acute response (usually within 24 hours) after transfer to dilute seawater. In the adult blue crab, one feature of this acute response is an increase in the activity of Na^+, K^+-ATPase in the gill lamellae, the primary site for osmoregulation (Towle, 1986). This enzyme is a key factor in the uptake of Na^+, which is critical for maintaining internal osmolality at a level that is higher than that of the surrounding medium (hyperosmoregulation). Animals that are maintained in dilute seawater for a longer period of time (8-18 days) undergo an acclimation response (Onken, 1998). The acclimation response involves an increase in the area of the osmoregulatory patches in proportion to the total gill lamellar surface area. The mechanisms that regulate either type of response to salinity changes have not yet been identified, especially for juvenile blue crabs.

Juvenile crabs have a larger surface-to-volume ratio than adults, and should have a far more difficult time maintaining the blood osmolarity as the salinity decreases. The relative metabolic rate of small organisms is higher than that of large organisms under similar conditions. The metabolic work associated with osmoregulation increases exponentially with the difference between blood and medium osmotic concentrations.

These considerations reflect a potentially huge metabolic cost of osmoregulation for juvenile crabs. No studies to date, however, have measured the extent of the osmoregulatory capabilities of postlarval blue crabs nor the metabolic costs of such osmoregulation.

Na^+, K^+-ATPase

The mechanism of salt uptake by the gills has been studied in a number of estuarine crustaceans, but the molecular components of the transport mechanisms are still a matter of active discussion. The unidirectional influx of Na^+ and Cl^- requires two steps: the translocation of ions from the water into the cytoplasm across the apical membrane of the gill epithelium cells, and the translocation of ions from the cytoplasm into the hemolymph across the basolateral membrane. Na^+ transport across the basolateral membrane is accomplished by the ouabain-sensitive Na^+, K^+-ATPase (Pequeux, 1995).

Na^+, K^+-ATPase, a membrane-bound enzyme first described by Skou, is generally considered to be a primary mediator of ion transport across cell membranes. The presence of Na^+, K^+-ATPase activity in the gills of estuarine and freshwater decapod crustaceans has been widely reported (Lucu, 1993; Piller, 1995; Rainbow, 2001; Towle, 1997). Many studies have pointed out a central role of this enzyme in active Na uptake. Like other shore crabs, the blue crab was dependent upon the Na^+, K^+-ATPase enzyme of the gills for active salt uptake to maintain a hyperosmotic hemolymph in the dilute estuarine waters (Towle, 1976). The posterior gills of adult *Callinectes* are the primary sites of ion uptake (Pequeux, 1995). The activity of the transport enzymes involved in this process is induced by acclimation of crabs to low salinities (< 25 ppt). Towle et al. (1997) reported the adaptive

changes in Na^+, K^+-ATPase activity in the gills of blue crabs. Furthermore, they suggested that the changes occurred at a rate which suggested modification of the catalytic rate rather than an alternation in the number of enzyme molecules in *C. sapidus*.

Studies of the biochemical basis of ion uptake have shown that Na^+, K^+-ATPase is present at high specific activities in gills and antennal gland of hyperosmotically regulating species. A larger enzyme activity in salt-transporting gills (posterior pairs) than in respiratory ones (anterior pairs), as well as changes of this activity as a function of the acclimation salinity, have also been reported. Copeland and Fitzjarrell (1968) found that in *Callinectes sapidus* the proportion of the total gill area given over to transporting epithelia was adaptively variable and, when transferred from normal to dilute sea water, these crabs doubled their areas of transporting epithelia in 1-3 weeks. This observation of the adaptive variations in permeability suggests that osmoregulatory ability should improve with time.

If a crab is acclimated (that is, kept for 2-3 weeks) in a dilute external medium in which it can hyperosmoregulate, the ionocyte patches in its gills increase in size. The activity of the enzyme which powers the cellular sodium pump in the crab's gills and other ion transport epithelia, the Na^+, K^+-ATPase, also increases in the gills of hyperosmoregulating crabs. Both short-term increases in ion pump activity and long-term increases in the number of cells and ion pumps in the ionocyte patches appear to occur when crabs are acclimated in dilute media.

Sodium and chloride are the major osmotic effectors in the hemolymph of *C. sapidus* and their concentrations are maintained out of electrochemical equilibrium, both in concentrated and diluted media, as do many other osmoregulating crabs. In blue crab, gills are presumed to be the sites of active transport of Na and Cl, in both concentrated and

diluted media. However, the mechanism of osmoregulation is not well understood, especially for juveniles.

Few data, other than those on survival, exist to shed light on the osmoregulatory capabilities in estuarine crustaceans. In blue crabs, there are data to suggest ontogenetic changes in osmoregulatory ability during the zoeal and megalops stages, but the data presented do not meet standards of statistical reliability. No other studies have investigated the induction of osmoregulatory capability in larval and postlarval blue crabs.

The present study includes the major kinetic characteristics of the Na^+, K^+-ATPase present in anterior and posterior gills of *C. sapidus*, data on Na^+, K^+-ATPase activities of juvenile crabs, relations between enzyme activity, carapace width and weight and temporal changes of enzyme activity, during acclimation to diluted sea water or sea water. The experimentation was undertaken to examine the control and function of the Na^+/K^+ pump likely responsible for osmoregulation in juveniles.

MATERIALS AND METHODS

Experimental Animals

Juvenile and subadult blue crabs, *Callinectes sapidus*, were collected by sweep net in euhaline to oligohaline regions of the Cape Fear River estuary. Subadult and adult crabs were collected by baited hoop nets; some adult crabs were purchased from a local commercial crabber.

All crabs were maintained in the laboratory in either full-strength sea water (1000 mOsm) or sea water diluted with distilled water to 150 mOsm. Crabs were fed biweekly on commercial hermit crab diet and the water was replaced following each feeding. Crabs were acclimated for at least one week to the desired salinity prior to use in any experimental protocol.

Na^+,K^+-ATPase Assays

Gill Na^+,K^+ - ATPase levels were measured using a modification of the microtiter plate-based assay of Mayer-Gostan and Lemaire (1991). Anterior and posterior gills were removed from 37 crabs acclimated to 1000 mOsm medium and from 38 crabs acclimated to 150 mOsm medium. Crabs ranged in weight from 0.17 to 171.58 g. Gills from larger crabs were treated individually; gills from smaller crabs were pooled into anterior and posterior groups. Gills were placed on ice in approximately 4 volumes of extraction medium (250 mM sucrose, 50 mM imidazole, 2 mM EDTA, 5 mM mercaptoethanol and 0.1% sodium deoxycholate, pH 7.4) and homogenized using a Teflon-glass homogenizer. The crude homogenate was centrifuged at 13,000 rpm for 10 min in a Fisher microfuge.

Supernatants were used for subsequent determinations. Protein concentrations of the supernatants were measured using the microtiter plate-based Bradford technique (BioRad).

For the ATPase assay, the following were pipetted into microtiter plate wells: 40µl distilled water, 50:l incubation medium (100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM Na_4EDTA, 500 mM Tris, pH 7.4) and 10µl of either anterior or posterior gill extract. The same extracts were also tested in the presence of ouabain by substituting 10µl of 10 mM ouabain for 10µl of distilled water. The reaction was begun by the addition of 10µl of 6 mM ATP. Extract blanks were run by substituting 10µl distilled water for ATP, and ATP blanks had water substituted for the extract. Standards were prepared by serial dilutions of 1 mM disodium phosphate to final concentrations of 1, 2, 3 and 5 nmol PO_4 per well along with a distilled water blank (0 nmol PO_4).

The reaction was terminated after 30 min. at 25°C by the addition of 150µl of color reagent prepared from stock solutions of ammonium molybdate (5.72% w/v in 6N HCl), polyvinyl alcohol (2.32% w/v), malachite green (0.0812%) and distilled water mixed in the proportions of 5:5:10:2.5 ml. Plates were mixed for 5 s and absorbance measured at 630 nm. The Na^+,K^+ - ATPase activity is the difference between the total ATPase activity and the ouabain-inhibited activity. Activity is expressed as nmol $PO_4 \cdot :g^{-1}$ protein $\cdot$ min^{-1}.

To compare the affinities of the gill Na^+,K^+ - ATPase for Na^+ and ATP between juvenile and adult crabs and between anterior and posterior gills, kinetic studies were performed. In separate experiments Na^+ and ATP concentrations were varied and Lineweaver-Burk plots were constructed and compared by linear regression analysis.

Acclimation Study

To assess the time-frame for acclimation of gill Na^+,K^+ - ATPase activity, 171 juvenile (<5g) crabs and 178 sub-adult (>5g) crabs, collected and maintained in sea water (1000 mOsm) in the laboratory for at least one week. A subset of each size class was sacrificed for gill ATPase assay, and the remainder were transferred to 150 mOsm dilute sea water. Crabs from both groups were sampled and sacrificed on days 1, 2, 3, 4, 5, 7, 10, 12 and 15 post-transfer for analysis of gill ATPase activity as described above.

Quantitative Na^+,K^+-ATPase mRNA Expression

Thirty juvenile crabs, ranging in body weight from 0.38 to 6.80 g (1.9 to 4.1 cm carapace width), were collected from a sea water habit and acclimated in the laboratory in full-strength sea water (1000 mOsm) for at least one week. On day 0, 10 crabs were removed for sampling and the remaining crabs were abruptly transferred to dilute sea water (150 mOsm). Crabs were sampled on days 2, 4, 6, 7 and 9 post-transfer. Gills were removed and frozen in liquid nitrogen. For mRNA extraction and quantification, gills were homogenized in TRIzol (Invitrogen) and the extracted RNA assessed and quantified by the ratio of absorbance at 260/280 nm, absorbance at 260 nm and agarose gel electrophoresis.

First-strand cDNA synthesis was accomplished using the Invitrogen Superscript II protocol modified by the inclusion of 5M betaine (Qiangen) in the reaction mixture and a reaction temperature of 48°C. Transcript abundance was analyzed with a Stratagene MX4000 real-time quantitative PCR system. Amplification was detected with the dye SYBR Green (Qiagen Quantitect), which fluoresces strongly upon binding to double-

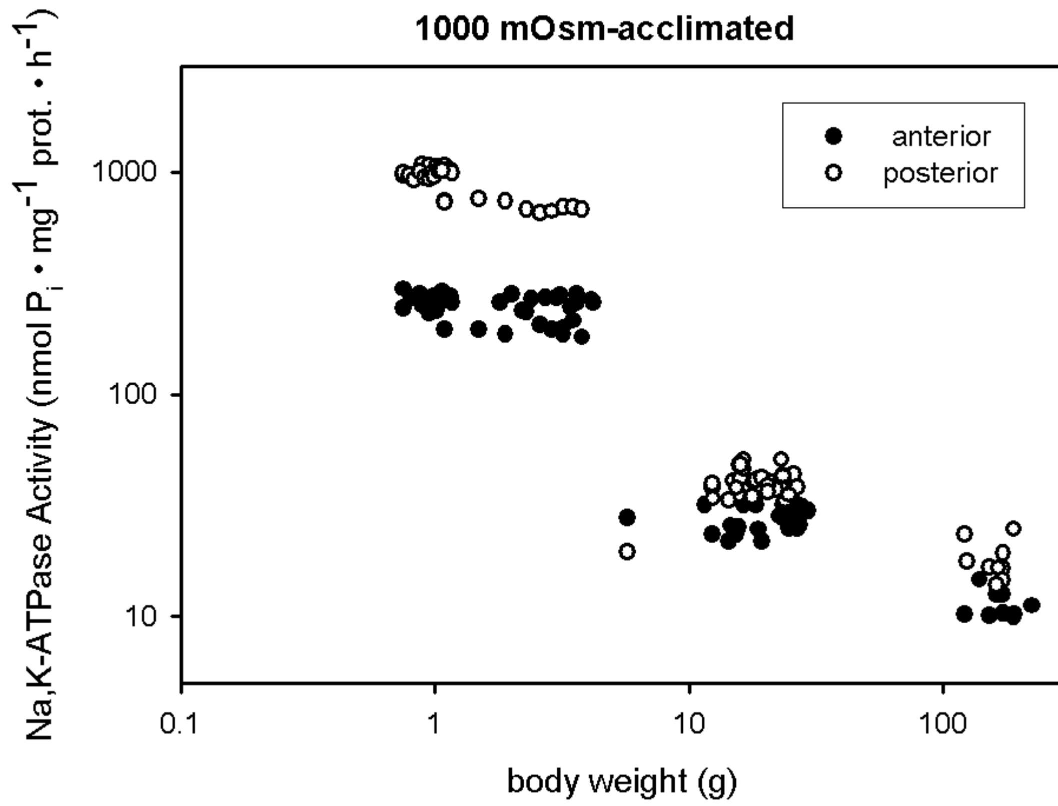
stranded DNA. Standardization was done empirically, using a dilution series of a reference template. The threshold cycle (Ct), the cycle number at which the fluorescent signal exceeds a minimum assigned value, is inversely proportional to the logarithm of transcript abundance, allowing normalization of experimental samples to the reference template. The instrument can detect less than two-fold differences in transcript abundance over five orders of magnitude.

RESULTS

The activity of gill Na^+,K^+ - ATPase activity showed an unusual, discontinuous distribution as a function of size. The sharp discontinuity in activity occurred at a body weight of approximately 5g (Fig. 1). Crabs with body weights < 5g had a substantially higher gill enzyme activity than larger crabs, both at 1000 and at 150 mOsm, and for both anterior and posterior gills. Anterior gills of small crabs had 10.6 times the Na^+,K^+ - ATPase activity compared to those of larger crabs in 1000 mOsm, and 9.5 times the activity at 150 mOsm. Posterior gills from small crabs at 1000 and 150 mOsm had 25.6 x and 15.6 x the activity, respectively, compared to the gills from larger crabs at those salinities (Figs. 1 & 2). All differences were highly significant ($P < 0.001$).

Crabs of all sizes showed significantly higher Na^+,K^+ - ATPase activity in posterior and anterior gills with acclimation to 150 mOsm medium compared to acclimation to 1000 mOsm (Figs. 1 & 2). Posterior gills from small crabs showed 35.6% more activity at 150 mOsm, while anterior gills had 79.5% more activity. For larger crabs, the activities in 150 mOsm acclimated crabs were 122.2% greater for the posterior gills and 100.3% greater for anterior gills. All differences in activity were highly significant ($P < 0.001$). While the largest crabs (> 100g) were included in the analysis of the latter group (> 5g), they had the lowest values for gill Na^+,K^+ - ATPase activity at 1000 mOsm. Moreover, there was no significant difference between the activities of posterior and anterior gills at this salinity. This group also had the largest difference in activity with acclimation to 150 mOsm (Fig. 1). Anterior gills demonstrated a 122.1% greater activity, while the activity of posterior gills was 331.7% higher ($P<0.001$ for both data sets).

Figure 1. Relationship between gill Na^{+},K^{+}-ATPase in posterior and anterior gills of *Callinectes sapidus* as a function of body weight. Crabs in the upper panel were acclimated to full-strength sea water, while those in the lower panel were acclimated in sea water diluted to 150 mOsm.


1000 mOsm-acclimated
anterior
posterior
Na,K-ATPase Activity (nmol P_i • mg^{-1} prot. • h^{-1})
1000
100
10
0.1
1
10
100
body weight (g)

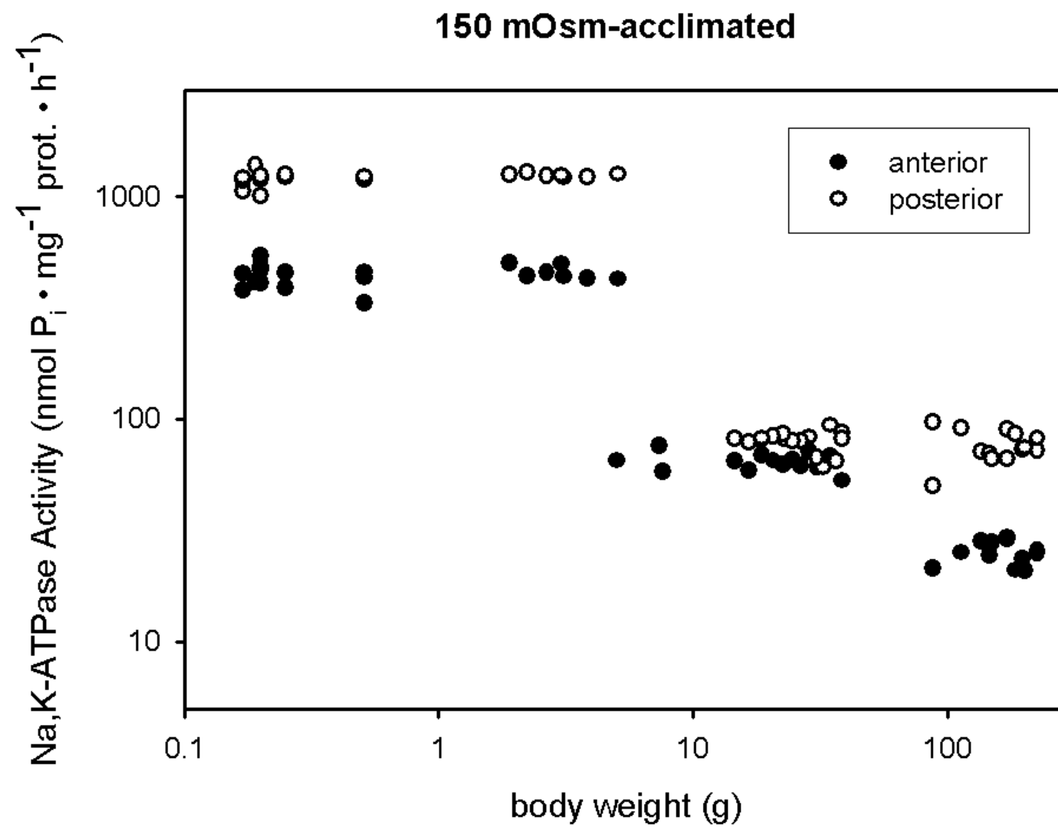
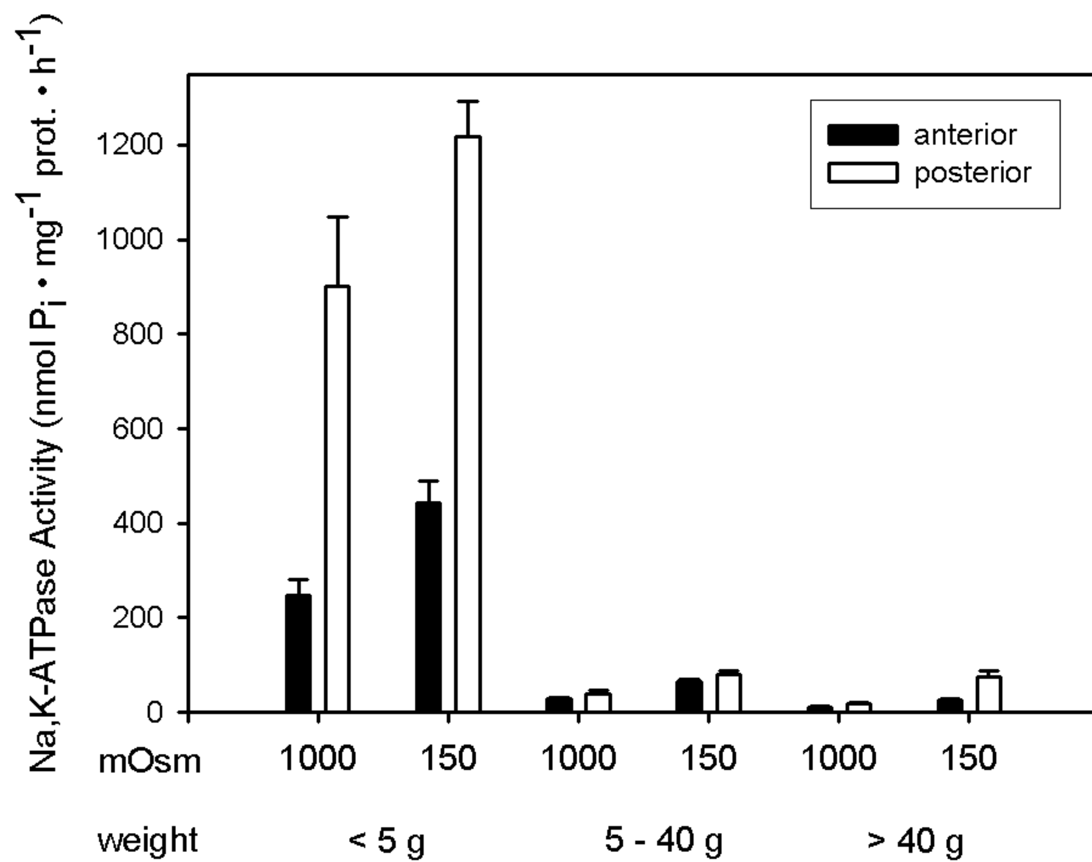

150 mOsm-acclimated
anterior
posterior
Na,K-ATPase Activity (nmol P_i • mg^{-1} prot. • h^{-1})
1000
100
10
0.1
1
10
100
body weight (g)

Figure 2. Mean (+ S.D.) values for *Callinectes sapidus* anterior and posterior gill Na^+,K^+-ATPase as a function of size and salinity, with animals grouped as <5g, 5-40g and >40g to reflect the break in the distribution if Fig. 1.

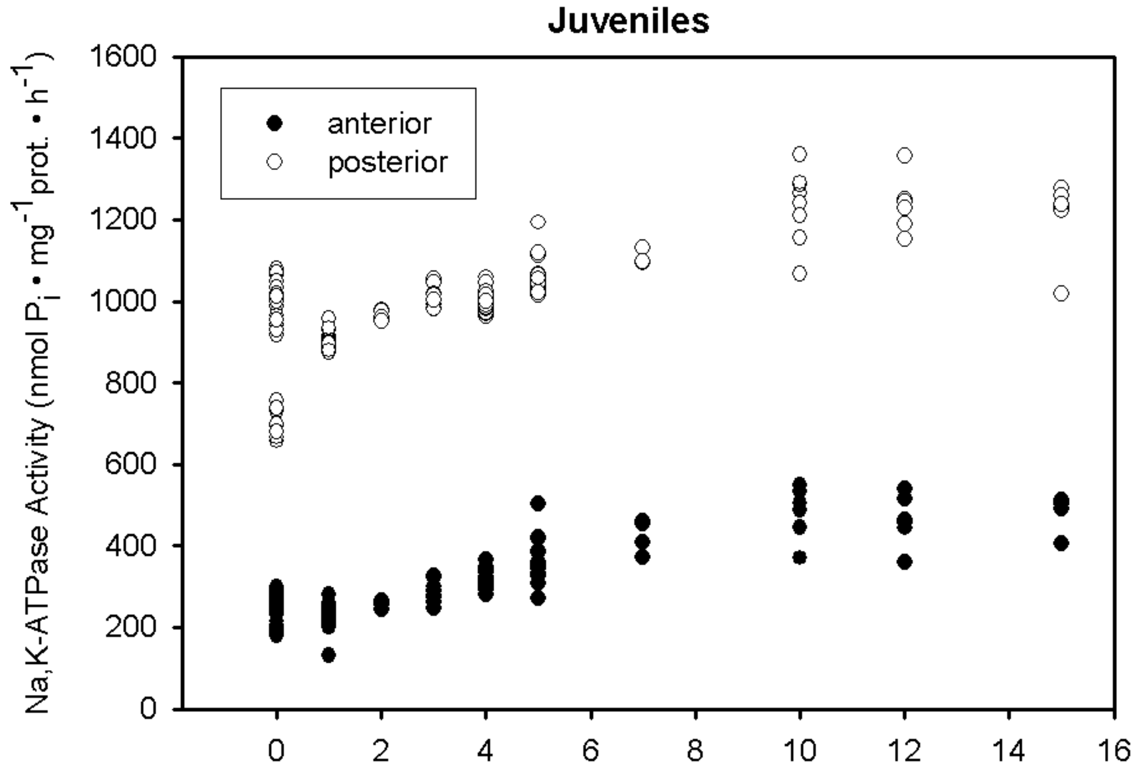

Na,K-ATPase Activity (nmol P_i • mg^{-1} prot. • h^{-1})
anterior
posterior
1200
1000
800
600
400
200
0
mOsm
1000
150
1000
150
1000
150
weight
< 5 g
5 - 40 g
> 40 g

Time course for changes in gill Na^+,K^+ - ATPase activity with during acclimation from 1000 to 150 mOsm was accomplished between seven and ten days following abrupt transfer(Fig. 3). While the timing of the increase in enzyme activity was similar in both juvenile (<5g) and sub-adult and adult (>5g) crabs, the magnitude of the increase was more pronounced in the larger crabs. However, absolute levels never approached that seen in juveniles, even at 1000 mOsm.

The increase in gill Na^+,K^+ - ATPase activity in juvenile crabs with acclimation to 150 mOsm was due, at least in part, to an increase expression of the mRNA coding for the enzyme. While there was no difference in the expression of the message for arginine kinase in either anterior or posterior gills following transfer from 1000 to 150 mOsm media, such was not the case for the Na^+,K^+ - ATPase mRNA (NAK). The latter showed an increase at day 2 post-transfer in both anterior and posterior gills. By day 4 post-transfer, the expression of NAK had returned to constitutive levels in the anterior gills but had increased to the maximum levels observed in the posterior gills, nearly seven times the levels observed at 1000 mOsm. The levels had decreased somewhat by day 6, but still were over four-fold higher than those at 1000 mOsm (Fig. 4).

The affinity of the Na^+,K^+ - ATPase for Na^+ in the posterior gills of juvenile crabs acclimated to 150 mOsm (K_m = 5.1 mM) was higher than that for anterior gills of juveniles (K_m = 13.0 mM) and that for either posterior or anterior gills of 150 mOsm-acclimated sub-adult crabs (K_m = 12.5 and 15.6 mM, respectively) (Fig. 5). On the other hand, there was no significant difference between the K_m for ATP for the Na^+,K^+ - ATPase from posterior gills of 150 mOsm-acclimated juveniles (1.2 mM) compared to sub-adults (0.9 mM) (Fig. 6). The affinity for the enzyme from gills of 150 mOsm-acclimated juveniles

Figure 3. Time course for the increase in *Callinectes sapidus* anterior and posterior gill Na^+,K^+ - ATPase activity following transfer of juvenile (upper panel) and sub-adult and adult (lower panel) crabs from 1000 mOsm medium to 150 mOsm medium. Each point represents an individual crab.


Juveniles
Na,K-ATPase Activity (nmol P_i • mg^{-1} prot. • h^{-1})
anterior
posterior
0
200
400
600
800
1000
1200
1400
1600
0
2
4
6
8
10
12
14
16

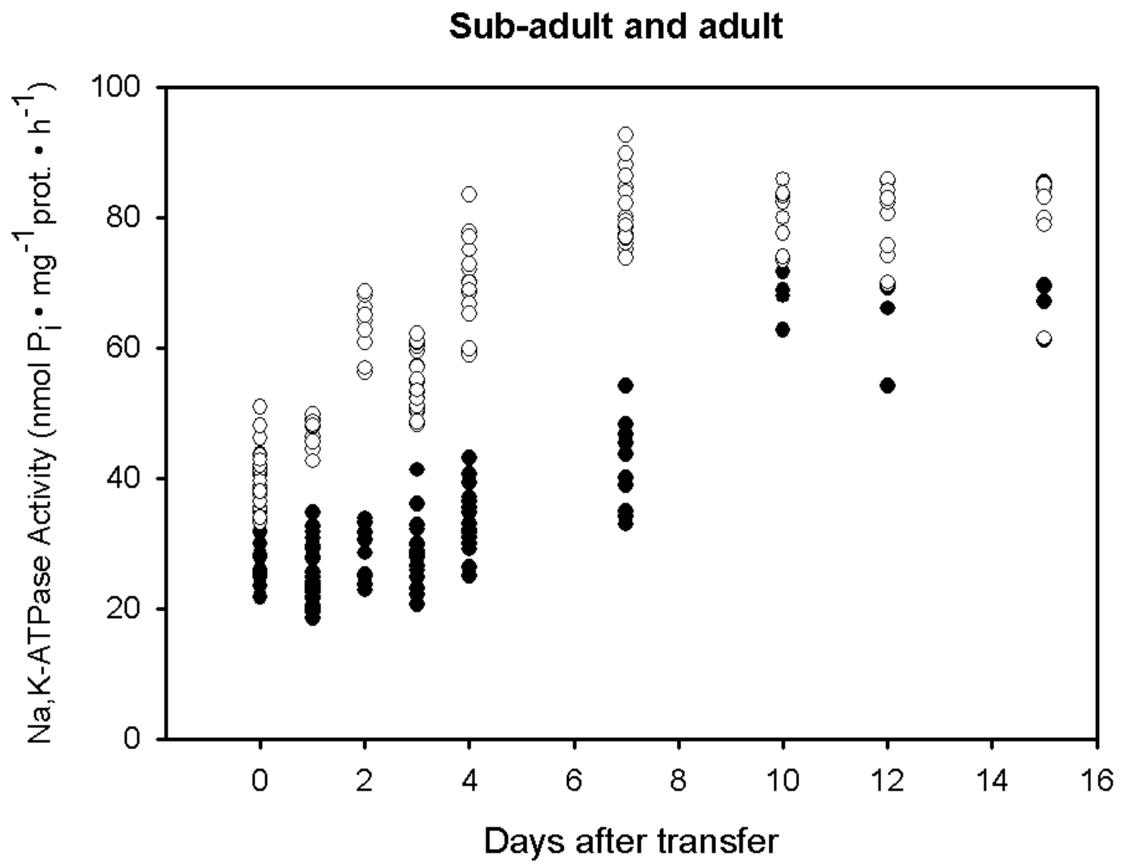
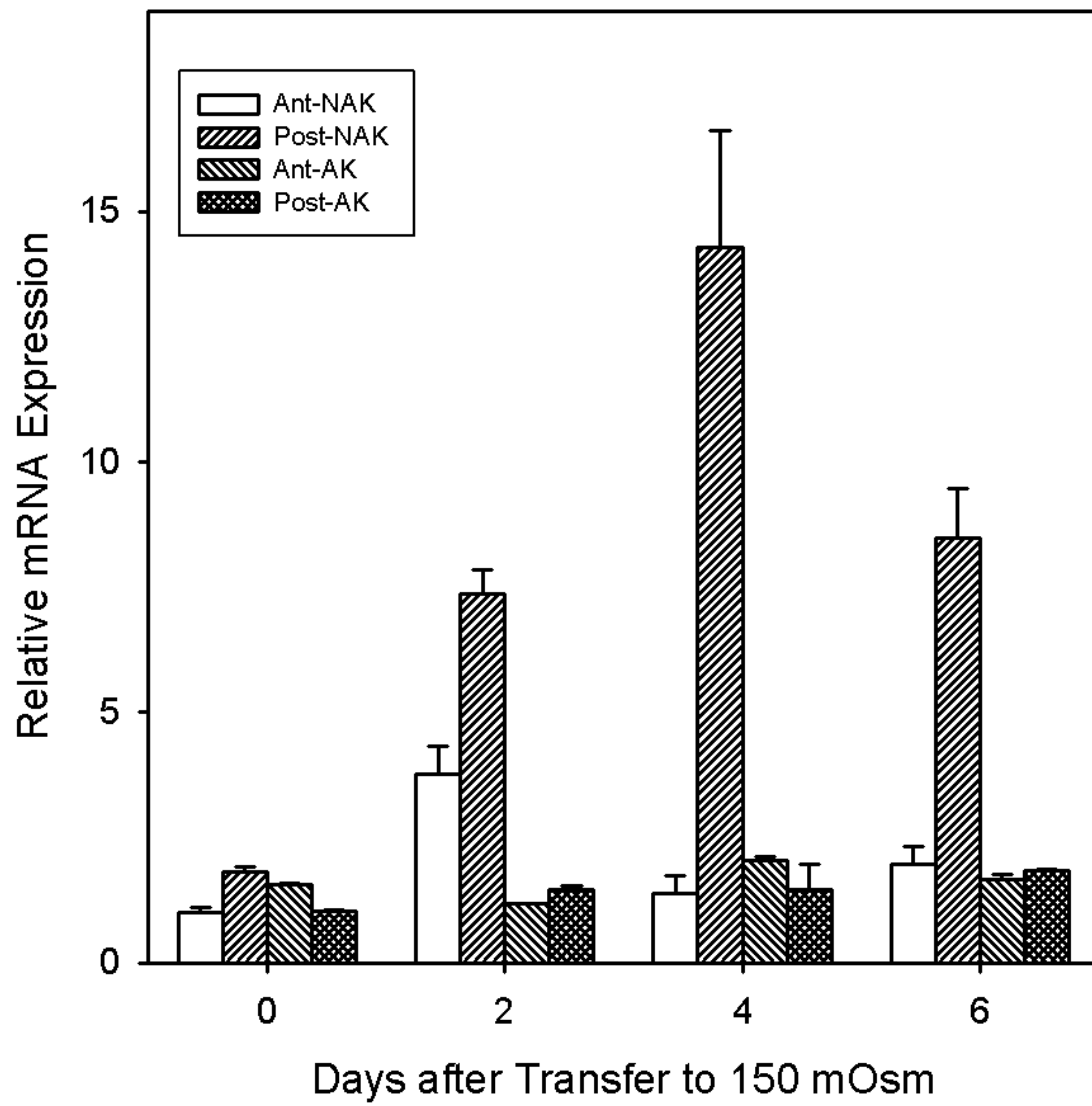

Sub-adult and adult
Na,K-ATPase Activity (nmol P_i • mg^{-1} prot. • h^{-1})
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
Days after transfer

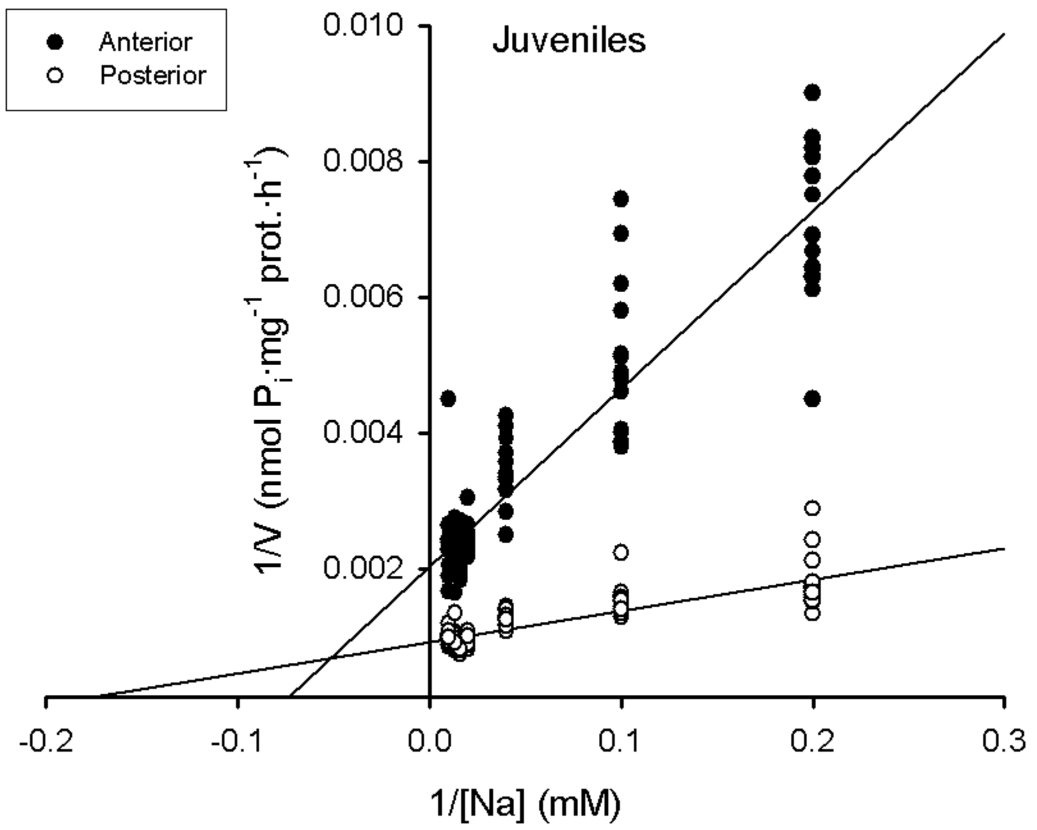
Figure 4. Relative mRNA expression of *Callinectes sapidus* gill Na^+,K^+-ATPase (NAK) and arginine kinase (AK), as determined by quantitative PCR, at various times following transfer of juvenile crabs from 1000 to 150 mOsm.



Ant-NAK
Post-NAK
Ant-AK
Post-AK
Relative mRNA Expression
0
5
10
15
0
2
4
6
Days after Transfer to 150 mOsm

Figure 5. Lineweaver-Burk plot of the Na^+-dependence of anterior and posterior gill Na^+,K^+-ATPase of juvenile (upper panel) and sub-adult (lower panel) *Callinectes sapidus* acclimated to 150 mOsm. Regression equations were:

Juvenile Anterior	$y = (26.17 \times 10^{-3})\, x + (2.02 \times 10^{-3})$	$r^2 = 0.840$
Juvenile Posterior	$y = (4.61 \times 10^{-3})\, x + (0.91 \times 10^{-3})$	$r^2 = 0.697$
Sub-adult Anterior	$y = (0.22)\, x + (0.01)$	$r^2 = 0.844$
Sub-adult Posterior	$y = (0.16)\, x + (0.01)$	$r^2 = 0.843$


Anterior
Posterior
Juveniles
1/V (nmol P_i·mg^{-1} prot.·h^{-1})
0.010
0.008
0.006
0.004
0.002
-0.2
-0.1
0.0
0.1
0.2
0.3
1/[Na] (mM)

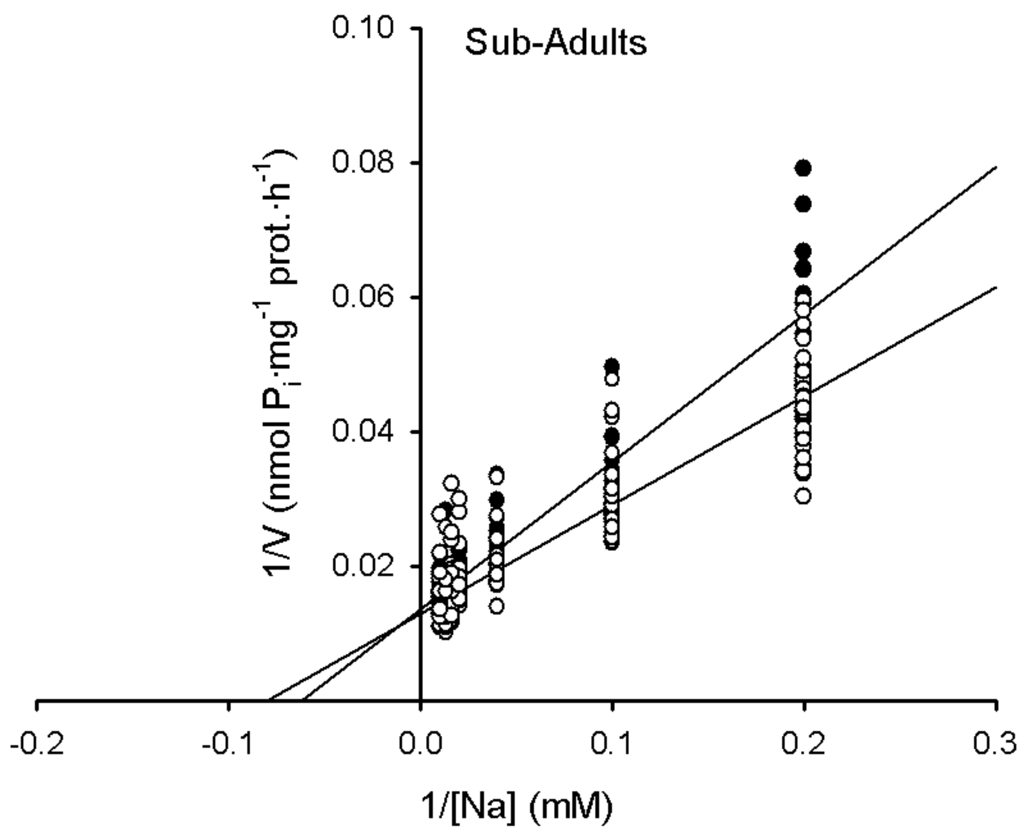
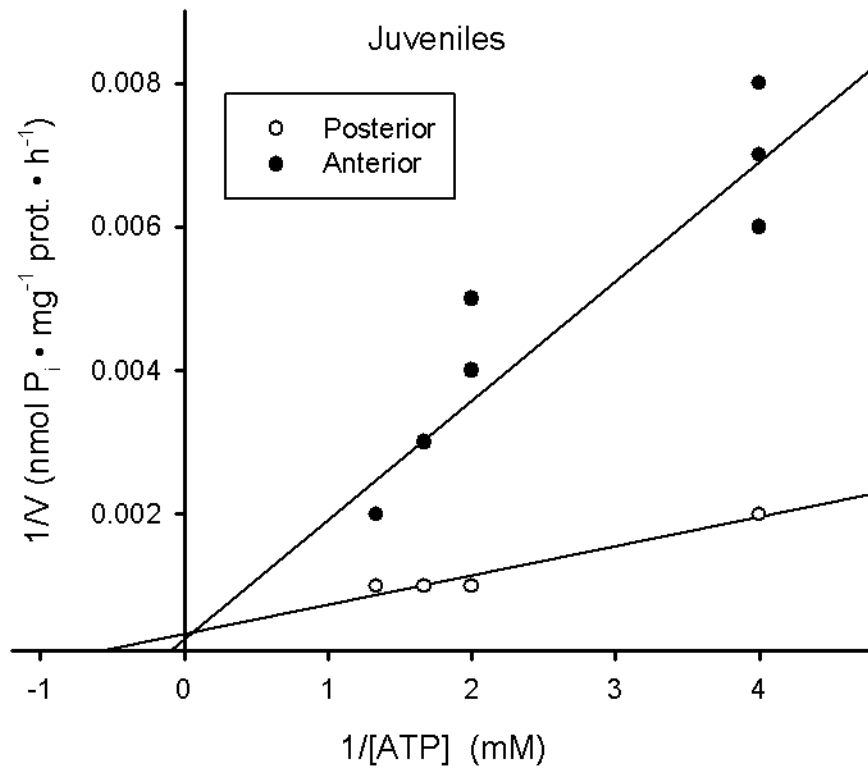
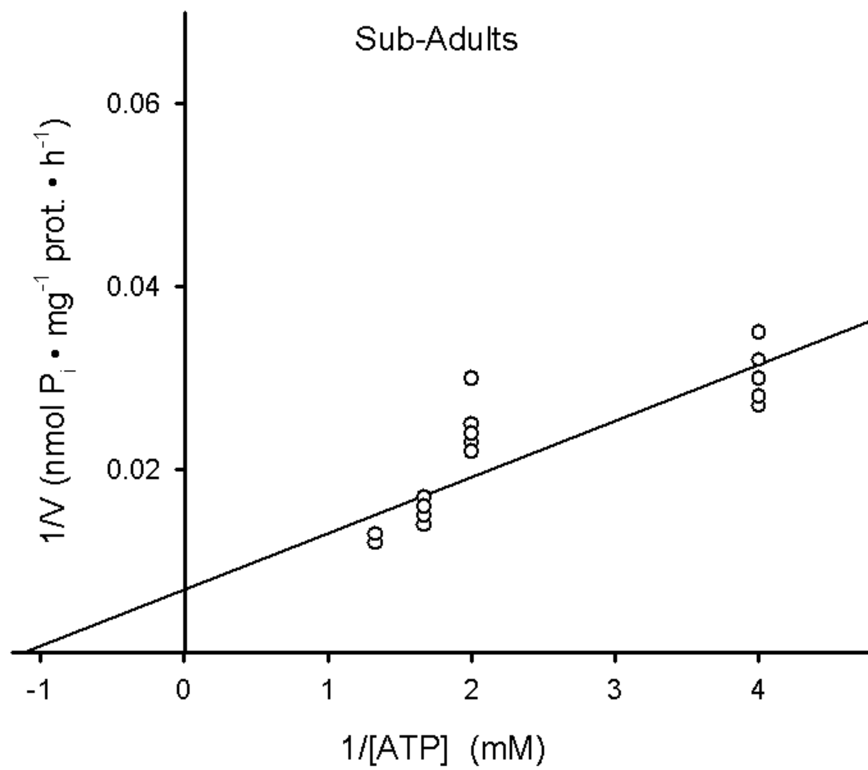

Sub-Adults
1/V (nmol P_i·mg^{-1} prot.·h^{-1})
0.10
0.08
0.06
0.04
0.02
-0.2
-0.1
0.0
0.1
0.2
0.3
1/[Na] (mM)

Figure 6. Lineweaver-Burk plot of the ATP-dependence of anterior and posterior gill Na^+,K^+-ATPase of juvenile (upper panel) and sub-adult (lower panel) *Callinectes sapidus* acclimated to 150 mOsm. Regression equations were:

Juvenile Anterior	$y = (16.65 \times 10^{-4})\,x + (2.55 \times 10^{-4})$	$r^2 = 0.884$
Juvenile Posterior	$y = (4.06 \times 10^{-4})\,x + (3.36 \times 10^{-4})$	$r^2 = 0.948$
Sub-adult Posterior	$y = (6.15 \times 10^{-3})\,x + (6.98 \times 10^{-3})$	$r^2 = 0.774$

There were no data for sub-adult anterior gill enzyme.


Juveniles
Posterior
Anterior
0.008
0.006
0.004
0.002
1/V (nmol P_i • mg^{-1} prot. • h^{-1})
-1
0
1
2
3
4
1/[ATP] (mM)


Sub-Adults
0.06
0.04
0.02
1/V (nmol P_i • mg^{-1} prot. • h^{-1})
-1
0
1
2
3
4
1/[ATP] (mM)

was appreciably lower (K_m = 6.5 mM). This value was similar to that for the Na^+,K^+ - ATPase isolated from the posterior gills of juvenile and sub-adult crabs acclimated to 1000 mOsm (8.0 and 4.9 mM, respectively) (Fig. 7).

There was no difference in the Na^+,K^+ - ATPase activity between male and female sub-adult and adult crabs in either the anterior or posterior gills. While both males and females displayed higher levels of Na^+,K^+ - ATPase activity at 150 mOsm relative to those at 1000 mOsm, there was no difference between the sexes at either salinity (Fig. 8).

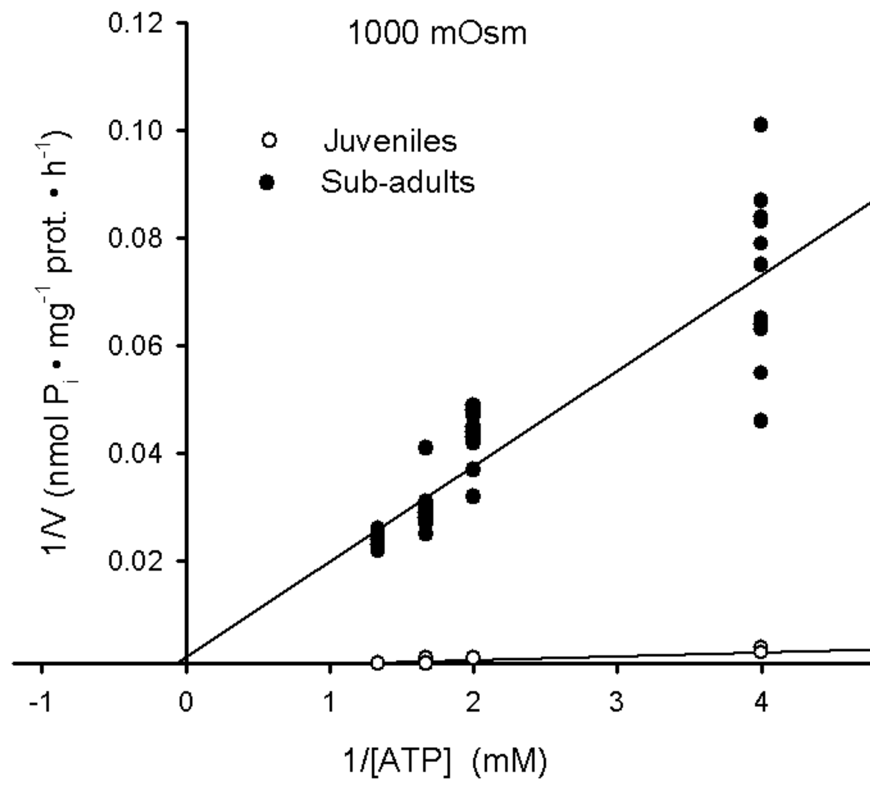
The gill Na^+,K^+ - ATPase from the anterior and posterior gills of small and large crabs at both salinities showed a fairly low sensitivity to inhibition by ouabain. The concentrations of ouabain that elicited 50% inhibition of enzyme activity was 0.436 ± 0.014 mM (mean ± S.D.) for anterior gills of juveniles and adults combined, 0.411 ± 0.075 for posterior gills, and 0.424 ± 0.052 when data were pooled for all isolated enzymes (Table 1).

Table 1. IC50 values for ouabain inhibition of gill Na,K-ATPase activity in adult and juvenile blue crabs acclimated to sea water or dilute sea water.

Salinity (mOsm)	Size	Ouabain IC50 (mM)	
		Anterior	Posterior
150	Juvenile	0.44	0.31
	Adult	0.43	0.49
1000	Juvenile	0.45	0.41
	Adult	0.42	0.44

Figure 7. Lineweaver-Burk plot of the ATP-dependence of posterior gill Na^{+},K^{+}-ATPase of juvenile and sub-adult *Callinectes sapidus* acclimated to 1000 mOsm (upper panel) 150 mOsm (lower panel). Regression equations were:

Juvenile 1000mOsm	$y = (7.47x10^{-4})\ x + (1.53x10^{-4})$	$r^2 = 0.849$
Juvenile 150mOsm	$y = (4.06x10^{-4})\ x + (3.36x10^{-4})$	$r^2 = 0.948$
Sub-adult 1000mOsm	$y = (17.46x10^{-3})\ x + (2.22x10^{-3})$	$r^2 = 0.818$
Sub-adult 150mOsm	$y = (6.15x10^{-3})\ x + (6.98x10^{-3})$	$r^2 = 0.774$


1000 mOsm
Juveniles
Sub-adults
1/V (nmol P$_i$ • mg^{-1} prot. • h^{-1})
1/[ATP] (mM)
0.12
0.10
0.08
0.06
0.04
0.02
-1
0
1
2
3
4

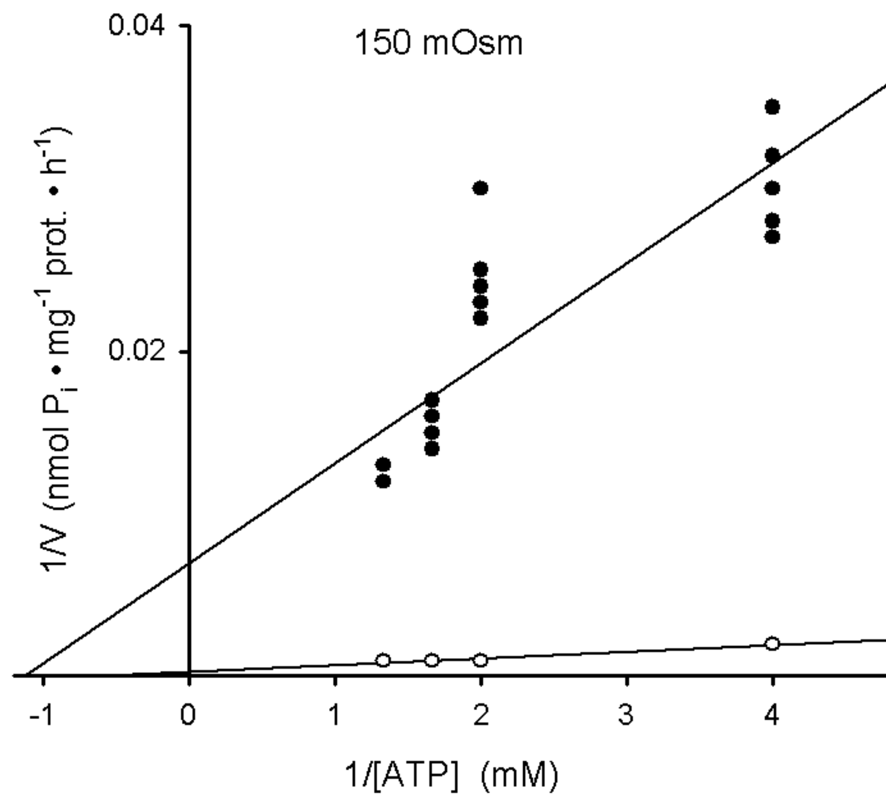
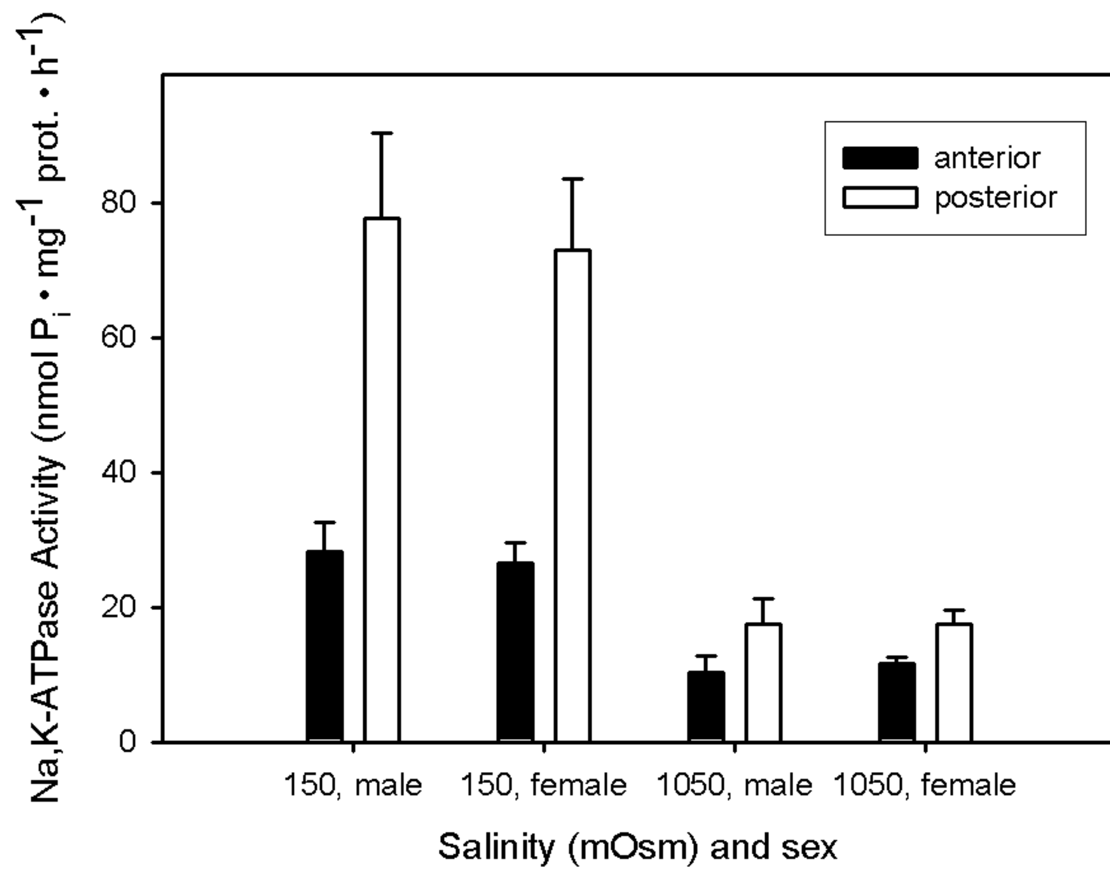

150 mOsm
1/V (nmol P$_i$ • mg^{-1} prot. • h^{-1})
1/[ATP] (mM)
0.04
0.02
-1
0
1
2
3
4

Figure 8. Mean (+S.D.) gill Na^+,K^+-ATPase activities from anterior and posterior gills of sub-adult and adult male and female *Callinectes sapidus* acclimated to sea water (1050 mOsm) or dilute sea water (150 mOsm).



Na,K-ATPase Activity (nmol P$_i$ • mg^{-1} prot. • h^{-1})
0
20
40
60
80
anterior
posterior
150, male
150, female
1050, male
1050, female
Salinity (mOsm) and sex

DISCUSSION

Adult blue crabs recorded fairly low levels of Na^+, K^+-ATPase activity in both anterior and posterior gills in seawater and dilute seawater. The posterior gills of adult *Callinectes* are the primary sites of ion uptake. The activity of the transport enzymes involved in this process is induced by acclimation of crabs to low salinities (< 25 ppt). The Na^+, K^+-ATPase shows expression of higher activities as the salinity decreases. In the experiment a significant increase in the Na^+, K^+-ATPase activity was observed in both the anterior and posterior gills after adult crabs acclimate to low salinity, and the levels of Na^+, K^+-ATPase activity in the posterior gills showed a marked increase at low salinities. These results are in accordance with the osmoregulatory performance of *C. sapidus* reported in literature (Pequeux, 1995; Lin, 2002; Guerin, 1992; Charmantier, 1995; Susanto, 2000; Towle, 1985; Walsh, 1990; Wanson, 1984), and point out the major role of posterior gills in the osmoregulation of this species, as described for other crab species.

Juvenile blue crabs have higher levels of Na^+, K^+-ATPase activity in both anterior and posterior gills relative to adults. Generally the crabs acclimated to 150 mOsm have a higher value of Na^+, K^+-ATPase activity in both anterior and posterior gills than the ones in 1000 mOsm. The juvenile posterior gills showed high enzyme Na^+, K^+-ATPase activity. The levels of Na^+, K^+-ATPase in the anterior gills of juveniles showed a marked increase at low salinities. This is particularly notable since the anterior gills in adults are largely relegated to respiratory work and usually display much lower Na^+, K^+-ATPase activity than posterior gills. Unlike adult crabs, juvenile blue crabs appear to utilize the anterior gills for ion uptake at low salinities in order to combat the increased electrofusive ion loss due to their large surface-to-volume ratio. These results demonstrated the different

osmoregulatory performance of juvenile blue crab in diluted media, and point out the major role of anterior gills in the osmoregulation.

The break in the distribution at around 5 g body weight is as perplexing as it is marked. There is no apparent, discrete change in the physiology or behavior of blue crabs that occurs at this size or stage. However, the sharp change in activity at or around this size range was also observed in the metabolic enzymes of the gills (Kinsey et al., 2003).

The Na^+, K^+-ATPase of the posterior gills both juvenile and sub-adult crabs demonstrated an increase in affinity for ATP when acclimated to low salinity. This implies that there are either isozymes that are preferentially expressed under different salinity regimes or that there is an allosteric regulation of the ATP binding site. In either case, the reduction in K_m at low salinities should make the enzyme more efficient.

The Na^+, K^+-ATPase of the posterior gills of juvenile crabs at low salinity had a higher affinity for Na^+ than that of the posterior gills of sub-adult crabs and than that for the anterior gills of either juvenile or sub-adult crabs. Again, the lower K_m should render the Na^+, K^+-ATPase of juvenile posterior gills more efficient at low salinities than that of larger crabs.

The IC_{50} values for ouabain observed in anterior and posterior gills of *C. sapidus* were similar to those reported for other crustaceans. They are also in accordance with the Na^+, K^+-ATPase sensitivity to this inhibitor along other taxonomic groups. For example, Na^+, K^+-ATPase from insects and chelicerates have higher sensitivity to ouabain (10 µM) than those from crustaceans tissues (100 µM). Values of *C. sapidus* were also in accordance with those reported by Neufeld et al. (1980), who demonstrated that the Na^+, K^+-ATPase from vertebrates are much more sensitive to ouabain than those from

brachyuran crustaceans. Unlike previous data (Engel and Eggert, 1974; Engel, 1977), however, the current study suggested that there was no difference in the osmoregulatory ability of males compared to females. The levels of gill Na^+, K^+-ATPase in anterior and posterior gills were the same for all crabs at a given salinity, regardless of sex.

In the present experiments we optimized the conditions for measuring Na^+, K^+-ATPase activity *in vitro*. Therefore, the values may reflect total Na^+, K^+-ATPase available; not all of this activity may be expressed *in vivo*. A rapid increase in Na^+, K^+-ATPase activity would make *de novo* synthesis of new Na^+, K^+-ATPase unnecessary. Furthermore, new enzyme synthesis would require an extended time interval for transcription and translation to occur. So in terms of time and economy of effort, the activation of preexisting pro-Na^+, K^+-ATPase would be an attractive hypothesis. However, the data suggested that crabs transferred to low salinity exhibited a gradual increase in enzyme activity, and that Na^+, K^+-ATPase activity was altered primarily by synthesis and degradation, rather than by modulation of the activity of existing enzyme. This conclusion was supported by the data demonstrating an increase in the expression for the Na^+, K^+-ATPase mRNA seen in anterior and, especially, in posterior juvenile crab gills following transfer to dilute medium. The time course for the increase in expression preceded by some one to three days the attainment of a new, higher steady-state level of Na^+, K^+-ATPase activity levels in the gills.

Juvenile crabs have a larger surface-to-volume ratio than adults, and should, therefore, have a far more difficult time maintaining blood osmolarities above that of the medium. It is possible that juvenile crabs have increased the relative amounts of this enzyme in order to compensate for the high loss rate of ions. They may display higher

levels in the posterior gills and even express the enzyme in the anterior gills that are normally relegated to respiration. The relative metabolic rate of small organisms is higher than that of large organism under similar conditions. The metabolic work associated with osmoregulation increases exponentially with the difference between blood and medium osmotic concentrations. Together, these considerations reflect a potentially high metabolic cost of osmoregulation for juvenile crabs. The increased osmoregulatory work, at least at the level of the gills, is confirmed by the extremely high levels of Na^+, K^+-ATPase in both anterior and posterior gills of juvenile crabs relative to sub-adults and adults. Some of the osmoregulatory cost may be ameliorated by increase enzyme efficiency as reflected by the higher Na^+ and ATP affinities of the Na^+, K^+-ATPase of the posterior gills of the juveniles.

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