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The effects of rapid salinity change on in vivo arginine kinase flux in the juvenile blue crab, *Callinectes sapidus*

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Abstract

The effect of acclimation salinity and salinity changes on the concentration of high-energy phosphate metabolites and arginine kinase (AK) flux was examined in vivo in juvenile blue crabs using ^{31}P-nuclear magnetic resonance (NMR). Crabs were acclimated for 7 days to a salinity of 5 or 35‰ and then placed in a flow apparatus that could sustain the animals while NMR spectra were acquired. Crabs were subjected to either hyperosmotic salinity changes, where an animal acclimated to 5‰ was exposed to a salinity of 35‰, or hyposmotic changes, which involved the reciprocal exchange. Neither acclimation salinity nor salinity change had a significant effect on the concentrations of arginine phosphate, inorganic phosphate or ATP. ^{31}P-NMR saturation transfer experiments were used to determine the effect of salinity on the forward and reverse flux of the AK reaction. There was no significant effect of acclimation salinity or salinity change on the flux rate through this reaction. This is in contrast to previous results, which showed that AK flux in isolated muscle was sensitive to prevailing osmotic conditions (Holt and Kinsey, J. Exp. Biol. 205 (2002) 1775–1785). The present study indicates that the integrated osmoregulatory capacity of the intact animal is sufficient to preserve cellular energy status and enzyme function during acute salinity changes.
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Keywords: Arginine kinase; Phosphagen kinase; Salinity; Crustacean; *Callinectes*; Saturation transfer

1. Introduction

The blue crab, *Callinectes sapidus*, inhabits salinities ranging from full strength sea water (35‰) to fresh water (Mangum and Amende, 1972; Lynch et al., 1973; Cameron, 1978). Like many euryhaline species, the hemolymph of blue crabs remains isosmotic with the environment at high salinities (above approx. 25‰) while the hemolymph is maintained hyperosmotic to that of the surrounding environment at lower salinities (Ballard and Abbott, 1969; Lynch et al., 1973; Mangum et al., 1985). The membrane-bound ion pumps that facilitate the blue crab's capacity for hyperosmoregulation at low salinity are principally located in the posterior gills (Mantel and Farmer, 1983; Towle and Weihrauch, 2001), although the ability to reduce membrane permeability and alter urine production may also facilitate osmoregulation (Robinson, 1982, 1994).

The pattern of osmoregulation in blue crabs leads to variation in hemolymph osmolarity that ranges from approximately 500 to 1000 mOsm l^{-1} as the environmental salinity that the crab is exposed to changes from 5 to 35‰, respectively,

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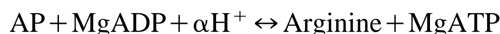
(Lynch et al., 1973; Mantel and Farmer, 1983; Piller et al., 1995). During salinity changes, the tissues must remain isosmotic with the hemolymph in order to minimize cell volume changes. This is principally accomplished by altering the intracellular concentrations of certain free amino acids and other compatible or counteracting solutes (Gérard and Gilles, 1972; Gilles, 1979). These compounds can be accumulated to high concentrations in cells without the detrimental effects on protein/DNA structure or function that often accompany changes in inorganic ion concentrations (e.g. Brown and Simpson, 1972; Clark and Zounes, 1977; Bowlus and Somero, 1979; Yancey et al., 1982; Pierce, 1982; Petronini et al., 1993; Sheikh-Hamad et al., 1994; Kültz and Chakravarty, 2001; Yancey, 2001). However, during rapid salinity changes the intracellular adjustments of organic osmolyte concentrations may occur over a period of hours or days, while hemolymph osmolarity may be altered more rapidly (Bartberger and Pierce, 1976; Gilles, 1979; Pierce, 1982). This may lead to a transient period where the osmotic constituents of the tissue and hemolymph are not matched. Under these conditions, fluxes of inorganic ions, which can disrupt protein function, may be the primary mode of cell volume regulation (Warren and Pierce, 1982). While the studies cited above as well as many others have clearly demonstrated the in vitro effects of osmotic conditions on enzyme function, the impact of changing environmental salinity on in vivo enzyme function is unclear.

The enzyme arginine kinase (AK) is a phosphagen kinase that is central to energy metabolism in crustacean muscle. AK catalyzes the reversible transfer of a high-energy phosphate from arginine phosphate (AP) to ADP to form ATP,



$$AP + MgADP + \alpha H^{+} \leftrightarrow Arginine + MgATP$$

where α represents a partial proton. This reaction functions as a temporal ATP buffer during transient periods of high energy demand, such as during burst contractions in muscle, and it may also serve as a spatial ATP buffer during periods of sustained, low-level energy demand (reviewed in Ellington, 2001). Using a ^{31}P-NMR magnetization transfer method, we have recently shown that in isolated swimming leg muscle of the blue crab, changes in the osmolarity of the external medium induced a nearly two-fold change in the rate of flux through the AK reaction (Holt and Kinsey, 2002). In this study the osmolarity of the external medium was varied in order to simulate the ionic changes that take place in the blood over the crab's natural range of environmental salinities. AK flux was found to increase when the muscle was exposed to a hyposmotic medium, while a decrease in flux was shown when the muscle was exposed to a hyperosmotic medium. This pattern led us to propose that the principal cause of the variation in AK flux was a direct effect of inorganic ions on the enzyme. Hyperosmotic conditions would potentially expose AK to relatively high concentrations of destabilizing, inorganic ions and relatively low concentrations of stabilizing, organic osmolytes. In contrast, hyposmotic treatments may result in relatively low levels of inorganic ions and high levels of organic osmolytes (Holt and Kinsey, 2002).

While this previous work was conducted using physiologically relevant simulations of the magnitude of changes in hemolymph osmolarity, the superfusion medium was changed instantaneously, which likely constitutes a more extreme osmotic challenge to tissues than is experienced in the intact organism (Holt and Kinsey, 2002). In contrast, the intact animal has the capacity to temporally buffer osmolyte concentrations in the hemolymph when the environmental salinity undergoes an acute change, which may moderate the effect of salinity change on protein function (Gilles, 1979; Towle et al., 1994). The present study addressed this issue by examining how salinity changes affect in vivo energy state and AK flux in juvenile blue crabs, which exploit low salinity habitats but are thought to be less tolerant of salinity fluctuations than are adults.

2. Materials and methods

2.1. Animal collection and maintenance

C. sapidus were collected using small nets, during low tide at River Road Park, near Wilmington, NC. The animals were maintained at room temperature in separate, air-equilibrated containers. Crabs were maintained at either 5 or 35‰ for 7 days prior to experimentation (subsequently referred to as acclimation salinity). Every other day, crabs were fed dried crab and lobster bites bought at a local pet store, and holding containers were cleaned.

2.2. Metabolite assays

Spectrophotometric measurements of the phosphogen, AP, were conducted in animals acclimated to 5 or 35‰. AP concentration could then be used as a reference to infer the concentration of ATP and inorganic phosphate (P_i) by comparing the relative ^{31}P-NMR peak areas of these three metabolites (see below). Whole crabs were freeze-clamped between metal plates cooled in liquid N_2. The frozen crabs were then divided into two subsamples by splitting the crabs sagitally, and each half was then weighed (one half was used for the AK activity assays described below). The crab sub-sample to be used for metabolite assays was added to 10 volumes of ice-cold 7% perchloric acid with 1 mM EDTA and immediately homogenized using a PowerGen 125 homogenizer. The homogenate was centrifuged for 15 min at $10\,000\times g$ in an Eppendorf 5415C microcentrifuge at 4 °C. The supernatant was titrated to pH 6.5 using 3 M KOH containing 50 mM PIPES, allowed to cool on ice for 10 min, and centrifuged again using the above conditions. The supernatant volume was then determined and arginine phosphate assays were performed immediately.

AP concentration was determined using an enzyme-coupled assay in a Pharmacia Ultrospec 4000 spectrophotometer as described in Holt and Kinsey (2002). The following assay medium was pipetted into a semi-microcuvette: 30 mM Tris/HCl, 2.5 mM $MgCl_2$, 2.5 mM D-glucose, 0.63 mM NADP, 75 units of glucose-6-phosphate dehydrogenase and 25 μl crab tissue extract. A baseline absorbance was established, which was followed by the addition of 1.5 units of hexokinase. The new absorbance was allowed to stabilize after the endogenous ATP was consumed. ADP (0.5 mM) and 2.5 units of AK were then added and AP concentration was determined from the change in absorbance.

2.3. NMR flow system

Juvenile crabs with carapace widths ranging from 10.8–16.4 mm (mean = 14.9 ± 1.1 mm) were placed sideways in a 10 mm NMR tube containing a medium equivalent to the acclimation salinity. Animal movement was restricted by two sections of elastic tubing that were placed above and below the animal. This allowed the animal to freely move its limbs but prevented it from moving along the long axis of the NMR tube. The NMR tube was attached to a recirculation system that provided the crab with constant flow of saline solution while the animal was inside the magnet. Masterflex peristaltic pumps outside of the NMR magnet maintained a constant flow rate of 10 ml min^{-1}. The superfusion medium was maintained at 20 °C by a 1016S Isotemp re-circulating water bath. Crabs were allowed to adjust to the NMR tube at their acclimation salinity for one hour prior to experimentation.

2.4. Nuclear magnetic resonance spectroscopy

In order to mimic the effects of environmental salinity changes on live crabs, four experimental salinity treatments were used. To determine the effect of different acclimation salinities on high-energy phosphate concentrations and AK flux, experiments were run using a superfusion medium equal to the acclimation salinity of 5 or 35‰. To determine the effect of salinity change, crabs were exposed to hyper- or hyposmotic conditions. Hyperosmotic treatments involved exposure of a crab acclimated to a salinity of 5‰ to a salinity of 35‰, while hyposmotic treatments involved the reciprocal protocol.

The flow system containing the live crab was lowered into the magnet of a Bruker 400 MHz DMX spectrometer and mated with a 10 mm NMR probe. The probe was tuned to the phosphorus precessional frequency of 162 MHz and the sample was shimmed used the proton signal arising from water to optimize the magnetic field homogeneity. Initial spectra were obtained to ensure that the crab was in good physiological condition, as determined by a high AP to ATP ratio and a small or absent P_i peak. For the initial spectra, 110 scans were acquired, using a 25 μs excitation pulse with a 1 s relaxation delay.

It is possible that energetic challenges associated with different acclimation salinities or a rapid salinity change may alter the concentrations of AP, ATP, and P_i in crabs. To address this issue, ^{31}P-NMR spectra were collected first on crabs acclimated to 5 or 35‰, and subsequently in a time series during the hypo- and hyperosmotic treatments described above. Completely relaxed spectra were taken using a 10 s relaxation delay and a 90° pulse so that the peak areas would reflect the relative concentration of each metabolite. Each spectrum was acquired as the average of 110 scans,

which required a total collection time per spectrum of 19 min. An initial spectrum was taken at the acclimation salinity and then the superfusion medium was switched to either a hyperosmotic or hyposmotic treatment. Peak areas for AP, ATP, and P_i were then integrated to yield the relative concentrations of each compound. These values were then normalized to the spectrophotometric measurements of AP to determine absolute levels of each metabolite.

^{31}P-NMR saturation transfer was used to measure the pseudo-first-order unidirectional rate constants for the AK reaction in both the forward ($k_{forward}$) and reverse ($k_{reverse}$) directions (Graham et al., 1986; Ellington and Wiseman, 1989; Holt and Kinsey, 2002). The resonances for either AP or the γ-P of ATP were selectively irradiated using a 10 s low-power pulse so that the net magnetization of the irradiated peak was equal to zero. Immediately following the low-power pulse, a 45 μs broadband excitation pulse (90° tip angle) was applied to allow for signal detection. Each spectrum was acquired by averaging 110 scans, which required 19 min. The $k_{forward}$ was determined by saturation of the γ-ATP peak, while the $k_{reverse}$ was measured by saturation of the AP peak. Control spectra were acquired to offset any partial saturation of the unsaturated peak during selective irradiation. In the control spectra the saturating pulse was applied on the opposite side of the unsaturated peak at the same frequency offset as in the corresponding saturated spectrum. The control spectra were compared to the spectra in which AP and γ-ATP peaks were selectively irradiated and areas under both the AP and γ-ATP peaks were determined.

The peak areas were used to determine the ratios of magnetization of the unsaturated metabolite resonance in the presence of the saturating irradiation at the exchanging site (M^+) to the magnetization of the unsaturated resonance in the control spectrum (M^o). The M^+/M^o ratio is related to the rate constant (k) for the AK reaction through the expression:

$$k=[1-(M^+/M^o)](T_1)^{-1}$$

Here, T_1 is the spin lattice relaxation time of the unsaturated resonance in the presence of saturation at the exchanging site (Graham et al., 1986). T_1 values of 2 s for AP and 0.9 s for ATP were used, which were previously determined for crustaceans in vivo at our experimental temperature of 20 °C (Butler et al., 1985), and are nearly identical to the values obtained for crustaceans in vivo by Briggs et al. (1985) at 23 °C (2.1 s for AP and 0.8 s for ATP). To determine the forward flux through the reaction (formation of ATP), the forward rate constant was multiplied by the spectrophotmetrically determined concentration of AP. The reverse flux for the AK reaction (formation of AP) was found by multiplying the reverse rate constant by the ATP concentration (derived by relating the NMR peak area of AP to that of ATP).

2.5. In vitro AK activity assays

Maximal activity of AK was measured spectrophotometrically to characterize potential effects of acclimation salinity and to compare the in vitro activity to the in vivo flux. The half of the frozen crab that was not used for determining AP concentration was used for enzyme assays. The frozen crab was homogenized in 9 volumes of ice-cold 50 mM imidazole (pH 7.0). The homogenate was centrifuged at 12 000×g at 4 °C and the supernatant was assayed for AK activity using an enzyme-coupled assay in a Pharmacia Ultrospec 4000 spectrophotometer. Temperature was maintained at 20 °C with a recirculating water bath. The assay medium consisted of 65 mM Tris/HCl (pH 8.0), 38 mM KCl, 13 mM magnesium acetate, 5 mM ATP, 1.25 mM phosphoenolpyruvate, 0.25 mM NADH and excess pyruvate kinase/lactate dehydrogenase. The rate of oxidation of NADH was determined by monitoring the initial decrease in absorbance over time at a wavelength of 340 nm. Basal ATPase activity was initiated by addition of the crab extract. The AK reaction was then initiated by adding 10 mM arginine, and the AK activity was determined from the decrease in absorbance, after subtracting the effect of ATPases.

The concentration of total protein was determined on the same extracts used for AK activity assay using the Bio-Rad dye-binding assay (Bradford method). Total protein was determined in triplicate for each sample using a 96-well microtiter plate and a Molecular Devices Spectra Max 250 microplate spectrophotometer. The total protein concentration for each sample was used to determine AK specific activity.

2.6. Statistical analyses

One-way analysis of variance (ANOVA) was used to test for significant effects of salinity

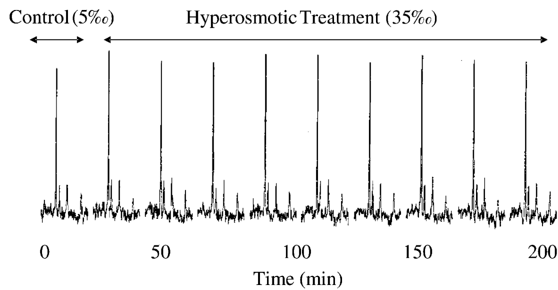


Fig. 1. Representative ^{31}P-NMR spectra time series during a hyperosmotic treatment. The tallest peak in each spectrum represents AP and the three smaller peaks to the right of the AP peak represent, from left to right, the γ, α, and β peaks of ATP. Each spectrum was collected in 19 min and the total experiment time was 3 h and 20 min. The initial spectrum shown was collected while the crab was in an acclimation salinity of 5‰, and subsequent spectra were taken after transfer to 35‰. Peak areas remained constant throughout the 3 h period. Similar results were observed for hyposmotic treatments.

treatments and repeated-measures ANOVA was used to analyze changes in high-energy phosphate compounds over time. Homogeneity of variance was assessed using Bartlett's test. Tukey's HSD test was used for pairwise comparisons. Data were analyzed using SAS-JMP statistical software version 4.04 (SAS Institute, Cary, NC) and all values are reported as means ± S.E.M.

3. Results

3.1. Salinity effects on high-energy phosphate concentrations

The spectrophotometrically measured AP concentrations were found to be 7.6 ± 1.29 μmol g wet weight^{-1} for animals acclimated to 35‰ and 6.6 ± 2.27 μmol g wet weight^{-1} for animals acclimated to 5‰. The mean values were not significantly different (Tukey's HSD, $P > 0.05$). A time series of ^{31}P-NMR spectra revealed that high-energy phosphate concentrations were similar at the two acclimation salinities and exhibited stability over the time course of our experiments during both hypo- and hyperosmotic treatments. An example of a time series is shown in Fig. 1. The peak areas for AP, γ-ATP, α-ATP, and β-ATP remained constant across each treatment, and the P_i peak, which would be elevated by a sizable energetic challenge, was typically not visible in ^{31}P-spectra. Fig. 2 shows the lack of change in mean metabolite concentrations over a period of 2 h for both hypo- and hyperosmotic experiments. Repeated measures ANOVA found no significant effect of time on the concentrations of AP ($F = 6.01$; DF = 4; $P = 0.15$) or ATP (mean integral of the α, β and γ-P peaks; $F = 1.60$; DF = 4; $P = 0.42$), and the mean AP/ATP ratio also did not change ($F = 1.41$; DF = 4; $P = 0.45$) during an 80 min time span for each salinity treatment (the last two time points in Fig. 2 could not be included in the repeated measures ANOVA due to missing values). There was also no significant effect of acclimation salinity on the AP/ATP ratios ($F = 0.45$; DF = 1; $P = 0.53$).

3.2. Arginine kinase fluxes

A representative set of spectra from a saturation transfer experiment that illustrates the phosphate

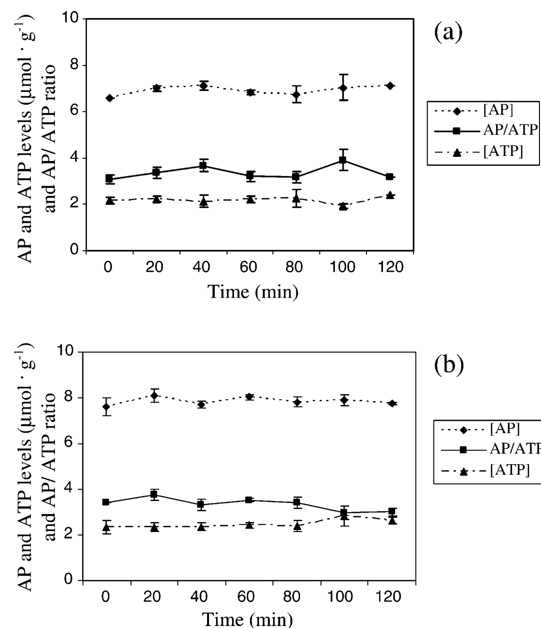


Fig. 2. Concentrations of metabolites and AP/ATP ratios over a period of 2 h during (a) a hyperosmotic salinity change (5–35‰ transfer) and (b) a hyposmotic salinity change (35–5‰ transfer). The data at the zero time point were collected while the crab was still at its acclimation salinity, while subsequent data were collected following transfer to a different salinity. These data were derived from NMR spectra times series like that shown in Fig. 1, where the initial [AP] was assumed to be 6.6 μmol g^{-1} for a 5‰ acclimation salinity and 7.6 μmol g^{-1} for a 35‰ acclimation salinity, based on the enzymatic analyses described in the text. The [ATP] was determined by the size of the ATP NMR peak relative to that of AP. AP concentration, ATP concentration, and the AP/ATP ratio did not significantly change during the experiments. In (A), $n = 4$ except for 100 min ($n = 3$) and 120 min ($n = 2$). In (B), $n = 3$ except for 100 min ($n = 2$), and 120 min ($n = 1$). Data are means ± S.E.M.

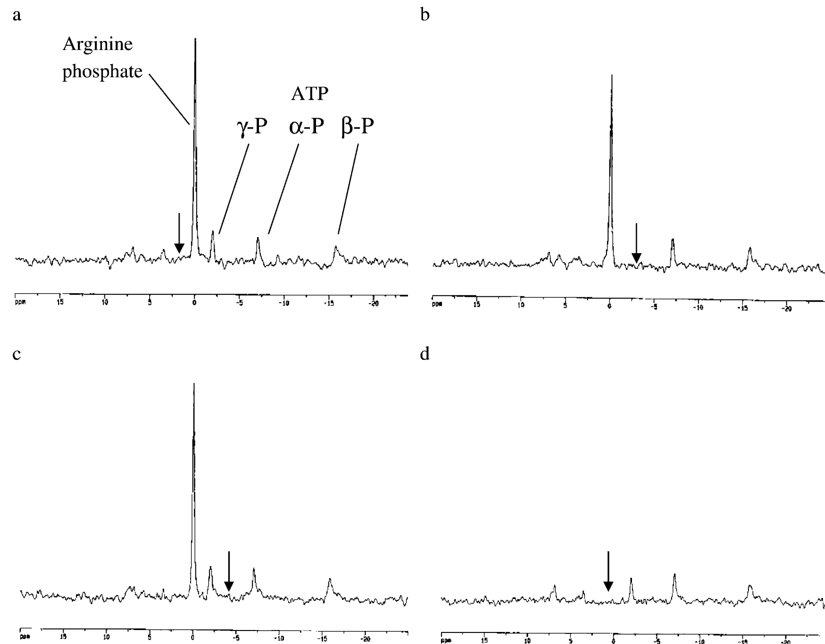


Fig. 3. A representative set of spectra used for measuring the psuedo-first-order unidirectional rate constant for the forward ($k_{forward}$) and the reverse ($k_{reverse}$) directions of the AK reaction. (a) A control spectrum for measuring $k_{forward}$ where saturating irradiation (indicated by the vertical arrow) is applied to a frequency to the left of the AP peak at a frequency offset that is equal to that between the AP peak and the γ-ATP peak. (b) A spectrum illustrating that when the γ-ATP peak is saturated there is a decrease in the height of the AP peak compared to that in the control spectrum in (a). This decrease is proportional to the rate of transfer of phosphate from AP to ADP, and is used to determine $k_{forward}$. (c) A control spectrum for measuring $k_{reverse}$ where saturating irradiation is applied to a frequency to the right of the γ-ATP peak at a frequency offset that is equal to that between the γ-ATP peak and the AP peak. (d) A spectrum showing a decrease in the height of the γ-ATP peak (relative to the control spectrum in c), resulting from saturation of the AP peak.

exchange catalyzed by AK is given in Fig. 3. The peak amplitude arising from the AP or γ-ATP resonance can be seen to decrease when the exchanging resonance is saturated. The $k_{forward}$, $k_{reverse}$, forward flux, and reverse flux values for each salinity treatment are presented in Fig. 4. One-way ANOVA applied to rate constants and flux measurements indicated no significant effect of salinity treatment for $k_{forward}$ (DF=3; $F=0.19$; $P=0.90$), $k_{reverse}$ (DF=3; $F=0.43$; $P=0.73$), forward flux (DF=3; $F=0.40$; $P=0.75$), or reverse flux (DF=3; $F=0.1$; $P=0.95$). Forward fluxes were similar to reverse fluxes, as expected for an enzyme close to equilibrium. The overall forward/reverse (F/R) ratio calculated from a grand mean of 0.55 for the forward flux and 0.47 for the reverse flux was 1.17. Animals acclimated to 5‰ had a F/R ratio of 1.04, while animals acclimated to 35‰ had a F/R ratio of 1.67. For animals that experienced a change in salinity from 5 to 35‰ the F/R ratio was 1.04, while those that were changed from 35 to 5‰ had a F/R ratio of 1.01.

3.3. In vitro AK activity

Spectrophotometric assays of AK maximal activity for the forward reaction yielded values of 25.9 ± 1.8 μmoles s^{-1} g wet weight^{-1} ($n=5$) for animals acclimated to 5‰ and 19.7 ± 5.1 μmoles s^{-1} g wet weight^{-1} ($n=4$) for animals acclimated to 35‰. There was no significant difference between the mean values (Tukey's HSD, $P>0.05$). These activities have the same units as the flux measurements, and the in vitro maximal activities were approximately 40 times higher than the in vivo flux rates (Fig. 4). When normalized to protein concentration, the maximal activity values were also not significantly different at the two

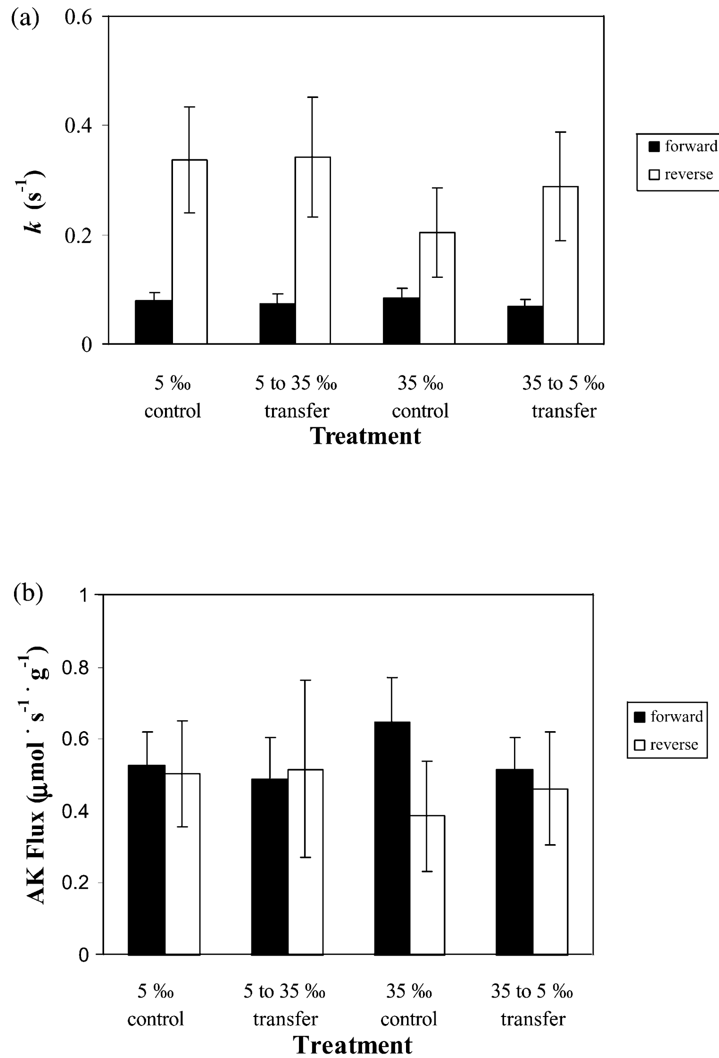


Fig. 4. Pseudo-first order unidirectional rate constants (a) and fluxes (b) for the AK reaction in crabs exposed to the 4 salinity treatments. Data are means ± S.E.M and $n=5$ for each salinity treatment.

acclimation salinities. The mean AK activity was 73.5 ± 13.2 μmoles s^{-1} mg protein^{-1} ($n=5$) at 5‰ and 69.9 ± 21.9 μmoles s^{-1} g wet weight^{-1} ($n=4$) at 35‰ (Tukey's HSD, $P>0.05$).

4. Discussion

There is a substantial body of literature indicating that salinity fluctuations, particularly hyposmotic challenges, are energetically costly to blue crabs. For instance, it has been demonstrated that a reduction in salinity leads to responses in the posterior gills, which are the principal site of active osmoregulation, that include increased rates of oxygen consumption (King, 1965; Engel et al., 1975; Piller et al., 1995) and glucose/amino acid oxidation (Pressley and Graves, 1983), a proliferation of transport epithelia cells (Aldridge and Cameron, 1982), and an upregulation of transport-related enzymes (e.g. Neufeld et al., 1980; Savage and Robinson, 1983; Péqueux, 1995; Henry, 2001). Additionally, indirect costs associated with salinity change also incur increases in metabolic demand. McGaw and Reiber (1998) have shown that in the blue crab the bulk of the increase in cardiac output that accompanies exposure to low salinity serves to enhance perfusion to the legs and mouthparts. This increased blood flow is associated with

behavioral responses to low salinity, such as increased movement and cleaning of the antennae, and not to osmoregulatory ion-pumping per se.

The current study, however, found no significant effects of acclimation salinity or rapid salinity changes on the concentrations of high-energy phosphate compounds or on AP/ATP ratios in the blue crab (Figs. 1 and 2). These results are similar to those found by Holt and Kinsey (2002) for isolated muscle from blue crab. If changes in salinity induced a sizable perturbation to the energetic status of cells in the organism, such as that typically caused by environmental challenges like hypoxia, then we would expect to see a decrease in the AP/ATP ratio and an increase in the P_i concentration (for reviews see Wasser et al., 1996; van den Thillart and van Waarde, 1996).

The lack of change in high-energy phosphate concentrations in the whole animal as well as in isolated muscles is not contradictory to the well-established ATP dependence of osmoregulation. Rather, these results imply that the osmoregulatory-induced increase in the rate of ATP demand is not sufficiently high to cause a discernible alteration in the whole-organism high-energy phosphate pool. AK activities and AP concentrations tend be highest in tissues with high rates of ATP demand such as muscle (Ellington, 2001), and crustacean muscle has particularly high AK activities and AP concentrations (e.g. Zammitt and Newsholme, 1976). Since muscle is the dominant tissue with respect to volume in the blue crab, it is likely that the bulk of the animal's total high-energy phosphate pool is located in muscle. It follows that the signal for the high-energy phosphate compounds in ^{31}P-NMR spectra is principally derived from the compounds in muscle. Since the concentrations in isolated blue crab muscle are not perturbed by extracellular osmotic changes (Holt and Kinsey, 2002), it is not surprising that whole animal metabolite concentrations are equally stable. Further, the ATP consumption that powers active ion-exchange associated with osmoregulation is largely confined to the posterior gills, which make up a very small fraction of the total metabolically active volume of blue crabs (Kinsey et al., 2003). There are also likely to be increases in the ATP demand of other physiological systems due to responses such as increased heart rate or increased movement of antennae or mouthparts, but the contribution of these tissues to the total high-energy phosphate pool is apparently too small to allow detection of localized changes in energetic status. It should be noted, however, that when gill tissues are measured in isolation, salinity changes do lead to the expected perturbations in the high-energy phosphate concentrations (Budd et al., 2000). Thus, the present results indicate that neither the direct costs of osmoregulation nor potential indirect perturbations to cellular energy status substantially impact the whole-animal pool of high-energy phosphate compounds.

The other principal finding was that AK rate constants and fluxes were not significantly altered by acclimation salinity or by rapid salinity change (Fig. 4). This is in contrast to our previous findings for isolated blue crab muscle, which had rate constants and flux rates that were proportional to the extent of the osmotic challenge (Holt and Kinsey, 2002). In this prior study, hyperosmotic conditions led to reductions in AK rate constants and fluxes, while hyposmotic treatments led to enhanced values. We suggested that the nearly 2-fold change in AK flux observed across the range of osmotic treatments was at least partially the result of a direct negative effect of inorganic ions on the enzyme. This conclusion was based on the fact that during rapid changes in blood osmolarity, cell volume regulation may be transiently achieved by compensatory changes in the concentrations of intracellular inorganic ions, which may perturb enzyme structure (Pierce, 1982). The accumulation or release of compatible or counteracting organic osmolytes in cells constitutes the principal means of long-term cell volume regulation, but this response may be implemented over a time course that is longer than that of our experimental treatments (Pierce, 1982; Holt and Kinsey, 2002). Thus, hyperosmotic conditions would lead to an influx of inorganic ions and hyposmotic conditions would lead to an efflux of these perturbing ions.

There are two likely mechanisms by which increasing concentrations of inorganic ions would lead to reduced rates of enzymatic flux. First, is the non-specific destabilizing effect that certain ions have on protein three-dimensional structure. In the blue crab, changes in the environmental salinity lead to variation in the intracellular concentrations of ions such as Na^+, K^+ and Cl^- (Gérard and Gilles, 1972) that are of the same magnitude as that required to dramatically impact enzyme function in vitro (Bowlus and Somero, 1979; Yancey et al., 1982; Yancey, 1994). As indicated above, this effect was suggested by Holt

and Kinsey (2002) to be the principle source of AK flux variation in isolated blue crab muscle subjected to osmotic challenges. An alternative possibility is that planar anions, such as Cl^-, may stabilize the abortive dead-end complex, enzyme-MgADP-arginine, similar to what has been seen for creatine kinase flux (McFarland et al., 1994). This would mean that as the intracellular concentration of planar anions like Cl^- increase during hyperosmotic treatments, AK flux would be reduced. However, the anion stabilization in AK is less pronounced than in creatine kinase, and Cl^- has not been shown to be an effective stabilizer of the dead-end complex (Anosike and Watts, 1975). Thus, the non-specific, destabilizing effect on protein structure appears to be the most likely source for salinity-induced changes in enzyme flux.

Why then did we not observe an effect of salinity change on AK flux in vivo that was similar to that found in isolated muscle? Unlike isolated muscle, the integrated osmoregulatory response in the intact juvenile blue crab is apparently adequate to fully preserve AK function even during rapid, dramatic changes in salinity. Blue crab hemolymph osmolarity varies by approximately 500 mOsm over the range of environmental salinities that the crab encounters (Mantel and Farmer, 1983; Piller et al., 1995). However, the ion-transporting posterior gills can temporally buffer hemolymph osmolarity, such that instantaneous changes in environmental salinity lead to more gradual changes in hemolymph osmolarity (Gilles, 1979; Towle et al., 1994). This slow change in hemolymph osmolarity may allow sufficient time for cells to increase or decrease the concentration of organic osmolytes so that potentially detrimental inorganic ions need not be the principal means of cell volume regulation. In the present study, the acute change in environmental salinity of 30‰ experienced by the animal may have led to a much less dramatic change in hemolymph osmolarity. Hence, the cells (and AK) would experience a considerably less substantial osmotic challenge than the experimental protocol might suggest, leading to a preservation of AK flux in all of the salinity treatments.

While the above explanation may adequately account for the discrepancy between the present in vivo results and our previous findings using isolated muscle tissues, the question remains as to the meaningfulness of the data obtained from isolated muscle described in Holt and Kinsey (2002). Isolating tissues always introduces bias in that it removes the tissue from an environment that provides, for example, circulating fuel molecules, extracellular signals such as hormones, and neural input. This may be particularly important when the tissue function that is being studied is substantially modified by responses at a higher level of biological organization. For example, Clemens et al. (1998) have demonstrated that in the lobster, *Homarus gammarus*, peripheral sensory feedback contributes to the control of stomatogastric activity in the gut of the intact animal, but these peripheral modifications to neural input are not observable in vitro. On the other hand, the in vitro analyses of Clemens et al. (1998) allowed them to identify those processes that were independent of peripheral input, and the authors point out the necessity of both types of experiments. Similarly, the initial study of Holt and Kinsey (2002) indicated that a vulnerability to osmotic change exists at the tissue level, while the present study revealed that compensations at higher levels of biological organization offset these effects. Thus, the in vivo and ex vivo experiments are complimentary and aid our understanding of the integrated physiological defenses against salinity change. It should also be noted that the study by Holt and Kinsey (2002) was, in fact, conducted at a higher level of organization (tissue) than had previously been examined, since the consequences of changes in the ionic environment on enzyme function were elucidated in studies that used in vitro manipulations of purified enzymes.

The in vitro AK activity was also not significantly altered by acclimation salinity, which is consistent with the results for AK flux. However, the in vitro values were approximately 40-fold higher than the flux measurements. This discrepancy is typically observed for AK (Briggs et al., 1985; Platzer et al., 1999; Holt and Kinsey, 2002) and reflects the fact that enzymes that are near equilibrium must have maximal velocities that are much higher than their flux rates (Suarez, 1998). The AK F/R flux ratios were very near a value of one, supporting the notion that AK is near equilibrium in vivo and similar to previous results (Graham et al., 1986; Holt and Kinsey, 2002).

In conclusion, we found that neither acclimation salinity nor acute salinity change had an effect on in vivo high-energy phosphate concentrations, AK flux rates, or AK activity. This suggests that in juvenile blue crabs a rapid change in environmen-

tal salinity does not constitute a substantial challenge to the whole-animal cellular energy status nor does it perturb enzyme function. This is in agreement with field observations that very small juvenile blue crabs effectively utilize low salinity habitats.

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