Partial Characterization of Na^+, K^+- ATPase and its Gill and Antennal Gland Osmoregulatory Role in the Crayfish, *Orconectes putnami*.

A Thesis

Presented to

the Faculty of the College of Science and Technology

Morehead State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

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by

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Director of Thesis

Master's Committee: _____, Chair

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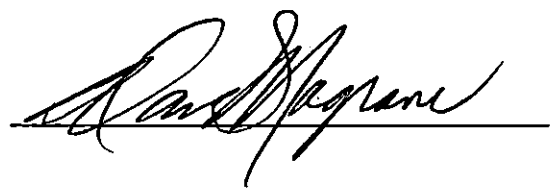
Director of Thesis: 

Crayfish that live in a hypotonic environment excrete copious amounts of very hyposmotic urine (Prichard and Kerley 1970). Ionic homeostasis must be maintained by absorption of ions across the gills, and reabsorption of ions from the urine by the antennal gland. The enzyme responsible for this absorption and reabsorption is Na^+, K^+- ATPase. This study was undertaken to partially characterize Na^+, K^+- ATPase in the fresh water crayfish, *Orconectes putnami* and determine the osmoregulatory mechanisms. *Orconectes putnami* were placed in distilled water, or following acclimation to distilled water into 8.75, 17.5, or 25.5 g NaCl / L . Three crayfish were sampled every two days. Antennal gland and gill Na^+, K^+- ATPase activities were determined as well as Na^+ concentrations in urine and hemolymph. Results show that antennal gland as well as gill Na^+, K^+-

ATPase activities decreased as salt concentrations in the media increased. As the concentration of the external media was increased from distilled water to 17.5 g/L of NaCl, the urine concentration increased from 0.165 mg/ml to 4.152 mg/ml Na^+ after ten days exposure. Hemolymph Na^+ concentrations demonstrated a slight increase (2.420 mg/ml Na^+ in distilled water to 3.162 mg/ml Na^+ in 17.5 g/L NaCl media for ten days) as the salt concentration in the media increased. This indicated that *Orconectes putnami* is able to tolerate and osmoregulate in saline conditions. This tolerance was not maintained, however, in 25.5 g NaCl / L as all crayfish placed in this media died within five days. Na^+, K^+- ATPase partial characterization was found to correspond well with previous studies.

Accepted by: _____, Chair

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Introduction

Osmoregulation in crayfish

Crayfish that live in a hypotonic environment must excrete copious amounts of hyposmotic urine (Pritchard and Kerley 1970). Even though crayfish living in fresh water demonstrate a reduced permeability to salts and water through their integument (Rudy 1967), ionic homeostasis must be maintained by the transportation of ions across the gills (Dickson et al. 1991, Ehrenfeld 1974, Kisiol 1988, Shaw 1960, Wheatley and Henry 1987) and reabsorption of ions, specifically Na^+, from urine across the antennal gland (Peterson and Loizzi 1974, Sarver et al. 1994, Sarver et al. 1988, Wheatley and Henry 1987). In intermolt, one of the primary concerns is sodium regulation (Wheatley and Gannon 1995). The enzyme responsible for this reabsorption of ions is Na^+, K^+- ATPase (Dickson et al. 1991, Ehrenfeld 1974, Horiuchi 1977, Kisiol et al. 1988, Peterson and Loizzi 1974, Shaw 1960). This enzyme is believed to power a sodium pump that is similar to the pump found in other animal cells (Holiday 1985). Henry and Wheatly (1988) concluded that crayfish acclimated to fresh water maintain ionic concentrations significantly above the ion concentration in the media through a combination of active ion uptake and then reabsorption of ions from urine.

The Na+, K+-ATPase enzyme

Na^+, K^+-ATPase, a heterodimeric protein, is composed of a large multi-membrane spanning α subunit and a small glycosylated membrane protein designated as the β subunit (DeTomaso et al. 1994). Hastings and Renolds (1979) determined the molecular weight of the catalytic peptide (α subunit) to be 106,400 ± 3,000 gm protein / mole complex and the molecular weight of the glycopeptide (β subunit) to be 3,600 ± 1,500 gm protein / mole complex. (See Figure 1) The Na^+, K^+- ATPase pumps three sodium and two potassium ions for every ATP hydrolyzed (Goldin 1979).

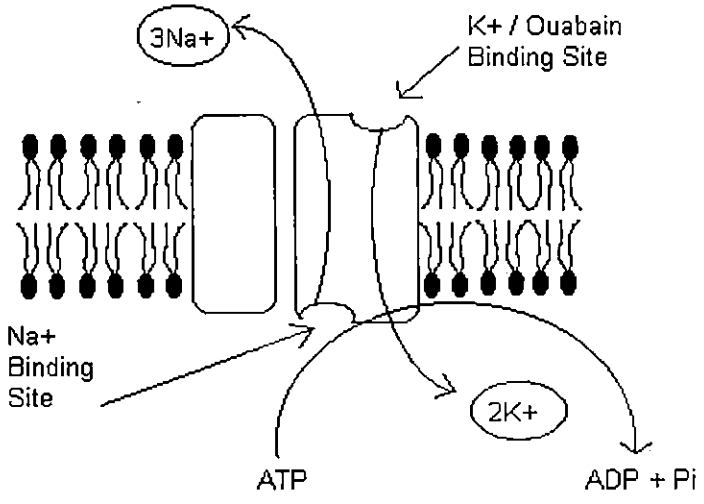

Figure 1. Generalized model of the Na^+, K^+-ATPase enzyme. Modified from Alberts et al. 1994.

It is widely accepted that the α subunit mediates the actual catalytic function of the enzyme (Lingrel et al. 1991), but the function of the β subunit has been debated. Geering (1991) noted that in the absence of β subunits, newly synthesized α subunits are not completely folded, and the α and β subunit are both required for correct movement from the endoplasmic reticulum to the cell membrane. Research by Kawamura and Noguchi (1991) suggested that at least one role of the β subunit is to promote the correct assembly of the α subunit into the cell membrane. If the β subunit is absent, irregular insertion of the α subunit into the cell membrane transpires, and no Na^+, K^+-ATPase activity occurs. Jaunin et al. (1993) also state that the β subunit plays a central role in post-translational processing and transport of the α subunit to the cell membrane. Through mutation and chimera experiments, it was determined that the transmembrane domain of the β subunit is important for assembly of the α subunit and that the β subunit NH_2-terminal transmembrane and the extra cytoplasmic domain are essential for high potassium affinity. The hypothesis that an interaction between transmembrane regions of the α and β subunits brings the subunits together and allows interaction of the ectodomains has been proposed by DeTomasso et al. (1994). They suggest that due to the speed and specificity of subunit assembly, individual subunits must possess and present domains that

recognize the complementary subunit and that the subunit exists in a stable, partially folded state before oligomerization. They also state that it is not known if oligomerization relies on mechanisms present in the endoplasmic reticulum. Their research demonstrates that the α and β subunits may exist in stable configurations within the cytoplasm of cells and that each subunit can be transported independently from the endoplasmic reticulum. They hypothesize that the α and β subunits are targeted to the cell membrane independently and that assembly is not an endoplasmic reticulum restricted activity action. The subunits may exist as independent membrane proteins and in Na^+, K^+-ATPase activity may be regulated by assembly independent of the endoplasmic reticulum. This may provide another mechanism for ion transport regulation (DeTomasso et al. 1994).

Sites of osmotic regulation

Wheatly and Henry (1987) reported that Na^+, K^+- ATPase activity, and therefore the enzyme, is uniformly distributed throughout gill sets two through seven in the euryhaline crayfish, *Pacifastacus leniusculus*. This conclusion arose from tissue preparations from entire gill sets. Dickson et al. (1991), utilizing silver staining and transmission electron microscopy concluded that

gill filaments are found not to be homogenous in structure and function with some filaments being devoted to ion transport and other filaments devoted to respiration. Ion transporting filaments in *Procambaras clarkii* were found to possess a thicker layer of epithelium than filaments dedicated to respiratory function. The Na^+, K^+- ATPase containing epithelium also demonstrated an increased basal folding and an increased number of mitochondria (Dickson et al. 1991). Furthermore, the ion transporting filaments were shown to be in the central body with the respiratory filaments in lateral rows in most crayfish gills (Dickson et al. 1991).

Reabsorption of ions from urine takes place in the antennal gland (Peterson and Liozzi 1974, Sarver et al. 1994, Wheatly and Henry 1987). Peterson and Loizzi (1974) found the highest specific activity of the Na^+, K^+-ATPase in the tubular portion of the gland (Figure 2), providing evidence of its role in urine dilution.

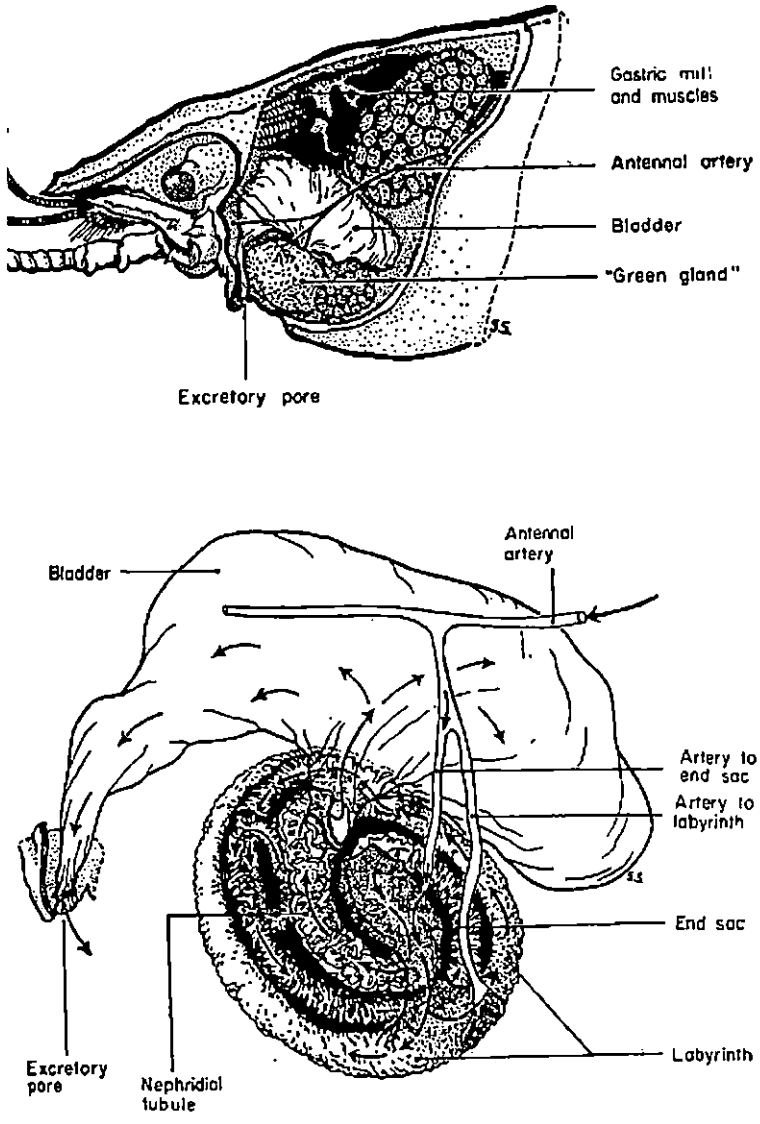

Figure 2. Lateral view of the structure and position of the antennal gland in the crayfish *Astacus pallipes*. Taken from Potts and Parry (1963).

Examples of Na^+, K^+- ATPase Enzyme Specific Activity (ESA)

Kerley and Pritchard (1967) conducted survival experiments and concluded that *Pacifastacus leniusculus*, naturally can tolerate step-wise acclimation up to seventy percent sea water, and by utilizing hyposmotic regulation can survive in the seventy percent sea water for at least twenty-one days. They also determined that in *Pacifastacus* osmotic regulation did not occur due to water shifting across body compartments. Only crayfish exposed to one hundred percent sea water experienced a gain in internal water volume. Wheatley and Henry (1987) found that the specific activities of Na^+ , K^+ - ATPases are two-fold higher in the antennal gland than in the gill of *Pacifastacus leniuscalus,* and that urine production was hyposmotic to hemolymph due to active resorption of electrolytes in the antennal gland. *Pacifastacus* were able to osmoregulate in 350 mosmol / kg sea water, but in 750 mosmol / kg, the crayfish became isosmotic. Pritchard and Kerley (1970), determined that when *Pacifastacus leniusculus* was acclimated to increasing salinities, the ionic concentration of the urine remained low in fresh water, twenty percent sea water, and forty percent sea water due to reabsorption of ions. Urine / Hemolymph ratios of sodium ions remained below one, ranging from .07 in fresh water to .266 in seventy percent sea water. This research also found that antennal gland Na^+, K^+- ATPase activity

inversely correlated with ion concentration in urine further lending evidence that Na^+, K^+- ATPase is responsible for reabsorption of ions from urine (Prichard and Kerley 1970). Henry and Wheatly (1988) observed a significant reduction in gill Na^+, K^+- ATPase activity when fresh water acclimated *Pacifastacus leniusculus* were placed in either 350 of 750 mOsm sea water for 48 hours. Antennal gland Na^+, K^+- ATPase enzyme specific activity (ESA) in fresh water acclimated animals did not significantly change after a 48 hour exposure to 350 mOsm sea water. However, when exposed to 750 mOsm for 48 hours, antennal gland Na^+, K^+- ATPase ESA significantly increased, but then decreased below fresh water Na^+, K^+- ATPase ESA levels in the third week (Henry and Wheatly 1988). Sarver et al. (1994) conducted studies on *Procambarus clarkii* and determined that this crayfish is a strong hyper osmoregulator in fresh water and twenty-five percent sea water, and is a weak hyper osmoregulator in fifty and seventy-five percent sea water. Antennal gland Na^+, K^+- ATPase ESA was found to be highest in *Procambarus clarkii* acclimated in fresh water and decreased significantly in the crayfish acclimated for two weeks or two months in fifty percent and seventy-five percent seawater. In the gills of a osmoregulating crustacean, such as the mud fiddler crab, *Uca pugnax*, Holiday (1985), concluded that changes in gill Na^+, K^+- ATPase ESA appear to only be important in osmotic

regulation. Neufeld et al. (1980) suggests that changes in gill Na^+, K^+-ATPase activity in the blue crab (*Callinectes sapidus*) are due to synthesis and degradation rather than activation of existing enzyme.

Effects of Cardiac Glycosides on Na^+, K^+- ATPase

Na^+, K^+- ATPase is inhibited by the interaction of a class of compounds called cardiac glycosides (Bonting 1970, Lingrel et al. 1991, Prosser 1991, Schmidt-Nielson 1990). Experimentally, the most widely utilized cardiac glycoside is ouabain due to its solubility in water (Bonting 1970, Prosser 1991). Other cardiac glycosides are soluble in alcohol, and alcohols have been shown to inhibit ATPases (Bonting 1970). The binding site of ouabain on Na^+, K^+- ATPase is believed to be the same as the binding site for K^+, since the addition of K^+ reduces the binding of ouabain as the ouabain and K^+ compete for the same binding domain (Akera et al. 1979). Removal of K^+ has been shown to reduce Na^+, K^+- ATPase activity by 92% with the resulting activity probably due to K^+ present in the tissue preparation (Holliday 1985). Ouabain has also been shown to reduce Na^+, K^+- ATPase activity by 87% in 30 mM K^+(Holliday 1985). Therefore, it can be assumed that ouabain will cause a complete inhibition of Na^+, K^+- ATPase in the

absence of K^+ (Holliday 1985). Bonting (1970) demonstrated complete inhibition of Na^+, K^+- ATPase by a concentration of 10^{-4} M ouabain. Sarver et al. (1994) found that 1 mM ouabain caused at least a 90% inhibition of Na^+, K^+- ATPase, and Koisiol et al. (1988) demonstrated at least 94% inhibition of Na^+, K^+- ATPase by 5 mM ouabain. Ouabain has no effect on Mg^{2+} ATPase (Bonting 1970, Hourichi 1977) further increasing the value of ouabain as a specific research tool for the study of Na^+, K^+- ATPase.

Research objectives

The basic objective of this research was to study osmoregulation in a local fresh water crayfish, *Orconectes putnami*. Questions addressed by the study were: 1. Does antennal gland as well as gill Na^+, K^+- ATPase decrease as environmental salinity increases? 2. How does urine and hemolymph Na^+ concentrations change with increasing environmental salinities? 3. Does the fluctuation in antennal gland and gill Na^+, K^+- ATPase correspond with urine and hemolymph Na^+ concentrations? The following experiments were then designed to provide answers to the questions addressed by the study. Crayfish were observed under increasing salt stresses by placement in distilled water, 8.75, 17.5, 25.5 g NaCl / L. Osmoregulation was examined by

performing Na^+, K^+- ATPase assays at two major sites of sodium regulation, the antennal gland and the gills. Urine as well as hemolymph Na^+ concentrations were taken simultaneously with gill and antennal gland samples. Urine:Hemolymph ratios were also calculated to observe the relative Na^+ content of urine and hemolymph.

Materials and Methods

Collection, treatment, and maintenance of crayfish

Crayfish (*Orconectes putnami*) were collected from Tygart's Creek in Carter County, Ky. The crayfish were identified according to Hobbs (1972) and then held in the animal care facility in Lappin Hall at Morehead State University. The crayfish were acclimated to distilled water that was changed every day, for a two week period preceding each experiment. The crayfish were maintained on a 12 Light :12 Dark photoperiod at approximately 25 ^{0}C, and were supplied with Gainesburgers dog food once a week, *ad libitum*, every seventh day.

Osmolarity of body fluids

Urine and hemolymph samples were taken using the method of Sarver et al. (1994). Urine was sampled from the nephropores of the crayfish aspiration with a plastic Pasteur pipette (Figure 3). Hemolymph was sampled through the arthrodial membrane of the ventral abdomen or from the base of various walking legs using a 1 cc syringe with a 27 gauge needle

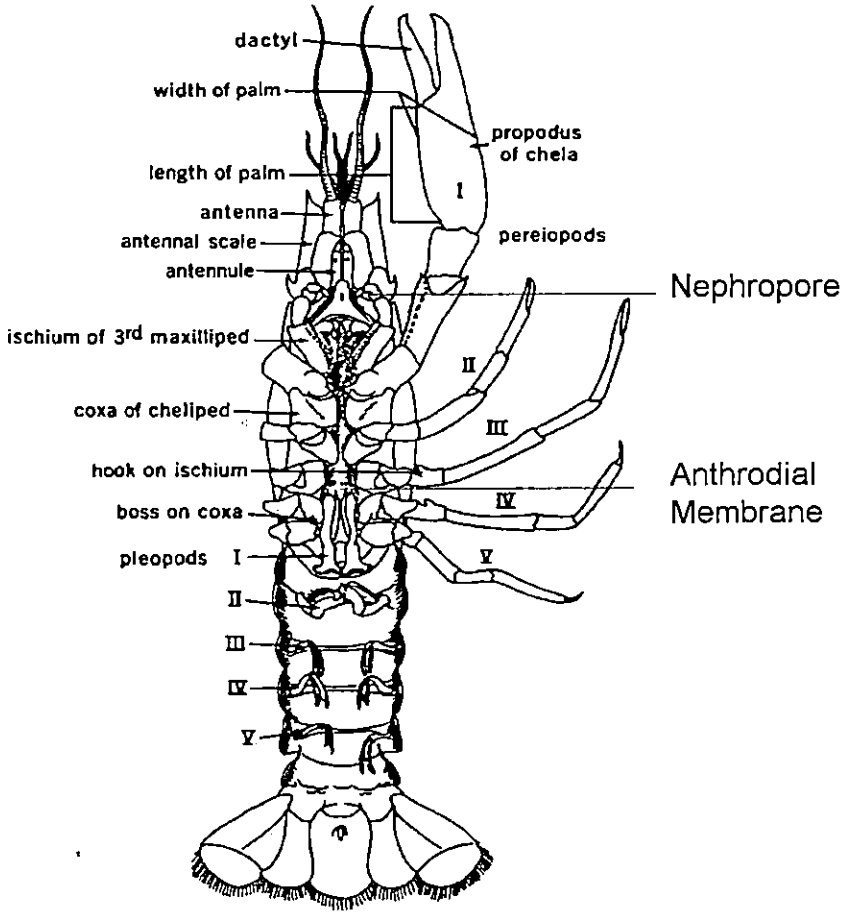

Figure 3. Ventral view of a generalized crayfish illustrating structures and common reference points. Modified from Hobbs (1972).

(Figure 3). Both hemolymph and urine samples were stored in 1.5 cc Eppendorf tubes and frozen at -20 ^{0}C until assayed. For analysis, the tubes of hemolymph were thawed and centrifuged at 16,000 x g for ten minutes at room temperature. After centrifugation, 6 µl of sample was diluted with 5 ml of deionized water. The sodium content of the urine as well as the hemolymph samples were then measured by flame emission at 389.5 nm using a Hitachi 170-30 Atomic Absorption Spectrophotometer. A Microna Voltmeter was connected to the spectrophotometer for greater precision and accuracy. Sodium chloride standards of 1.0, 0.7, 0.5, and 0.1 ppm were prepared using water that was purified using a Sybron\Barnstead NANOpure-A 4-holder system.

Crayfish sacrifice and preparation of homogenate

Crayfish were sacrificed by removal of the head posterior to the eyestalks with a scalpel. Entire antennal glands were harvested when possible. Excess tissue was removed. The paired antennal glands were then rinsed in ice cold sucrose homogenizing buffer [0.25 M sucrose, 6 mM ethylene diamine tetra-acetic acid (EDTA) according to Sarver et al. 1994]. Gill tissue was sampled from the gills attached to the right and left, second

and third walking legs. The gill tissue was also treated in cold sucrose supplemented with EDTA. Homogenates were prepared with an Omni 5000 International homogenizer using a 5 mm x 95 mm flat bottom generator while keeping the homogenate on ice. Protein concentration of the homogenate was measured by colorimetry using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard.

Optimization studies

Two optimization studies were conducted as modified versions of the methods of Sarver et al. (1994). The first study examined the effect of various potassium concentrations on antennal gland Na^+, K^+-ATPase activity with a constant $Na^+ + K^+$ concentration of 130 mM. A summary of the media for the K^+ optimization study is shown in Table 1. The second study examined the effect of various magnesium concentrations upon the Na^+, K^+-ATPase with a constant ATP concentration of 5 mM. Table 2 indicates the contents and amounts of the substrates present in the Mg^{2+} optimization study.

Table 1. Summary of media utilized in the potassium optimization study

Media	K^+	Na^+	Mg^{2+}	ATP	Imidazol
1	1mM	129mM	10mM	5mM	20mM
2	3mM	127mM	10mM	5mM	20mM
3	5mM	125mM	10mM	5mM	20mM
4	7mM	123mM	10mM	5mM	20mM
5	10mM	120mM	10mM	5mM	20mM
6	20mM	110mM	10mM	5mM	20mM
7	30mM	100mM	10mM	5mM	20mM
8	50mM	80mM	10mM	5mM	20mM

Table 2. Summary of media utilized in the magnesium optimization study

Media	K^+	Na^+	Mg^{2+}	ATP	Imidazol
1	30 mM	100mM	0mM	5mM	20mM
2	30 mM	100mM	1mM	5mM	20mM
3	30 mM	100mM	2mM	5mM	20mM
4	30 mM	100mM	4mM	5mM	20mM
5	30 mM	100mM	6mM	5mM	20mM
6	30 mM	100mM	8mM	5mM	20mM
7	30 mM	100mM	10mM	5mM	20mM
8	30 mM	100mM	14mM	5mM	20mM

Acclimation study

Polycarbonate containers (1' x 2') were filled with 1 liter of either distilled water, 8.75 g NaCl / L, 17.5 g NaCl / L, or 25.5 g NaCl / L. Twenty crayfish were added to each container. Three crayfish from each media were sacrificed every two days after placement in the various environments. Urine, hemolymph, antennal, and gill samples were all taken from each crayfish upon sacrifice.

Na^+, K^+-ATPase assay

The enzyme assay used to measure the specific activity of Na^+, K^+-ATPase was modified from the methods used by Kosiol et al. (1987), Holliday (1985), and Sarver et al. (1994). Two media were used in the study. Each media contained specific concentrations of sodium, potassium, ATP, magnesium, and imidazole. The first medium contained 100.0 mM Na^+, 30.0 mM K^+, 5.0 mM ATP, 5.0 mM Mg^{2+}, and 20.0 mM imidazol. The phosphorous liberated by this media was a measure of all ATPases in the homogenate. The second medium ,which did not contain K^+, assayed all the ATPases except Na^+, K^+-ATPase. This medium contained 130 mM Na^+, 5.0 mM ATP,

5.0 mM Mg^{2+}, 20.0 mM imidazole, and 1.0 mM ouabain. The ouabain was added to the second media to keep any K^+ in the homogenate from stimulating Na^+, K^+-ATPase (Bonting 1970, Holiday 1985, Horiuchi 1977, Sarver et al. 1994). The pH of each media was adjusted to 7.25 by the addition of Tris. Sixty-seven µl of fresh homogenate was added to 200 µl of incubation media and incubated for fifteen minutes in a 30 ^{0}C water bath. The reaction was stopped by the addition of 800 µl of 10% TCA and the phosphorus level in each tube was measured. The enzymatic specific activity of Na^+, K^+- ATPase was calculated by taking the difference in phosphate liberated by each homogenate, and noted as mg P_i X mg^{-1} protein X hr^{-1}. The liberated phosphorus was determined immediately after the addition of the 10% TCA and subsequent centrifugation at 16,000 x g for ten minutes. The phosphorus was determined using the method described by Bonting (1970). One ml of the supernatant was added to one ml of color reagent. The color reagent was composed of a 1% ammonium molybdate solution in 1.15 M H_2SO_4 with 40 mg of $FeSO_4$ added for each ml of ammonium molybdate solution. After the addition of the $FeSO_4$ the color reagent is stable for two hours (Bonting 1970). The developed color was measured using a Fisher Spectro Master model 415 spectrophotometer at 700 nm. Standards of 0.50, 0.25, 0.125, 0.0625, 0.031 mg/ml Pi were used.

Statistical analysis

Student's unpaired *t*-test was used to determine if a significant change occurs between value means. The t-test as well as all graphical data was performed using PSI-Plot Version 4.53. In all graphs each point represents the mean ± the standard error of measurements from 3 crayfish. In Figures 6-9, asterisks indicate a significant difference ($p<.05$) from the distilled water environment. In Figures 10-12, asterisks indicate a significant difference ($p<.05$) from the previous sample.

Results

Optimization studies

The effect on antennal gland Na^+, K^+- ATPase activity of varying K^+ concentration with a constant Na^+ + K^+ concentration of 130 mM is shown in Figure 4. As K^+ concentration increaced the Na^+, K^+- ATPase activity increased until a peak is reached at 20-30 mM K^+. At K^+ concentrations above 30 mM the enzyme activity decreased.

The effect on Na^+, K^+- ATPase activity of varying Mg^{2+} concentrations with a constant ATP concentration of 5 mM is shown in Figure 5. As Mg^{2+} concentration increased there was an increase in enzyme until a peak is attained at 4-6 mM Mg^{2+}. As the Mg^{2+} concentration was increased above 6 mM, the Na^+, K^+- ATPase activity demonstrated a gradual decline.

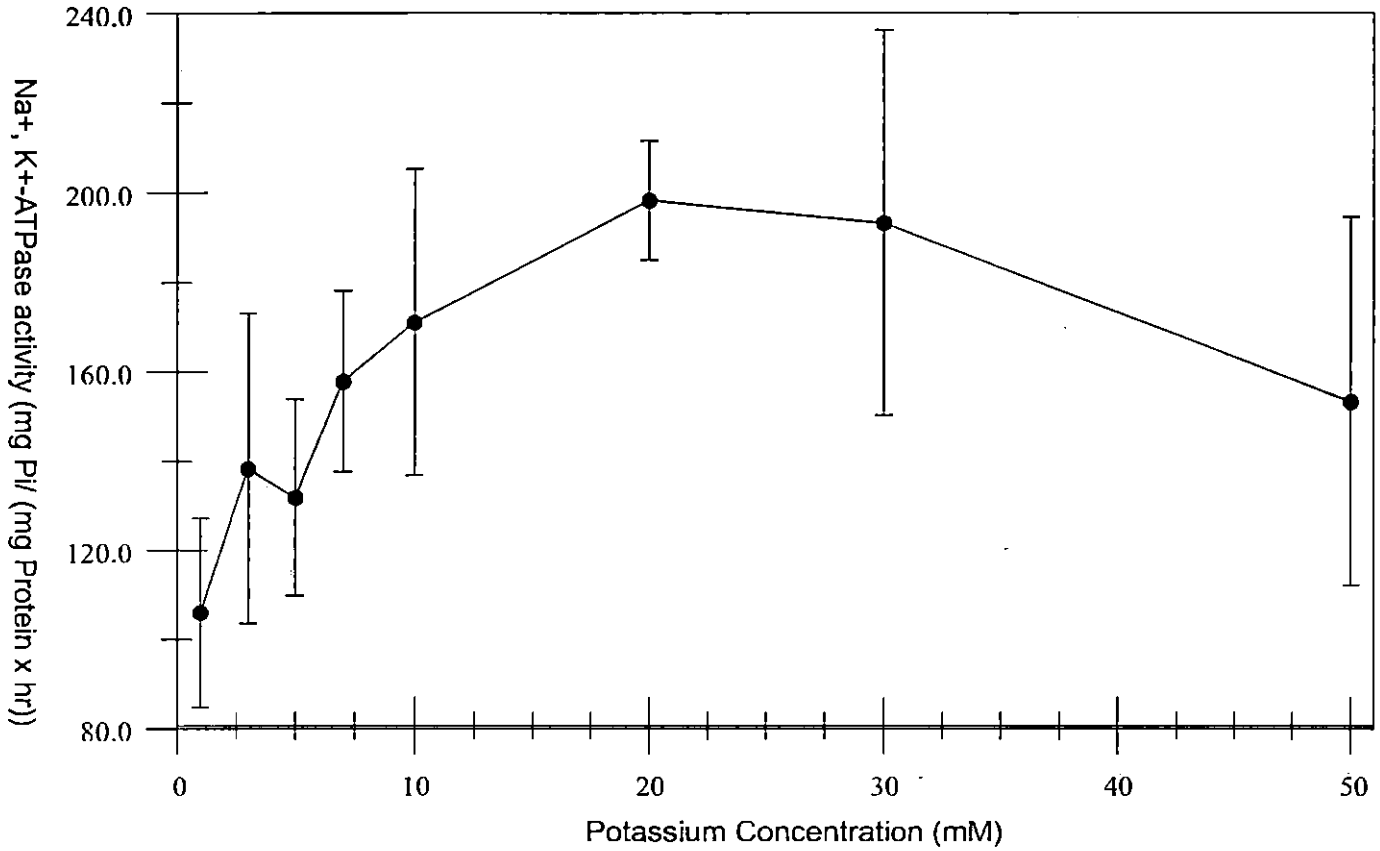

Figure 4. Effect of varying K+ concentration on antennal gland Na+, K+ -ATPase activity with a constant Na+ + K+ concentration of 130 mM

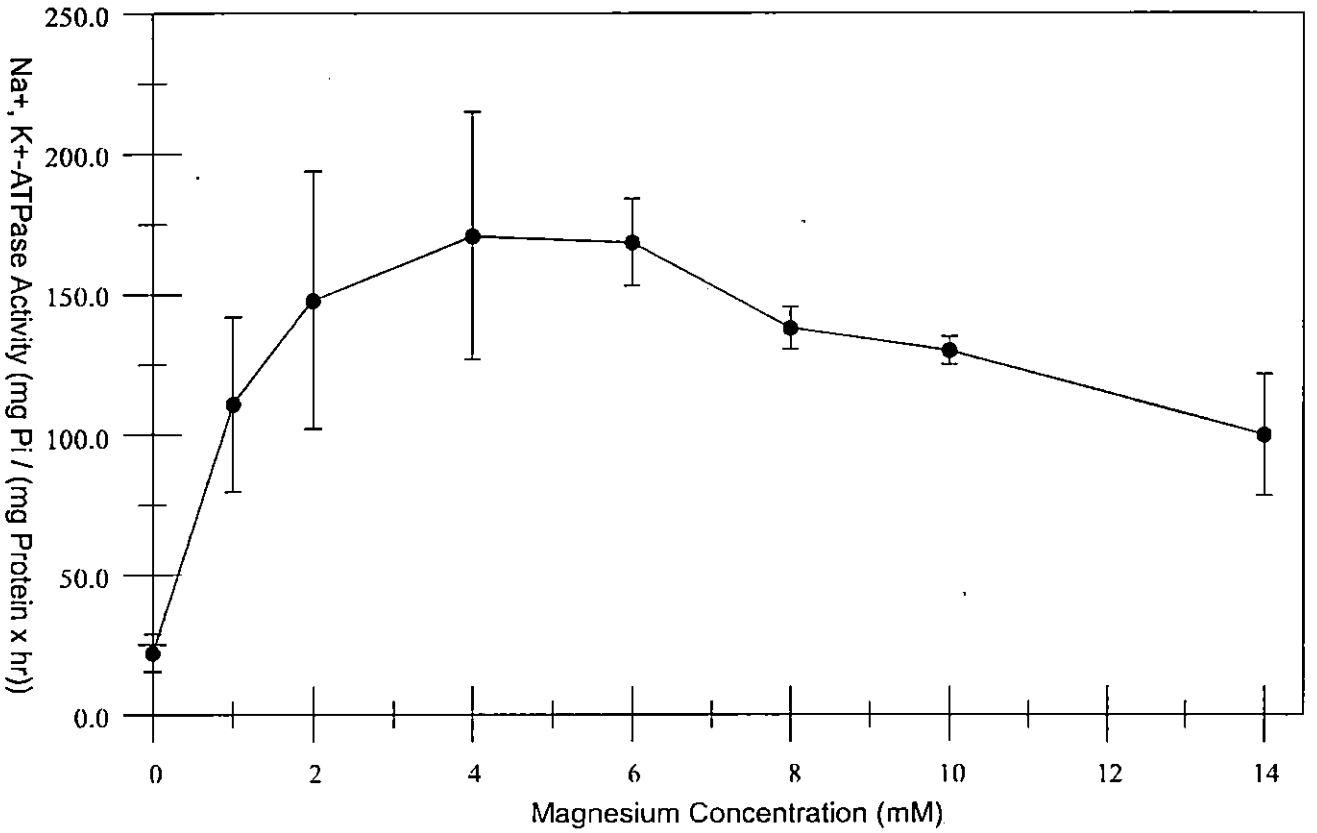

Figure 5. Effect of varying Mg2+ concentration on antennal gland Na+, K+ -ATPase activity with a constant ATP concentration of 5 mM

Antennal gland Na^+, K^+- ATPase activity

The effect of increasing doses of NaCl on antennal gland Na^+, K^+- ATPase activity is shown in Figure 6. It can be seen that when control organisms were placed in distilled water over a 10 day period, the Na^+, K^+- ATPase activity was highly variable. Fluctuations as great as 100 units (mg Pi / (mg Protein x hr)) of Na^+, K^+- ATPase activity were seen. Crayfish in all salinities demonstrated an increase in Na^+, K^+- ATPase activity at two days exposure. All crayfish in a media of 25.5 g NaCl / L died before the end of day five, and therefore, sampling for this environment did not continue past day four. After day two, there was a general decrease in Na^+, K^+- ATPase activity in the 17.5, and 25.5 g NaCl / L. The only significant difference from distilled water occurred at day eight in the 8.75 g ($T=2.81$, $p=0.094$) and 17.5 g NaCl / L ($T=5.42$, $p=0.023$), when the distilled water sample's enzyme activity increased for the second time.

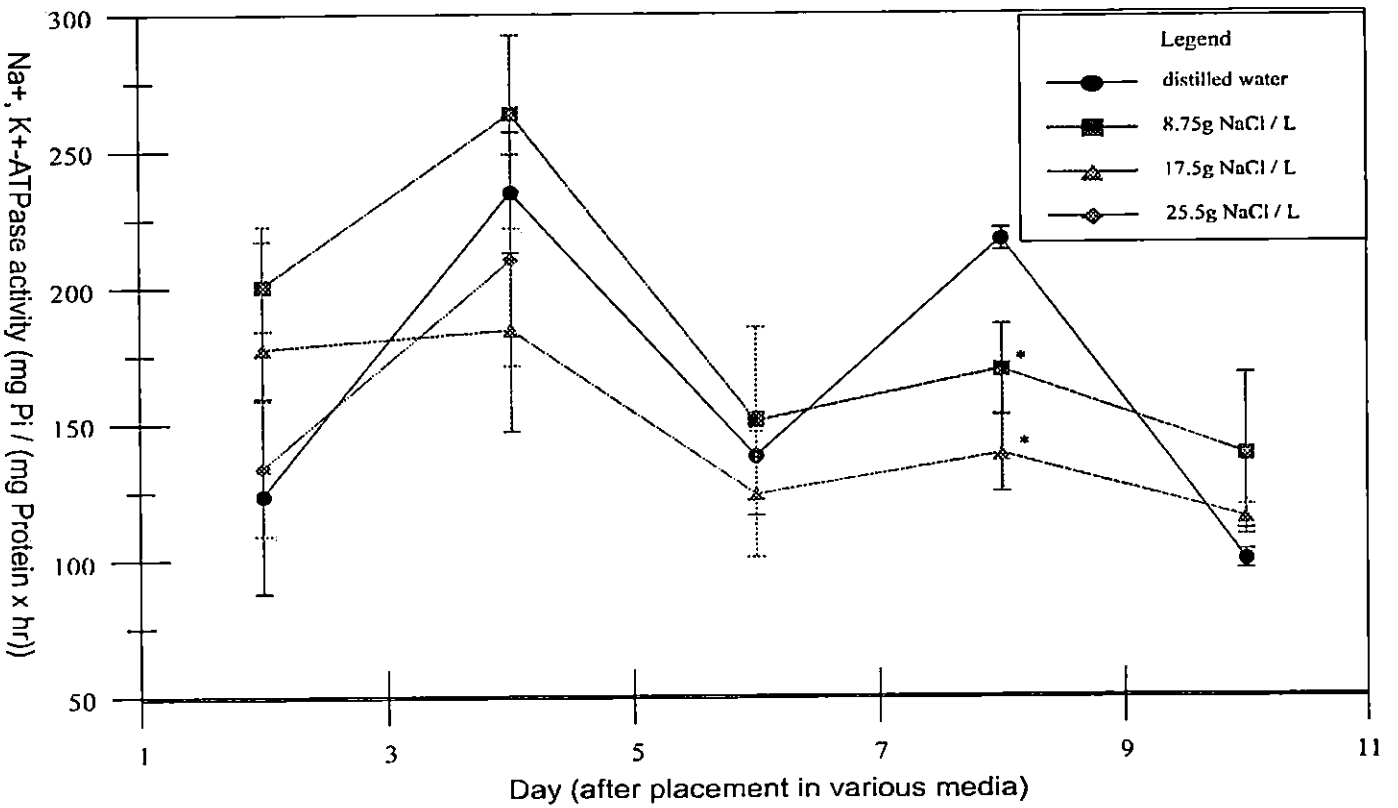

Figure 6. Antennal gland Na+, K+-ATPase activity after placement in various environments

Gill Na^+, K^+- ATPase activity

Gill Na^+, K^+- ATPase activity demonstrated an initial increase at day four in all media except 17.5 g NaCl / L (Figure 7). The distilled water and 8.75 g NaCl / L sample had a peak of ATPase activity at day 4. They each exhibited a decline in the enzyme activity before stabilizing at day 6 for the distilled water and day 8 for the 8.75 g NaCl / L samples. The 17.5 g NaCl / L sample's activity gradually declined through the experiment. A significant difference in gill Na^+, K^+- ATPase activity occurred at day four in the 17.5 g ($T=3.51$, $p=0.050$) and 25.5 g NaCl / L ($T=4.74$, $p=0.012$) media in comparison with the distilled water medium. A significant difference in gill Na^+, K^+- ATPase activity also occurred at day eight in 8.75 g NaCl / L ($T=4.90$, $p=0.021$), and at day ten in 17.5 g NaCl / L ($T=3.94$, $p=0.017$) in comparison with the distilled water medium.

Hemolymph and urine Na^+ concentrations

Sodium concentrations in hemolymph and urine following exposure of crayfish to various media for 10 days are shown in Figure 8 and 9 respectively. All media, in comparison to distilled water, at all days contained a significant increase in Na^+.

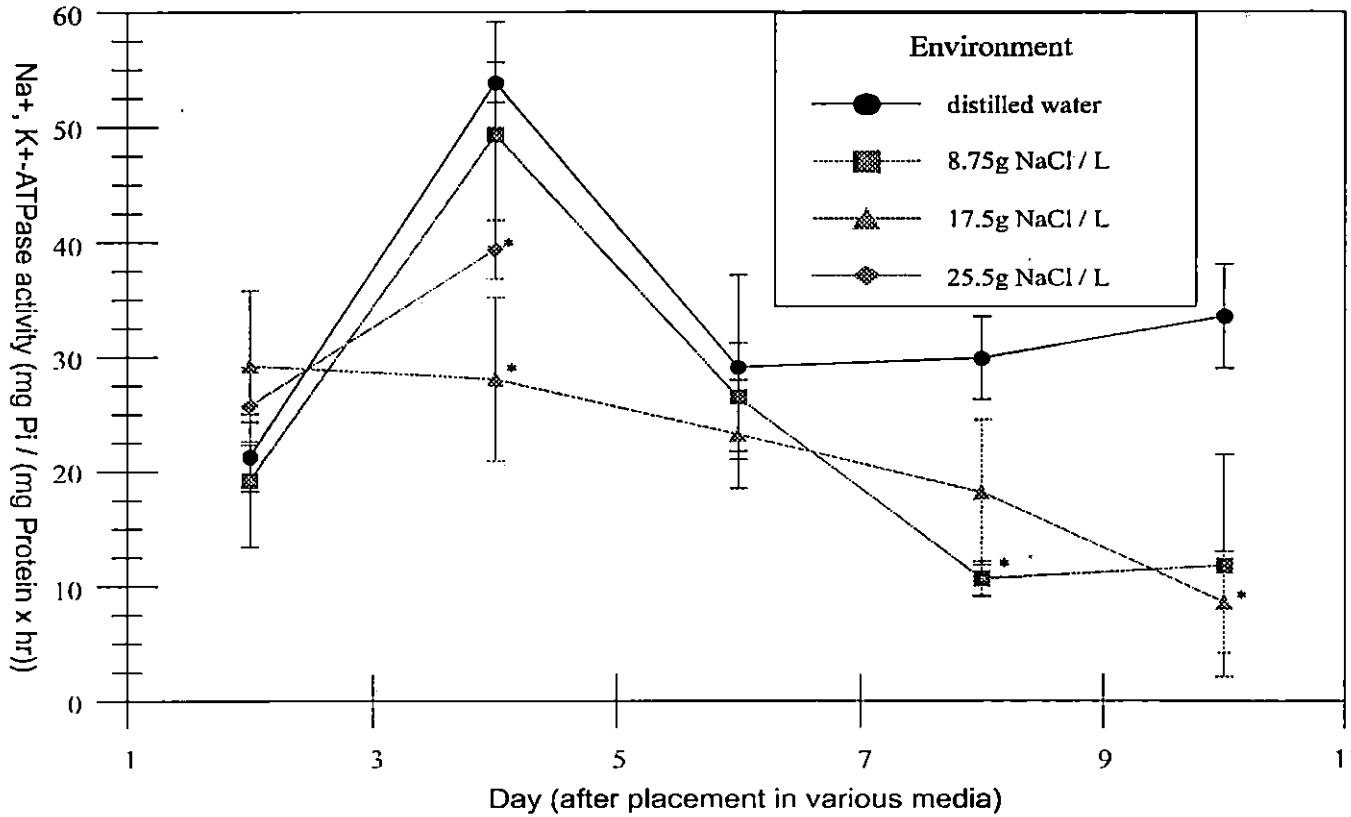

Figure 7. Gill Na+, K+-ATPase activity after placement in various environments

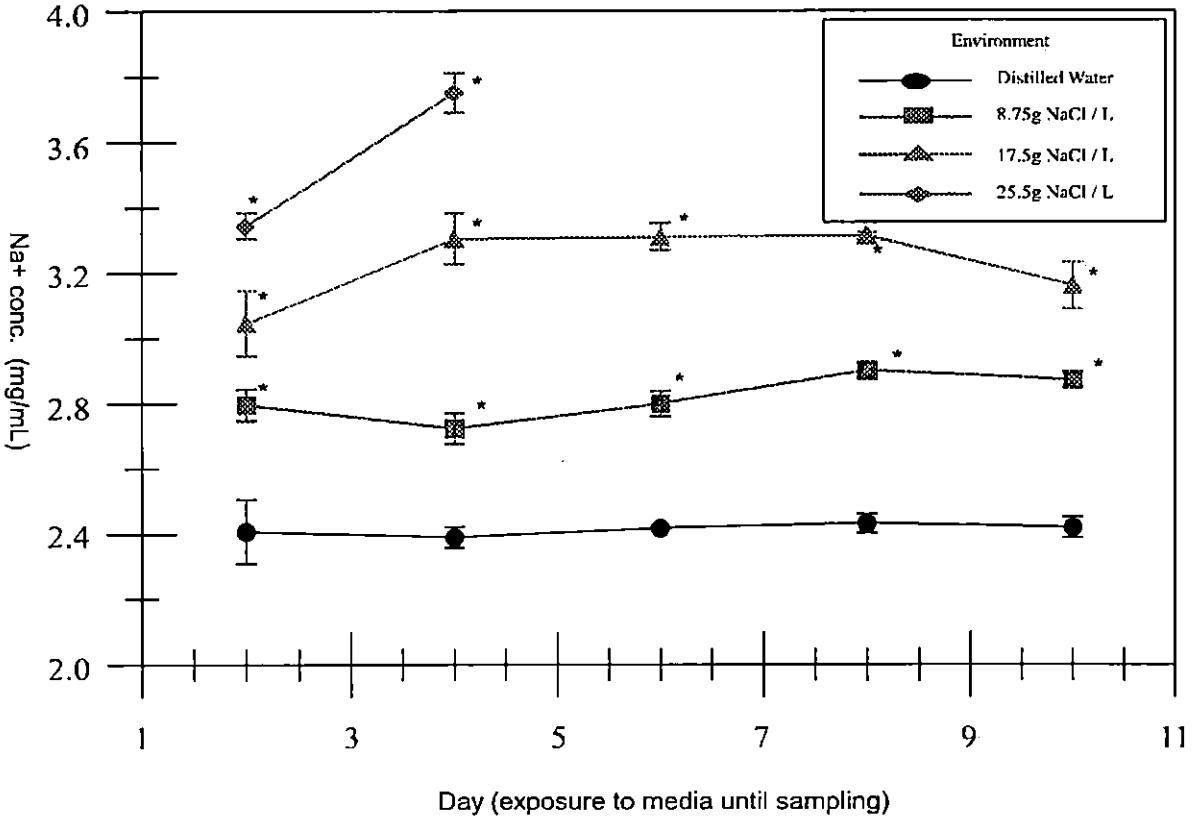

Figure 8. Hemolymph Na+ concentration sampled by aspiration from the nephropores after placement in various media

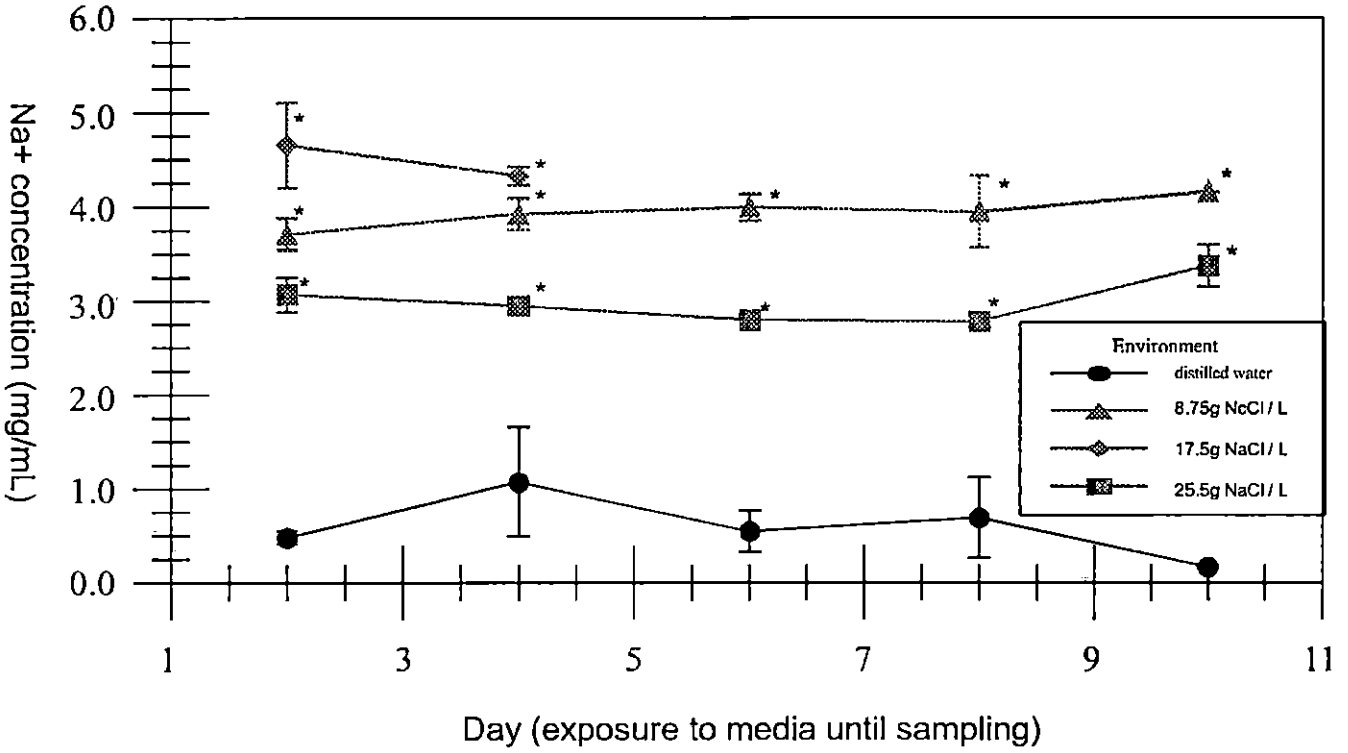

Figure 9. Urine Na+ concentration after placement in various media

Na^+, K^+- ATPase activities and Na+ concentrations after exposure to various media

Antennal gland and gill Na^+, K^+- ATPase activity with urine and hemolymph Na^+ concentrations for all crayfish in distilled water are illustrated in Figure 10. There was significant increase in gill ESA from days two to four (T=-9.32, p=0.001), and a significant decrease from days four to six (T=3.02, p=0.039). Antennal gland Na^+, K^+- ATPase activity in distilled water increased significantly from day two to four (T=-2.64, p=0.069) and from day six to eight (T=-4.82, p=0.0317), but decreased from days four to six (T=3.55, p=0.020), and from days eight to ten (T=22.23, p=.000). Hemolymph as well as urine Na^+ concentrations did not differ significantly from day to day during this acclimation to distilled water (See Figure 10). In both the 8.75 g NaCl / L media (Figure 11) as well as the 17.5 g NaCl / L media (Figure 12) there was no difference in the antennal gland or gill Na^+, K^+- ATPase activities. Figures 11 and 12 also show no significant difference in urine or hemolymph Na^+ concentrations. In the 25.5 g NaCl / L media (Figure 12) a significant increase occurred from day two to four in the gill Na^+, K^+- ATPase ESA (T=-3.26, p=0.035).

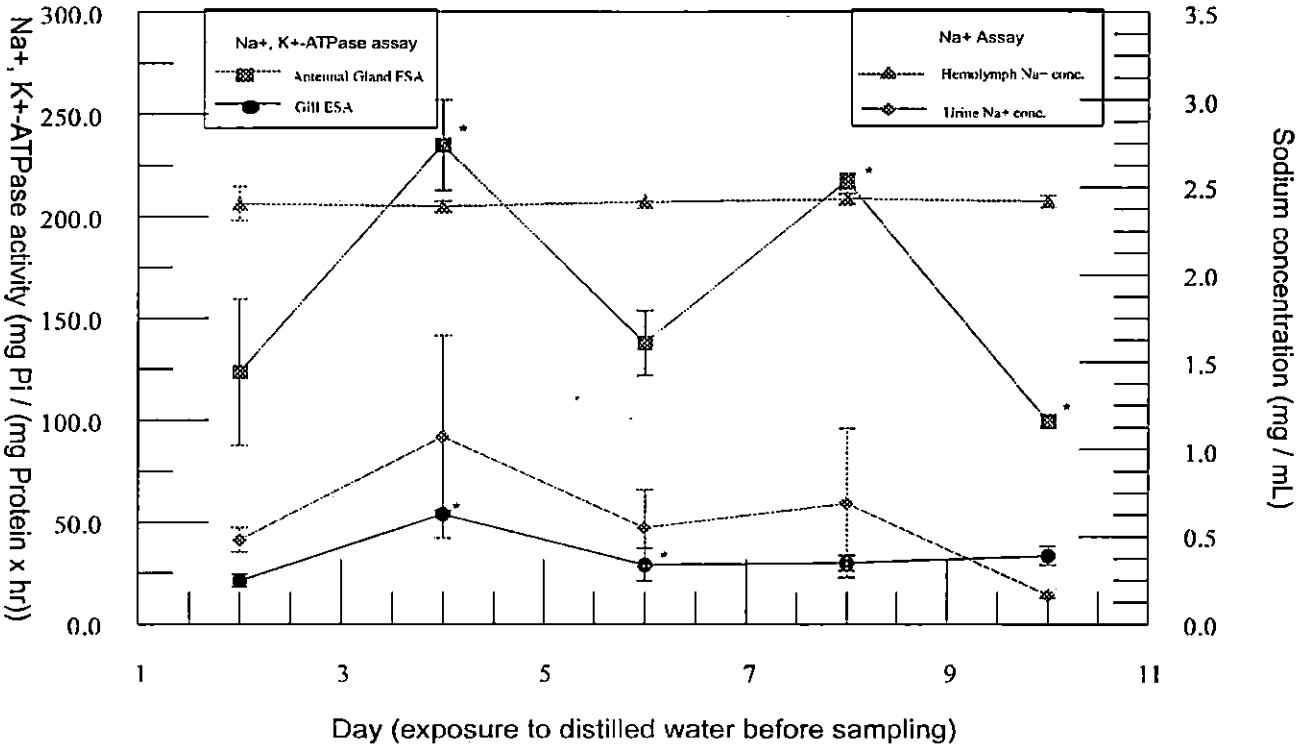

Figure 10. NA+, K+-ATPase from gill and antennal tissue as well as Na+ concentrations from hemolymph and urine sampled after exposure to distilled water

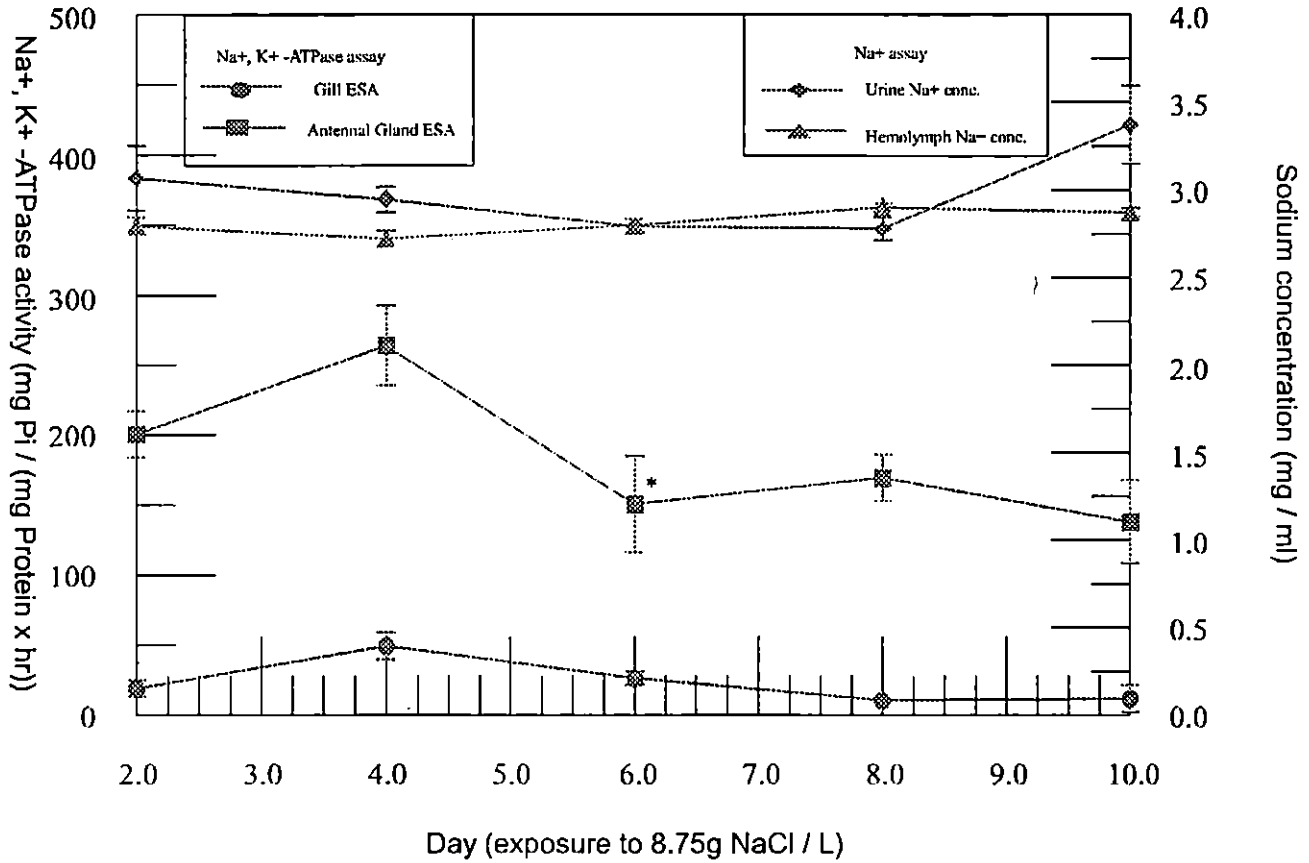

Figure 11. Na+, K+-ATPase activity from gill and antennal tissue as well as Na+ concentrations from hemolymph and urine sampled after exposure to 8.75 g NaCl / L

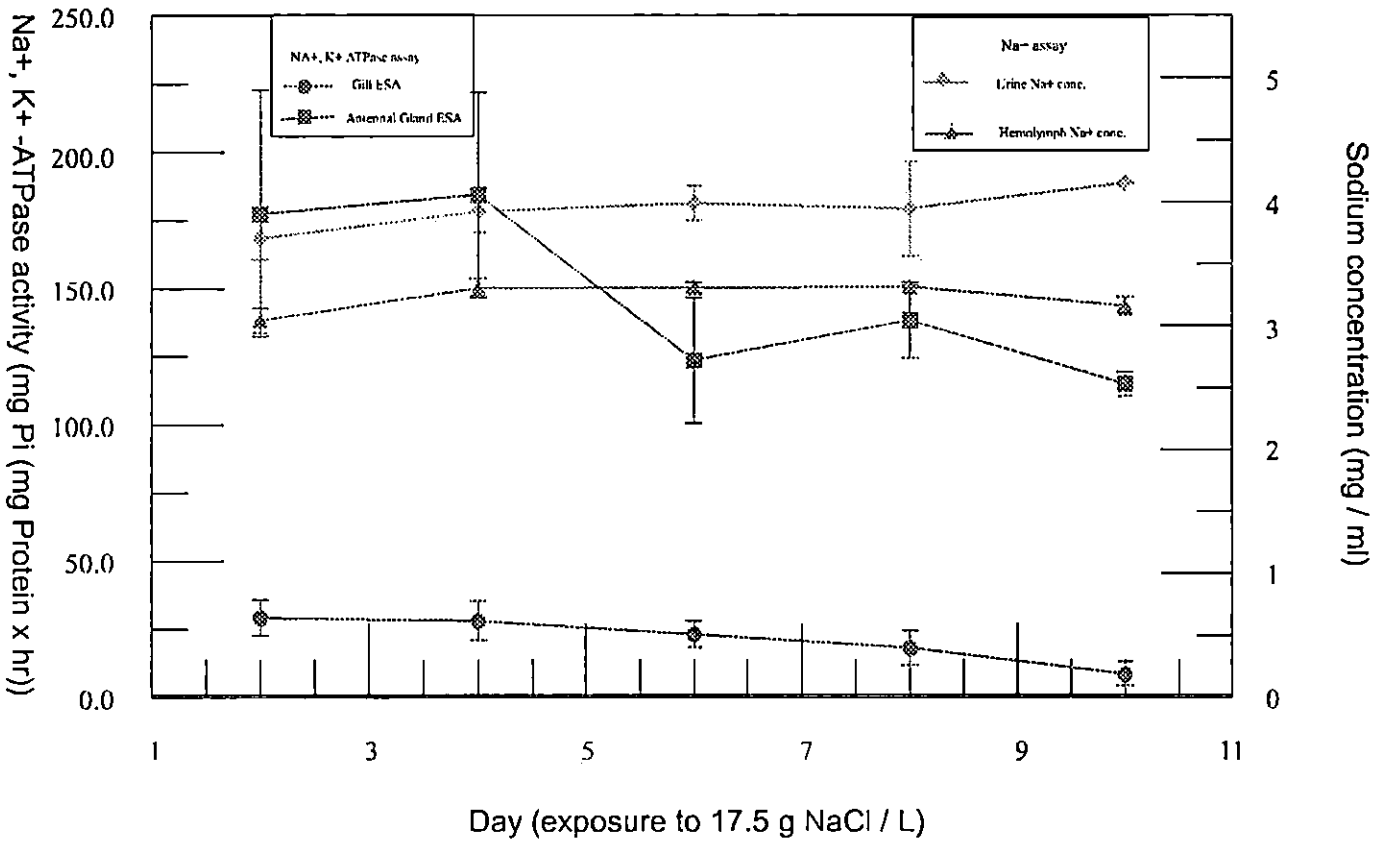

Figure 12. Na+, K+-ATPase activity from gill and antennal tissue as well as Na+ concentrations from hemolymph and urine sampled after exposure to 17.5g NaCl / L

Media and body fluid Na^+ content

Comparison of Na^+ concentrations in crayfish body fluids and various media after acclimation for two days and 10 days are presented in Figure 13 and 14. At two days (Figure 13) as well as ten days exposure (Figure 14) urine Na^+ concentrations remained relatively level. Urine Na^+ concentrations were hyposmotic to the hemolymph until a media concentration of 2.8 mg/ml of Na^+ was reached, then urine became hyperosmotic to the hemolymph.

Urine to hemolymph Na^+ ratios

Urine:Hemolymph ratios of crayfish in distilled water were all less than one (.068-.448), indicating hyperosmotic hemolymph. The crayfish that were acclimated to 8.75, 17.5, and 25.5 g NaCl / L demonstrated a (U:H) ratio of around or greater than one (.958-1.391), indicating a switch to a concentrated urine (Table 3).

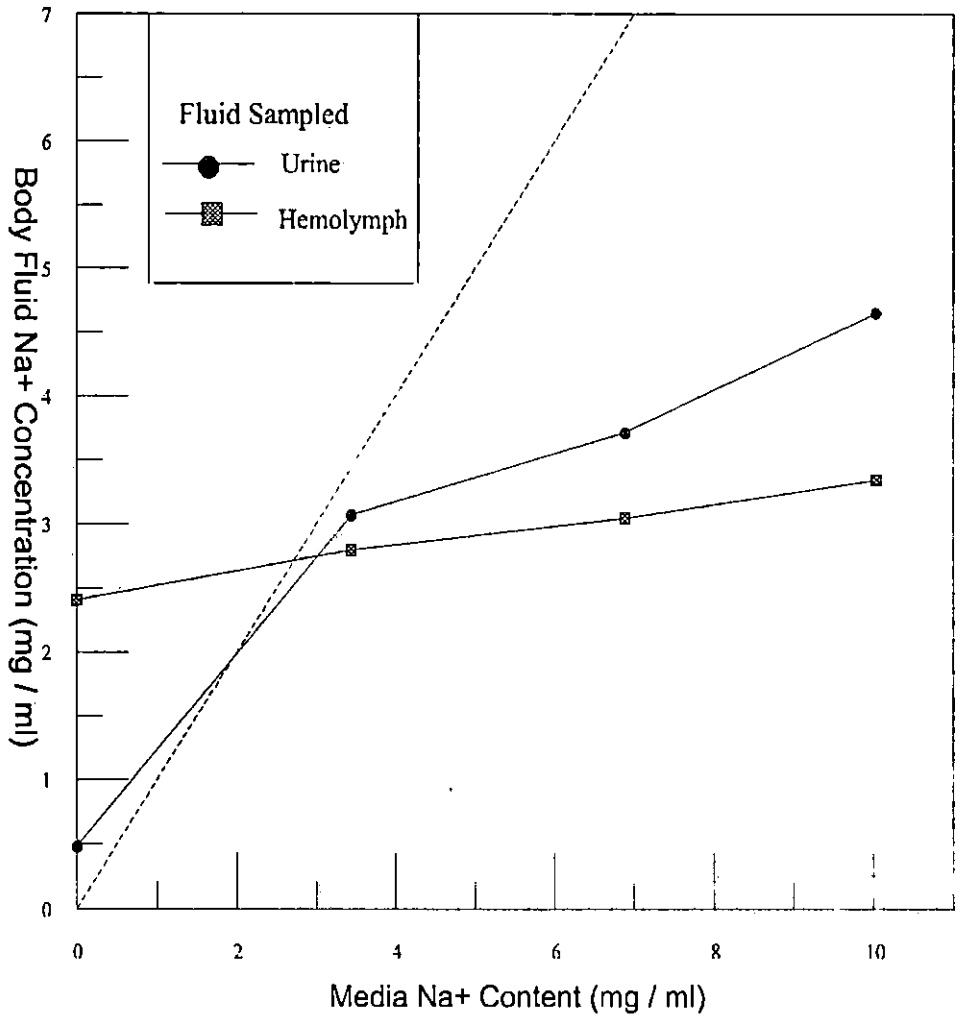

Figure 13. Osmotic performance of *Orconectes putnami* after acclimation in 0.0, 3.44, 6.89, 10.03 g Na^+ / L for two days

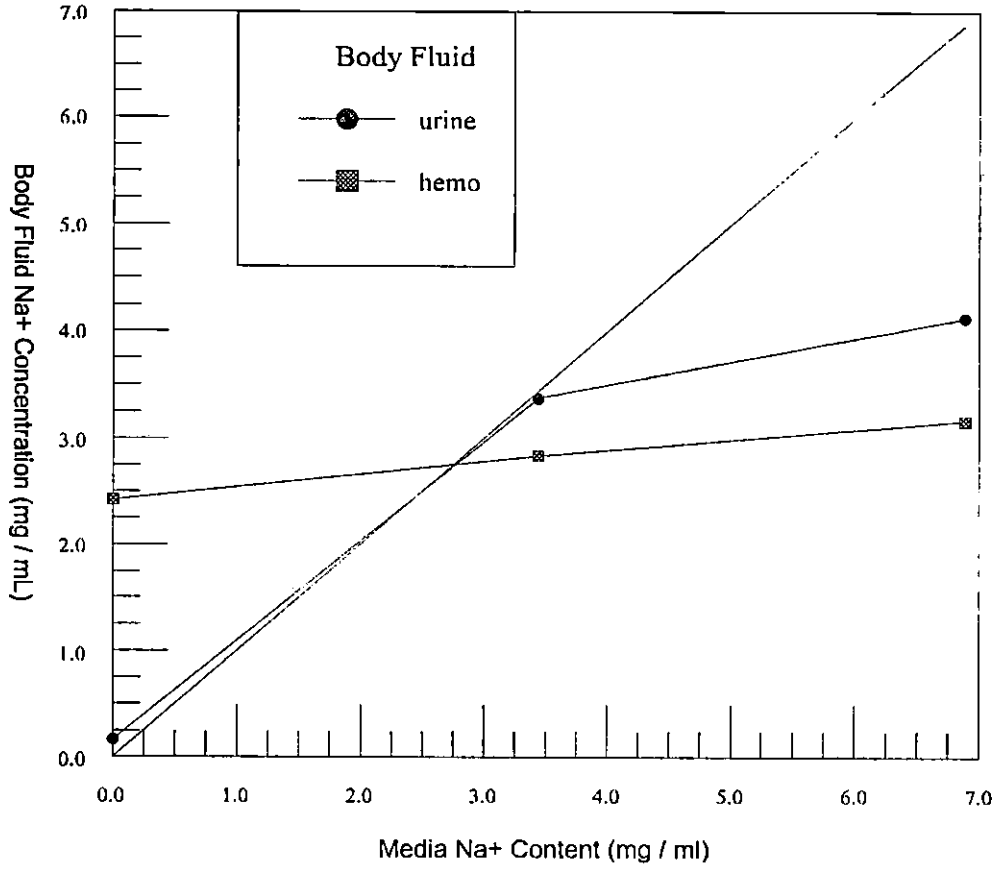

Figure 14. Osmotic performance of *Orconectes putnami* after acclimation in 0.0, 3.44, 6.89, 10.03 g Na^+ / L for ten days

Table 3. Urine:Hemolymph (U:H) Na^+ ratios for *Orconectes putnami* in various media

Day	Distilled Water	8.75 g NaCl / L	17.5 g NaCl / L	25.5 gNaCl / L
2	.200	1.098	1.218	1.391
4	.448	1.081	1.187	1.153
6	.227	.998	1.206	
8	.284	.958	1.190	
10	.068	1.173	1.313	

Discussion

A partial optimization study was conducted to confirm the conditions for the Na^+, K^+-ATPase enzyme assay. For the optimization study antennal glands were utilized. The optimum K^+ concentration in *Orconectes putnami* was found to be 20-30 mM (Figure 4), and optimal Mg^{2+} concentration was 4-6 mM (Figure 5). These values obtained are similar to those for *Procambarus clarkii* gill (Horiuchi 1977), *Procambarus clarkii* antennal gland (Sarver et al. 1994), *Uca pugnax* (mud fiddler crab) gill (Holliday 1985), and *Orconectes limosus* gill (Kosiol et al. 1988). Therefore, the enzyme assay utilized in later experiments was identical to Sarver et al (1994) and Holliday (1985) except the pH of the assay was at 7.25 instead of 7.4 and 7.2 respectively.

Orconectes putnami demonstrated an ability to survive in distilled water, 8.75 g NaCl / L and 17.5 g NaCl / L, as low mortality rates of less than two per week occurred in these media. *Orconectes putnami* did not survive very well in 25.5 g NaCl / L media as several crayfish died each day and all crayfish (20 total) were dead after five days exposure to this medium.

Kirschen (1992) demonstrated that *Astacus* (a fresh water crayfish) has a very low integumental permeability to ions as well as water, but there

was some diffusion across the membrane. In fresh water, this diffusion resulted in a 5% body weight per day gain of water, an amount of water that must be eliminated by means of the urine. This was supported by Kerley and Pritchard (1967) as they found that there was no change in tissue water content or blood volume in crayfish stepwise acclimated to 70% sea water.

Reabsorption of around 95% of sodium ions from the urine occured in the antennal gland (Kirschen 1992, Wheatly and Gannon 1985) to maintain ionic balance in the crayfish. As the external salt concentrations increased, the kidney function was reduced (Pritchard and Kerley 1970) and the antennal gland ESA decreased (Holliday 1985, Sarver et al. 1994). In this experiment utilizing *Orconectes putnami,* as well as in *Procambaras clarkii* (Sarver et al. 1994), the urine became more concentrated as the external salt concentration increased (Figure 9). In media with very high salt concentrations, urine production may decrease (Pritchard and Kerley 1970) or urine production may shut down altogether (Wheatly and Henry 1987). If the kidneys of a crayfish are subjected to an increasing osmotic stress, the crayfish would become dehydrated unless the crayfish decreased the amount of urine produced (Pritchard and Kerley 1970). Urine samples in *Orconectes putnami* in the present study became increasingly difficult to obtain as around 500 µl were obtained by aspiration from crayfish in distilled water, while only

around 10 µl was obtained from crayfish kept in 17.5 g NaCl / L media.

Antennal gland Na^+, K^+-ATPase activity (Figure 6) demonstrated a decrease in activity in 8.75 g NaCl / L as well as the 17.5 g NaCl / L medium. There was a greater decrease in activity observed in the 17.5 g NaCl / L medium when compared to the 8.75 g NaCl / L medium. Sarver et al. (1994) also obtained a reduction in Na^+, K^+-ATPase activity in *Procambarus clarkii* antennal glands in crayfish that were transferred from fresh water to 50% sea water. Henry and Wheatly (1988) also demonstrated a similar decrease in *Pacifastacus leniusculus* antennal gland Na^+, K^+-ATPase activity in crayfish transferred from 350 mOsm media to 750 mOsm, as well in crayfish transferred from freshwater to a medium of 750 mOsm media. Wheatly and Henry (1988) also found a significant reduction in antennal gland Na^+, K^+-ATPase activity in *Pacifastacus leniusculus* while existing in 750 mOsm water.

A peak in antennal gland Na^+, K^+-ATPase activity occurred in all media at day four of exposure to the various media (Figure 6). Sarver et al. (1994) also experienced a peak at day four in activity of *Procambarus clarkii* antennal gland after transfer from fresh water to 50% sea water. Henry and Wheatly (1988) experienced a peak in *Pacifastacus leniusculus* Na^+, K^+-ATPase antennal activity at day two of acclimation from a medium of 350

mOsm to 750 mOsm media and acclimation from fresh water to 750 mOsm sea water. This peak has not been explained in the literature, but may be a physiological response to the initial shock of the changing of the media, and is deserving of further study.

Gill Na^+, K^+-ATPase activity (Figure 7) also demonstrated a decrease in activity in *Orconectes putnami* acclimated to 8.75 g NaCl / L and 17.5 g NaCl / L media. This decrease was also observed in *Pacifastacus leniusculus* by Henry and Wheatly (1987). Holliday (1985) observed a significant increase in gill Na^+, K^+-ATPase activity when *Urca pugnax* were transferred from 100% to 10% sea water. This provides further evidence that this change in Na^+, K^+-ATPase activity is due to osmotic conditions. In *Orconectes putnami*, dilute urine was produced in media of low salt concentrations with hyperosmotic hemolymph production. As salt stress increased the hemolymph sodium ion concentrations in the hemolymph did increase significantly from distilled water controls (Figure 8), but the largest change occurred in the urine. Urine sodium ion concentrations increased sharply in increasing media salinities. Hemolymph remained more concentrated than the urine until around 2.75 mg/ml Na^+ in the medium, at which the urine becomes more concentrated than the hemolymph as the crayfish switches to hyposmotic hemolymph production (Figures 13, 14).

This is demonstrated by Urine:Blood (Hemolymph) Na^+ ratios that exceed one as the crayfish subjected to 8.75 g NaCl / L, 17.5 g NaCl / L, 25.5 g NaCl / L media (Table 3).

Orconectes putnami appears able to regulate internal ion concentrations by the means of Na^+, K^+-ATPase. The simultaneous decrease of Na^+, K^+-ATPase activity in the antennal gland as well as the gills (Figures 10-12) together with an increase in hemolymph and urine Na^+ concentrations, demonstrate a physiological response to osmotic stress. Also, the production of large amounts of dilute urine at low external salinities and production of a reduced concentrated urine at high external salinities suggests that *Orconectes putnami* is able to regulate ion balance. However, more work needs to be preformed examining Na^+, K^+-ATPase activity in *Orconectes putnami* exposed to distilled water, as well as the peak in Na^+, K^+-ATPase activity at day 4.

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