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Altered Na,k-Atpase Isoform Expression In Artemia Franciscana In Response To Hypersaline Environments

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38 Pages

The Na,K-ATPase (NKA) is an essential membrane pump that helps to establish cell ion gradients, and regulate intracellular salt in many organisms. One such species, *Artemia franciscana* (brine shrimp), extreme halophiles which live in hypersaline environments, express 2 distinct α-catalytic subunits of the NKA. One of these subunits, α2-(KK), has two key lysine substitutions located within the cation binding sites. Prior work has demonstrated this specific subunit may be directly involved in brine shrimp adaptation to their extreme environments. However, the precise molecular and physiological effects of α2-(KK) have not been entirely elucidated. I determined through immunohistochemistry that my initial hypothesis that the NKA may be expressed apically in the gut epithelia was wrong. However, further exploration of the brine shrimp showed that the salt regulation organ, metepipodites, have distinct NKA expression and cell morphology. I also identified RNA expression of the α2-KK subunit was upregulated and apparent ion affinities were altered, when brine shrimp were reared in a hypersaline environment.

KEYWORDS: Na,K-ATPase, *Artemia franciscana*, Salinity, Na,K,Cl-cotransporter, Chloride Cells, Metepipodites

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J.L.D.

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CHAPTER I: INTRODUCTION

NA,K-ATPase

Na,K-ATPase (NKA) is a plasma membrane (PM) bound transporter which shuttles 3 Na^+ out of the cell and 2 K^+ in, at the expense of 1 molecule of ATP. This 3:2:1 stoichiometry is seen in all orthologs of the NKA. This transporter is a member of the P-type ATPase super-family of membrane pumps which is phosphorylated at a conserved aspartic acid (**D**KTG) within the phosphorylation domain of the pump structure[1]. The NKA is a heterotrimeric protein composed of three subunits, a catalytic α-subunit, a regulatory trafficking β-subunit, and a tissue-specific auxiliary FXYD subunit[2]. The reaction mechanism of the NKA proceeds through a series of phosphorylated and dephosphorylated conformational states with cations bound and unbound, demonstrated by the Post-Albers scheme (Fig. 1). When the ion binding sites are intracellularly open (E1) the enzyme is bound by ATP and poised to bind Na^+ in the forward direction. Once 3 Na^+ ions bind and become occluded, the conserved aspartate becomes phosphorylated by ATP with the concomitant release of ADP during the conformational transition from E1 to E1P. Following a spontaneous conformational change, the NKA opens extracellularly with a substantially lower affinity for Na^+ which results in extracellular Na^+ release and the switch to the E2P conformation. Once extracellularly open with empty binding sites, 2 K^+ ions bind and expose the catalytic aspartylphosphoanhydride to nucleophilic attack by H_2O, thus dephosphorylating the NKA and occluding K^+ (E2[$2K^+$]). The final conformational change and deocclusion of K^+ intracellularly is the rate-limiting step of the cycle, but can be accelerated substantially by low affinity binding of ATP, forming E1 with ATP bound to begin another cycle[1].

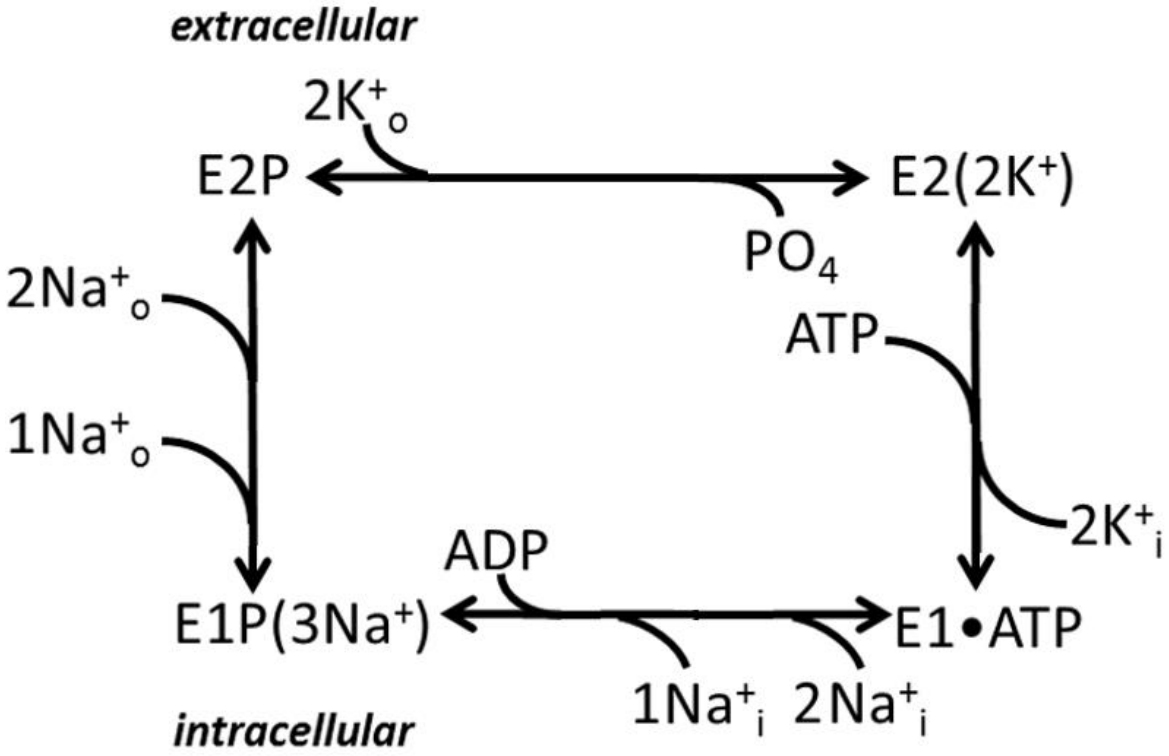

Figure 1. Post-Albers scheme of the catalytic cycle of NKA. The enzyme progresses through E1 and E2 states as the cycle proceeds to pump 3 Na^+ out of the cell (extracellular space) and brings 2 K^+ into the cytoplasmic side.

Role of Na,K-ATPase in adaptation to salt water

The NKA plays an important role in the osmoregulation of marine animals, in particular *Fundulus heteroclitus* (teleost fishes, bony fishes)[3]. Teleost fish gills have been studied extensively for their ability to extrude Na^+ and Cl^- from the gills directly into the surrounding seawater. Similarly, freshwater adapted *Anguilla rostrata* (American eels) have been shown to extrude Na^+ into the surrounding environment in combination with increased NKA activity,

when exposed to saltwater[3]. When the NKA exclusive inhibitor (ouabain) was applied to American eels, there was a 90% decrease in Na^+ efflux in the gills compared to control[3]. Additional adaptations for Na^+ extrusion are the single-stranded tight junctions found between adjacent chloride cells and accessory cells of fish gill epithelia[4]. Tight junctions are multi-stranded with high electrical resistance, preventing passive ion diffusion, with the exception of fish gill epithelia[4]. Na^+ efflux in teleost fishes has been shown to be inhibited by a paracellular cation shunt inhibitor, 2,4,6-triaminopyrimidine (TAP), across the opercular epithelium in isolated preparations[4].

Net loss of Na^+, in teleost fish, is achieved mainly in specialized cells of the gill epithelium called chloride cells[3]. These cells are characterized by their high mitochondrial content and excess surface area of the basolateral membrane[5]. Within the basolateral membrane of these chloride cells, ion regulation occurs in part by the NKA establishing a steep Na^+ gradient used by a series of secondary active transporters. Of these secondary active transporters, the Na^+,K^+,2Cl^- Co-transporter (NKCC) utilizes this Na^+ gradient to import K^+ and Cl^- from the hemolymph into the epithelial chloride cell[5]. Apically, Cl^- leaves the cell via facilitated diffusion through a Cystic fibrosis transmembrane conductance regulator (CFTR)-like channel. The excess excretion of Cl^- creates a positive trans-epithelial potential (TEP), which drives Na^+ extrusion from the body through specialized low-resistance single-stranded tight junctions (Fig. 2)[6,7]. Near these single-stranded tight junctions there is also an accumulation of Na^+ due to a tapering of the passage between lateral membranes of connected cells, which further facilitates Na^+ extrusion out of the body.

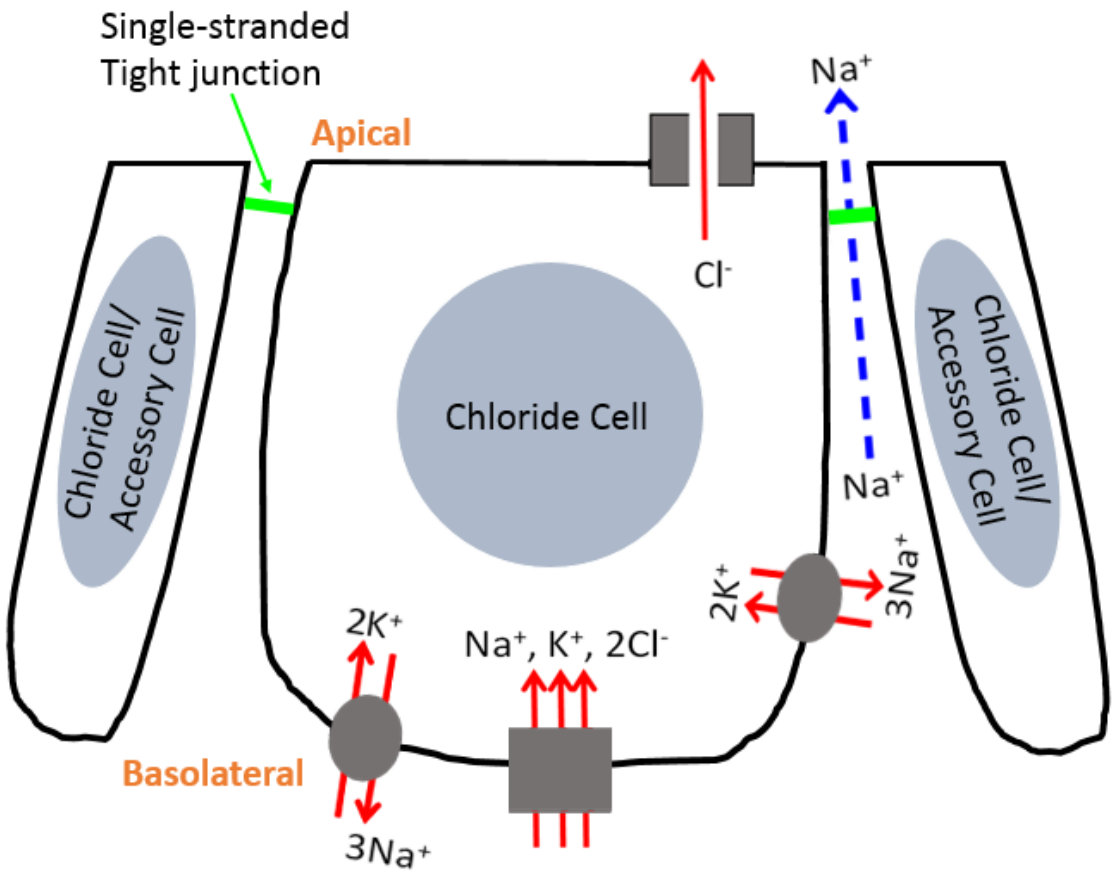

Figure 2. Simplified diagram of ion transport in the teleost gill epithelium. In the basolateral membrane the NKA establishes a Na^+ gradient and works in concert with the NKCC to import Cl^- into the cell from the hemolymph. Once inside the chloride cell, Cl^- is exported from the body through CFTR-channels, in doing so establishes a positive TEP. The positive TEP is then used to extrude Na^+ through a paracellular single-pass tight junction in a passive manner.

Physiology of salt tolerance in *Artemia franciscana*

Artemia franciscana (brine shrimp) are unique in their ability and adaptations to tolerate extreme salinities, even up to concentrations as high as 4M NaCl[8]. One adaptation is a cuticle impregnable to the surrounding medium, allowing for strict ionic and osmotic intake control via the gut through active swallowing [9]. As such, in hypersaline environments large amounts of Na^+ are passively absorbed into the gut epithelial cells and actively pumped into the hemolymph via the basolateral NKA[9]. Although these organisms absorb large quantities of Na^+ from an environment 100-times more concentrated externally than internally, the internal hemolymph only increases 6-fold in osmotic pressure when compared to an isosmotic environment[9]. However, in nauplii (or juvenile) stage, Na^+ and Cl^- excretion occurs at a special neck organ known as the salt gland or maxillary gland[8]. Upon reaching adulthood, this organ disappears and excretion occurs in metepipodites, organs attached to the swimming legs (phyllopodia) (Fig. 3)[10].

These brine shrimp metepipodites may have functional convergence with the aforementioned gills of teleost fishes with regard to their chloride cells[11]. One key morphological characteristic of these chloride cells is that they are rich in mitochondria, another adaptation to saline environments[11]. In brine shrimp, Cl^- is far from its electrochemical equilibrium across the apical membrane, internally (hemolymph) vs. externally (gut), suggesting it is actively transported out of the organism[11]. On the other hand, Na^+ is close in equilibrium to its electrochemical gradient across the body wall, and localized pockets of higher electrical potentials (aided, in part by the positive TEP generated by Cl^- exit) must exist where Na^+ is excreted passively along its electrochemical gradient, as is the case in teleost fishes[6,11,12] The chloride cells of fish gills are notable in that they form a crypt on the apical membrane shared by other chloride cells or accessory cells. These crypts serve as regions of concentrated Na^+ and

electrical current across the epithelia, in part driving Cl^- secretion[13]. There is evidence for the metepipodites having high permeability to chloride ions, and show differential localization of Cl^- permeability at the apical surface of the metepipodites[11].

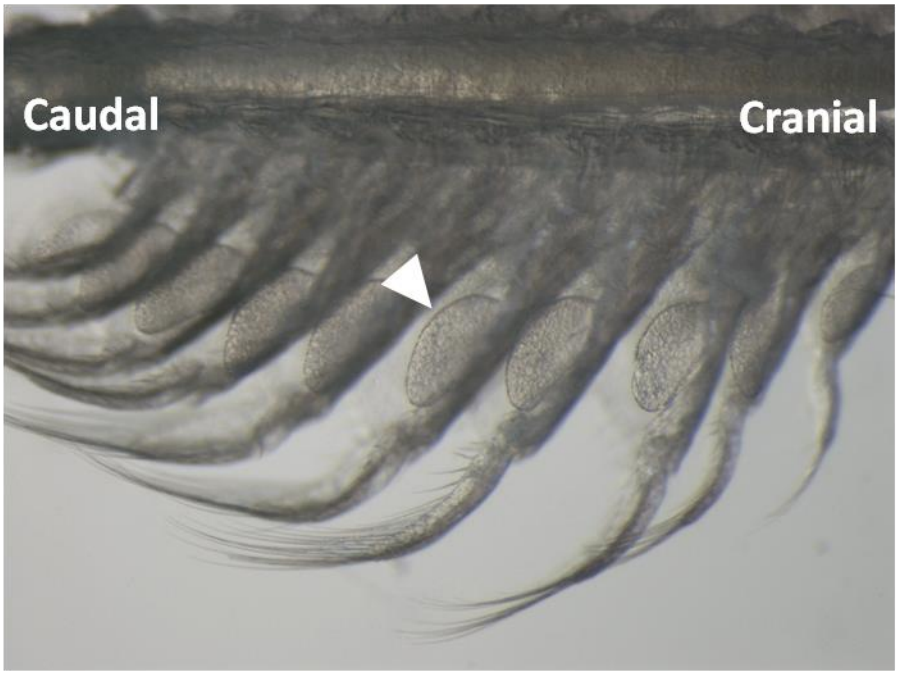

Figure 3. Metepipodites of an adult brine shrimp, adapted to 2M NaCl conditions. Right side is cranial, left is caudal. Arrow indicates a metepipodite on a phyllopodia.

Double lysine mutation in the α2 form of Na,K ATP-ase in brine shrimp

Brine shrimp have 2 isoforms of the NKA, a constitutively expressed α1 and lesser expressed α2 which share 74% amino acid identity with different RNA expression patterns[10]. The α2 isoform has 2 distinct positively charge lysines in place of asparagine in α1 (α1(NN)) within the ion binding regions at N324K and N776K (α2(KK)), which alter affinity and potentially stoichiometry[10]. This prediction stems from the thermodynamic limit of the energy released from hydrolysis the gamma phosphate of ATP. As extracellular [NaCl] approaches saturation, the energy provided by 1 ATP is insufficient to transport 3 Na+ ions against this gradient. Therefore to overcome the large Na^+ gradient it seems that the brine shrimp NKA would need to alter its canonical 3Na:2K:1ATP stoichiometry.

However, work by Jorgensen and Amat[10] proposed the possibility of a reduced stoichiometry from 3Na:2K:1ATP a2(KK). This hypothesis was supported by our lab, demonstrating the a2(KK) has a reduced stoichiometry to 2Na:1K:1ATP[14]. While introducing either one of the lysine mutant alone displayed reduced ion affinity, when both lysine residues were introduced together (i.e. like found in endogenous a2(KK)) there appears to be a partial recovery of ion affinity[11,14]. Our leading hypothesis is that structurally the K324 pushes (via ionic repulsion) the epsilon amino group of K776 into one of the ion binding sites shared by both Na^+ and K^+, thus providing a pseudo-cation constantly bound within one of the ion binding sites. Consequently, all binding sites are occupied by cations as the a2(KK) pump proceeds through the reaction cycle, but site occupied by $–NH_3^+$ of K776 is never released. It is a surrogate Na^+ ion during the E1-P to E2-P transition and a surrogate K^+ ion during the E1[$2K^+$] to E1 transition, thus reducing the number of Na^+ and K^+ ions required for transport by 1 each[14].

NKA's role in the mechanism of osmoregulation in brine shrimp

In marine teleosts fishes, the NKA is known to function as part of the gill's salt regulatory physiology, establishing the Na^+ gradient for the NKCC to use for the import of Cl- from the hemolymph to eventually be secreted out of the body by the CFTR-channel[6]. This same mechanism may be used analogously in the metepipodites of brine shrimp[11]. This mechanism in combination with the NKA α2(KK) with a reduced stoichiometry may play a pivotal role in the ability for brine shrimp to survive in such harsh environments. However, the localization of the α2(KK) remains unknown, but one such location may be on the apical membrane of the gut epithelium (i.e. immediate Na^+ extrusion upon passive entry into the gut epithelial cells). However, the location and function is more likely similar to the known physiology of salt secretion in the gills of marine teleost fish.

In this work, I attempted to elucidate differences in the localization and expression of *A. fransiscana* NKA between hypersaline and saline adapted animals. Throughout my work, I identified; *(i)* The NKA is only found on the basolateral surface, rather than apical, in the brine shrimp gut epithelia. *(ii)* There exists adaptations within the osmoregulatory salt organs, metepipodites, in hypersaline adapted animals similar to those found in the chloride cells of teleost fishes. *(iii)* Total RNA of both α1 and α2, identified an increased expression of α2 in hypersaline adapted animals. *(iv)* Variable ion affinities of the NKA was demonstrated between hypersaline and saline adapted animals. My work here provides further insight into how the differential localization and expression of the NKA help *A. fransiscana* adapt and survive in such harsh environments.

CHAPTER II: MATERIALS AND METHODS

Brine Shrimp Rearing and Maintenance

Following the manufactures protocol, approximately 225,000 nauplii (1 g of brine shrimp cysts), were hatched in 1 L of 1X instant ocean artificial sea water (SW) with vigorous aeration and constant light for 24 hours. After hatching, nauplii were divided and reared in separate aquariums with either low salt (0.5X SW, ~250 mM NaCl) and high salt (1X SW with additional 1.5 M NaCl, final [NaCl] ~2 M). Aquariums were maintained at 26-28°C under continuous light with aeration provided near the surface for O_2 supply. Feeding began 12 hours post-hatching with spirulina algal powder. Once reaching adulthood, animals were fed every other day.

Tissue Fixing and Sectioning

Adult brine shrimp in each condition were collected and fixed in 4% paraformaldehyde (PFA) until processing (minimum fixation 12 hours). Dehydration occurred through a series of alcohol washes beginning with 25%, 50%, 75% and completed in two washes in 100% ethanol. Upon dehydration, tissue was cleared with histoclear medium prior to paraffin embedding (graded series of histoclear:paraffin). Paraffin embedding was performed at 58°C-60°C to maintain paraffin in its liquid state. Tissue was transferred to a mold filled with liquid paraffin and solidified on dry ice for 15-20 minutes in preparation for sectioning. Individual sections were cut in 20 µm increments and transferred to poly-L-lysine coated slides, heated at 58°C for 20 minutes and stored at room temp (RT)[15].

Staining and Imaging

Paraffin sectioned slides were incubated in the following order for 10 minutes each: 3X in 100% histoclear, 3X in 100% ethanol and then rehydrated stepwise in 80% Ethanol, 50% ethanol, and finally ddH2O. Slides were then treated at 90-95°C for 15-20 minutes in sodium citrate buffer for antigen unmasking and then rinsed in dH_2O. Sections were permeabilized in Tris-buffered saline + 0.1% Tween-20 (TBST) with gentle agitation for 10 minutes, then blocked for 1 hour in 10% Bovine Serum albumin (BSA) in Tris-buffered saline TBS at RT. Primary antibody incubation occurred overnight at 4°C with gentle agitation in TBS with 1% BSA. Primary antibodies raised in rabbit, anti-NKA α-subunit (antibody α5, Developmental Studies Hybridoma Bank (DSHB)) and anti-cytochrome c oxidase (i.e. complex IV of the electron transport system in mitochondria (COX IV)) antibody (20E8C12, Abcam catalog no. ab14744), were used in 1:100 dilution. Secondary antibodies goat-anti-mouse Alexa 568 secondary antibody (Thermofisher Catalog #A-11004), phalloidin-Alexa 488 (Thermofisher Catalog #A12379), wheat-germ agglutinin-Alexa 488 (Thermofisher Catalog #W11261), and/or to-pro3 (Thermofisher Catalog #T3605) for counterstaining, were washed 3X in TBS in 0.025% triton and incubated for 1 hour at RT[16]. Slides were then washed 3X in TBS, mounted in vectashield fluormount mounting medium and imaged with a Leica Sp2 confocal microscope.

Images were imported into Adobe Photoshop to be manually assembled for labelling, overlays, and max projections in RGB format, with levels adjusted as needed for clarity.

NKA purification and activity from brine shrimp

Purification of the NKA from adult brine shrimp was carried out as described by Peterson *et al.* 1978, with modifications[16]. Homogenizations and resuspensions were performed on ice

while spins were performed at 4°C. Brine shrimp were homogenized using a Beckman dounce homogenizer in buffer HS (1X protease inhibitor, 250 mM sucrose, 25 mM Imidazole, 1 mM EDTA pH 7.4) and spun at 500 x *g* for 5 minutes. Supernatant was collected and the pellet was resuspended in one half of the starting volume of buffer HS and spun for an additional 5 minutes at 500 x *g*. Total supernatant was amalgamated and spun at 1000 x *g* for 15 minutes, and the supernatant fraction was collected and remaining pellet was resuspended in ¼ of the initial volume and re-spun at 1000 x *g* for 15 minutes. Both supernatants from the 1000 x *g* spin were pooled and spun at 27,000 x *g* for 2 hours. The final supernatant was removed and the pellet was resuspended in buffer HS.

Protein concentration was determined via Bradford assay as previously described[18], and then NKA activity, along with $[Na^+]$ and $[K^+]$ dependence (i.e. apparent affinity ($K_{0.5}$)) were assessed using a colorimetric ATPase assay[19]. To determine the $K_{0.5}$ for $[Na^+]$ and $[K^+]$ in enzyme preparation, protein was added in triplicate with ATPase solution (2.4mM EGTA, 14.5mM $MgCl_2$,16mM $Tris_2ATP$, 239.5mM imidazole, pH 7.2) in either increasing $[Na^+]$ with constant 24.5 mM KCl (for the $K_{0.5}$ of Na^+), or increasing $[K^+]$ with constant 135 mM NaCl (for the $K_{0.5}$ of K^+), with and without ouabain. The final volume of each well was brought up with 50mM Tris buffer (pH 7.4), then incubated 37°C for 15 minutes. The enzyme reaction was stopped in stopping solution (2ml H_2O, 2ml 1M HCl, 0.13g Absorbic acid, 200µL 10% NH_4MoO_4), brought up with ACG solution (2% Na-arsenite, 2% Na-citrate, 24mM Glacial acetic acid), incubated for additional 5 minutes at 37°C and read at 800nm.

RNA Extraction and RT-PCR

RNA isolation was performed using Trizol reagent according to the manufacturer's directions (TRIZOL Catalog No. 15596). Briefly, samples were homogenized in 600μL of trizol in a Beckman dounce homogenizer. Insoluble material was removed with a 12,000 x *g* spin for 10 minutes at 4°C. Samples were incubated at RT for 10 minutes and directly following, chloroform was added, equal to 20% of the starting volume of trizol. Tubes were shaken and incubated for 3 minutes at RT and spun at 12,000 x *g* for 15 minutes at 4°C. The aqueous phase was removed and pipetted into a new microcentrifuge tube with isopropyl alcohol added, equal to 50% of the starting amount of trizol. Sample was vortexed and centrifuged at 7,500 x *g* for 5 minutes at 4°C, supernatant was then discarded and pellet was left to air dry for 10-15 minutes, and resuspended in RNase free dH_2O. Quantitation was determined using a nanodrop spectrophotometer.

Reverse transcription reaction was performed with equal amounts of RNA and High-Capacity cDNA RT kit master mix (Applied biosystems catalog no. 4368814) consisting of 10x RT buffer, 25x dNTP mix, 10x random primers, reverse transcriptase enzyme, and dH2O. Reaction was run in a thermocycler for the following steps: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, 4°C hold. Following quantitation was determined using a nanodrop spectrophotometer and the cDNA was stored at -20°C until qRT-PCR was performed.

Real time quantitative PCR

Primers were designed at regions of dissimilarity between the α1 and α2 isoforms to prevent nonspecific amplification (Table 1). Quantitative real-time PCR was performed with cDNA and SYBR green master mix containing SYBR green dye, AmpliTaq Gold DNA

polymerase, dNTPS with dUTP, passive reference dye and buffer components (Applied Biosystems, catalog #4309155). Standard curves were created in a series of four 10-fold dilutions in a 50μL reaction volume, run in triplicate. Primers were added at a concentration of 15μM and template DNA was added from a 2000μg/ml stock cDNA prep. The reactions were read in an Applied Biosystems 7300 Real Time PCR System under the following conditions: 2 minutes at 50°C for 1 cycle, 10 minutes at 95°C for 1 cycle, 40 cycles of 95°C for 15s, 60°C or 30s, 72°C for 30s, and finally 10 minutes at 72°C for 1 cycle.

Bumetanide treatment assay

Bumetanide stock solution (100mM) was prepared in DMSO and added to 10mls of artificial sea water/NaCl mixtures depending on rearing conditions to treatment concentrations of 25, 50, 75, and 100μM bumetanide, with a control using a matching [DMSO]. Brine shrimp were added to 6-well tissue culture wells in each respective treatment dosage of bumetanide diluted at room temperature for 24 hours and then assayed for the response to bumetanide. Responses were scored as either no effect, or significantly impaired swimming/lethality.

Table 1. Primers for RT-qPCR of the a1(NN) and a2(KK) subunits

Gene	Accession #	Primer sequence	Amplicon size (bp)
α1(NN) subunit of Na,K-ATPase	X56650.1	F: 5'-CAGAGGAATTGAAAGAGGCC-3' R: 5'-AGCCCAGCAAATCTCAATCC-3'	172
α2(kk) subunit of Na,K-ATPase	P17326.1	F: 5'-AAAATTGTCACAGCAGACACC-3' R: 5'-GACCCAGTTGACATCTCTGC-3'	193
Tubulin α-chain	AF078670.1	F: 5'-GACAAATCGATTGGTGGAGG-3' R: 5'-CCACGGGCATAGTTGTTAGC-3'	203
		F: 5'-GGTACCGGATCTGGGTTCAC-3' R: 5'-ATAGATGGCCTCGTTGTCG-3'	202

Western Blot

A 7.5% polyacrylamide gel was run with 40ug amounts of both membrane purified high- and low-salt brine shrimp treatment groups with 0.5ug purified Na,K-ATPase from sheep kidney for control. The transfer occurred on PVDF membrane in 10mM CAPS, 10% methanol for 3 hours at 180mA using a bioRad blot system. The membrane was then was blocked with soy milk for 10 minutes at RT. The blot was rinsed 3X in PBST and then incubated overnight at 4°C with 1:1000 mouse-anti-NKA IgG Following primary antibody incubation, the blot was washed 3X in PBST at RT and then incubated with 1:5000 goat-anti-mouse IgG HRP-conjugate for 1 hour at room temperature. Following secondary antibody incubation, blot was washed 3X in PBST at RT and incubated with chemiluminescent substrate (ThermoFisher Scientific SuperSignal™ West Pico Chemiluminescent Substrate catalog no. 34080) for 5 minutes then imaged with a gel-doc system.

CHAPTER III: RESULTS

Localization of NKA in the brine shrimp

In order to determine the cellular membrane localization of the NKA in brine shrimp gut epithelia, immunohistochemical analysis was performed on adult tissue sections. Using an antibody against avian NKA α-subunit (antibody α5, DSHB) I labeled transverse sections of adult brine shrimp gut epithelia and the resulting images revealed distinct and consistent basolateral localization in both 2M and 250mM total NaCl-reared brine shrimp (Fig. 4A and 4C). Staining for F-actin identified the boundaries of the cell membrane, providing a clear picture of NKA within the epithelial layer of the gut (Figs 4B and 4D). F-actin staining wasn't typical and likely due to the treatment of the tissue or the characteristics of brine shrimp actin, however the stain still revealed the extent of the cells boundaries.

Cell morphology in the metepipodites

I immunostained the legs in an attempt to identify morphological differences between cell types (i.e. phyllopodia vs. metepipodites), however initially identified similar nuclei content in both metepipodites and phyllopodia (Fig 5C). The next logical step was to attempt to identify whether mitochondrial differences exist between these cells. Using an anti-COX IV antibody, a membrane bound mitochondrial protein in the electron-transport chain, and observed significant differences between the two cell types. The metepipodites were clearly identifiable due to the excess mitochondrial content compared to surrounding phyllopodia (Fig. 5B). Additionally, wheat germ agglutinin staining (WGA), which stains membrane-associated sugars, revealed an altered membrane architecture in the metepipodites relative to the phyllopodia, in part due to the amount of mitochondria present within these cells (Fig 5A, 5D, and 5E). It isn't clear if the

increased signal in the metepipodites is due to membrane amplification or altered membrane sugars.

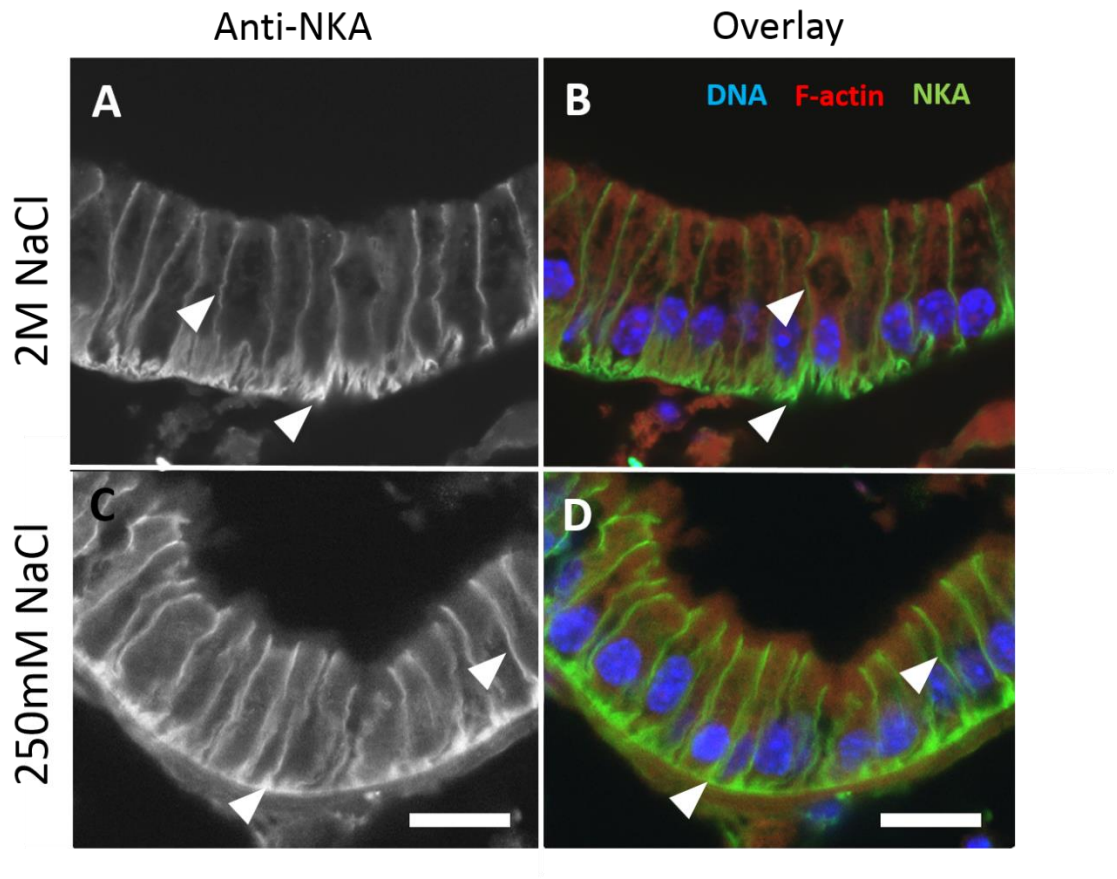

Figure 4. Immunolocalization of NKA on the membrane of gut epithelial cells. With staining for NKA (green), F-actin (red), and DNA (blue), and scale bar representing 20µm. A,B) 2M NaCl adapted brine shrimp epithelial cells showing NKA localization in the basolateral membrane. C,D) 250mM NaCl adapted brine shrimp epithelial cells showing NKA localization in the basolateral membrane.

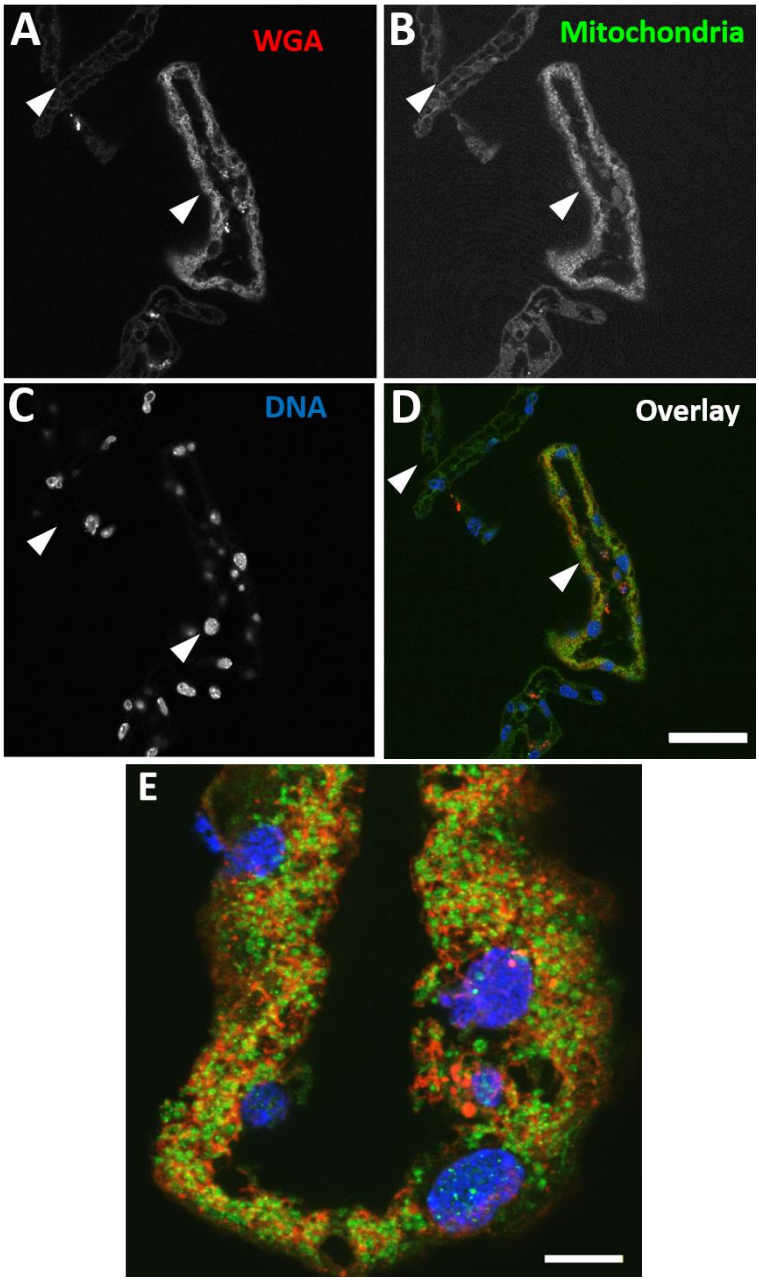

Figure 5. Staining of mitochondria and membranes of epipodite sections. Stained for mitochondria (green), membranes (red) and DNA (blue). Scale bar represents 50µm. Arrows indicate tissue sections from metepipodites and phyllopodia. A) WGA staining only. B) Mitochondria staining only. C) DNA stain only. D) Overlayed metepipodite image. E) Overlayed image of metepipodite, scale bar represents 10µm.

Initial staining of the metepipodites demonstrated the NKA expression, again localized the basolateral membrane, indicating that the NKA is pumping Na^+ into the hemolymph space and not directly out of the organism as hypothesized. This indicates that the a2(KK) form of the subunit is not being used to adapt to extreme salinities by differential membrane localization. Instead, the metepipodites appear to have a highly amplified basolateral membrane, as indicated by the extent of NKA staining (Fig. 6). Staining for the apical membrane and basolateral membrane was attempted with an anti-Drosophila crumbs antibody (DSHB, Cq4) and an anti-Drosophila discs-large antibody (DSHB, 4f3), respectively. Staining was not successful. Additional staining for the apical membrane Cl^- channel was attempted using anti-rabbit CFTR antibody (Abbiotec, 200186) however this was also unsuccessful.

Expression of 2 forms of NKA in brine shrimp

In order to attempt to identify the expression of two distinct α-subunit isoforms of the NKA, a western blot of whole animal homogenate from both conditions using anti-α NKA antibody revealed two distinct bands between 105-110 kDa. The two bands represented both α1(NN) and α2(KK) isoform, based on kDa size and comparison to purified kidney (Fig. 7). Although protein bands were not directly analyzed, identical protein concentrations were added to each lane, revealing a darker bands in the 2M NaCl group, as was expected based on increased expression of the NKA in response to increased salinity.

NKCC inhibition

Bumetanide, a specific inhibitor of NKCC, was added to the medium of brine shrimp adapted to 2M NaCl and 250mM NaCl[21]. Figure 8 shows an increase in response (i.e. reduced

movement and/or death) to bumetanide in brine shrimp adapted to 2M NaCl compared to those adapted to 250mM NaCl. At 50-75μM bumetanide, there was an approximate 50% response rate for the high-salt adapted brine shrimp only. Brine shrimp adapted to 250mM NaCl did not show a dose response to bumetanide (Fig 8). Additionally, 2M adapted brine shrimp had increased water loss (Fig 9) including shrinkage and an upward tail curling which greatly inhibited movement.

Ion affinities of ouabain-sensitive purified membrane fractions

Figure 10A shows the normalized average values for the $K_{0.5}$ of Na from brine shrimp adapted to both 2M and 250mM NaCl, measured by ATPase assay. There was no detectable difference in $K_{0.5}$ of the NKA fractions between the 2 groups while both showed well defined curves (R^2 = 0.95) for both datasets. Figure 10B shows the $K_{0.5}$ for K^+ in both 250mM and 2M adapted brine shrimp. The $K_{0.5}$ of the 250mM fraction was higher than that from the 2M NaCl fraction (3.78mM > 0.7mM K) , indicating a reduced affinity for K^+ compared to the 2M NaCl fraction.

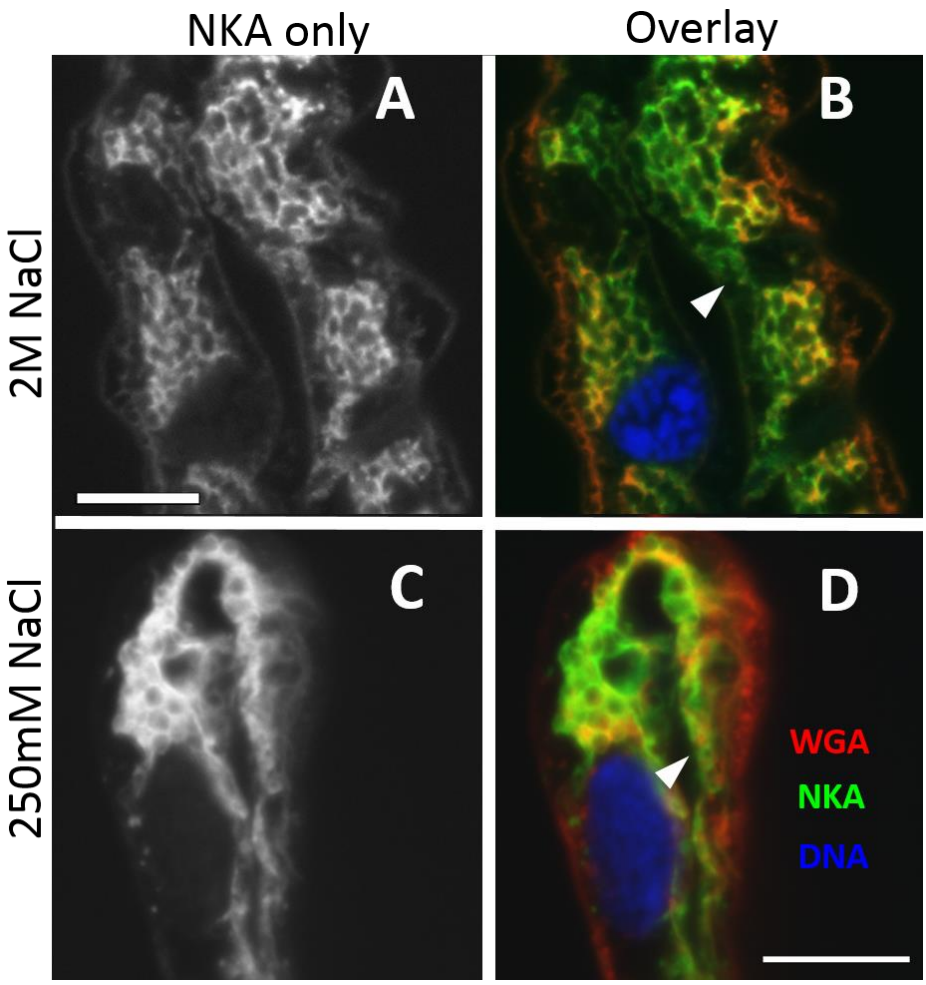

Figure 6. Immunolocalization of NKA in the metepipodites of brine shrimp. With staining for membrane (red), NKA (green) and DNA (blue). Arrows indicate the basolateral side of the metepipodite, facing the hemolymph. A, B) Brine shrimp reared in 2M NaCl, revealing NKA localized to the basolateral membrane. C,D) Brine shrimp reared in 250mM NaCl, revealing basolateral localization of NKA. Scale bar represents 10µm.

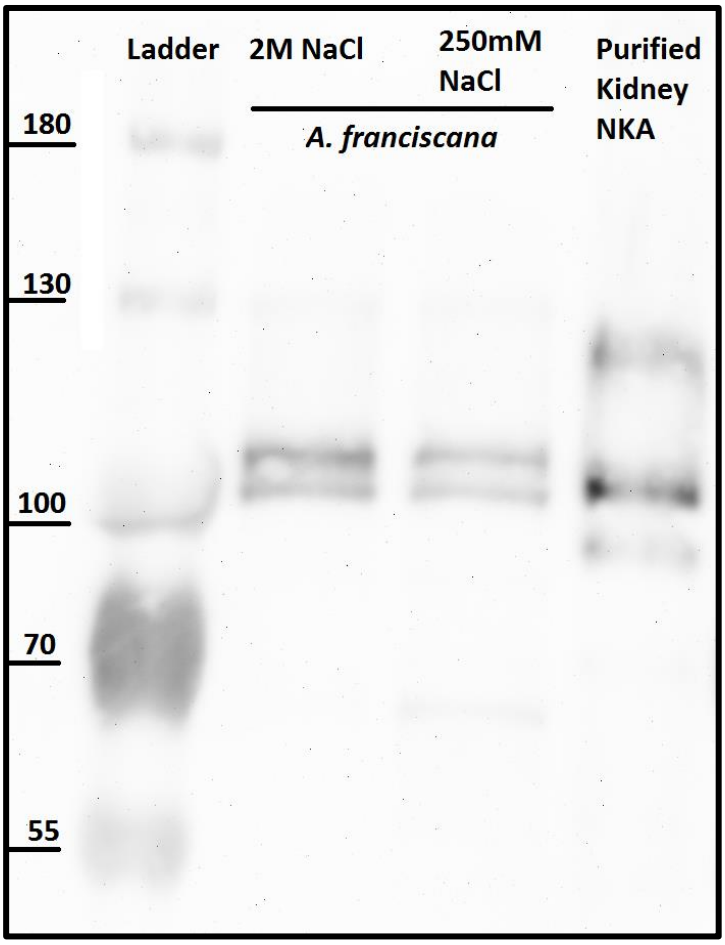

Figure 7. Western blot of NKA from brine shrimp adapted to 2M or 250mM NaCl. Probed with antibody for α-subunit of NKA. Lane 1 contains a protein ladder, lane 2 contains whole-animal homogenate from 2M NaCl-adapted brine shrimp, lane 3 contains whole-animal homogenate from 250mM NaCl-adapted brine shrimp, and Lane 4, NKA control. The two bands in lanes 2 and 3 represent the two different forms of the α-subunit, α1(NN) and α2(KK).

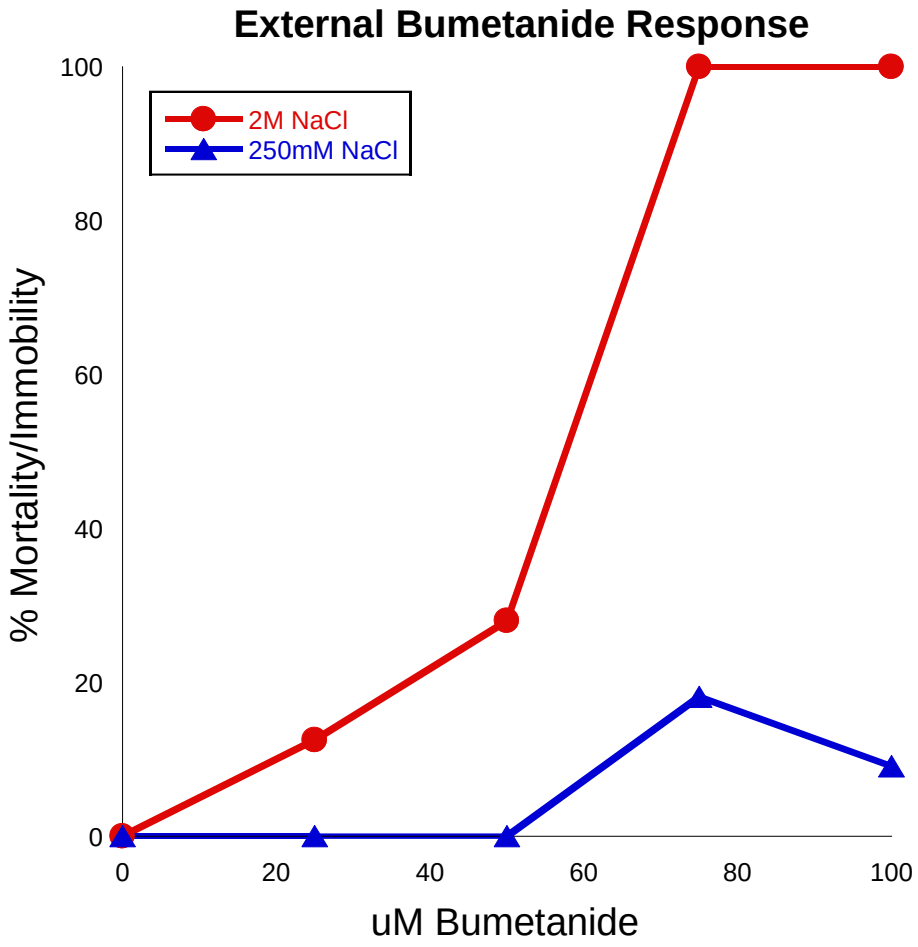

Figure 8. Dose response of bumetanide in 2M or 250mM adapted brine shrimp. Results were either response (impairment/lethality) and nonresponse. Dosing occurred over 24 hours.

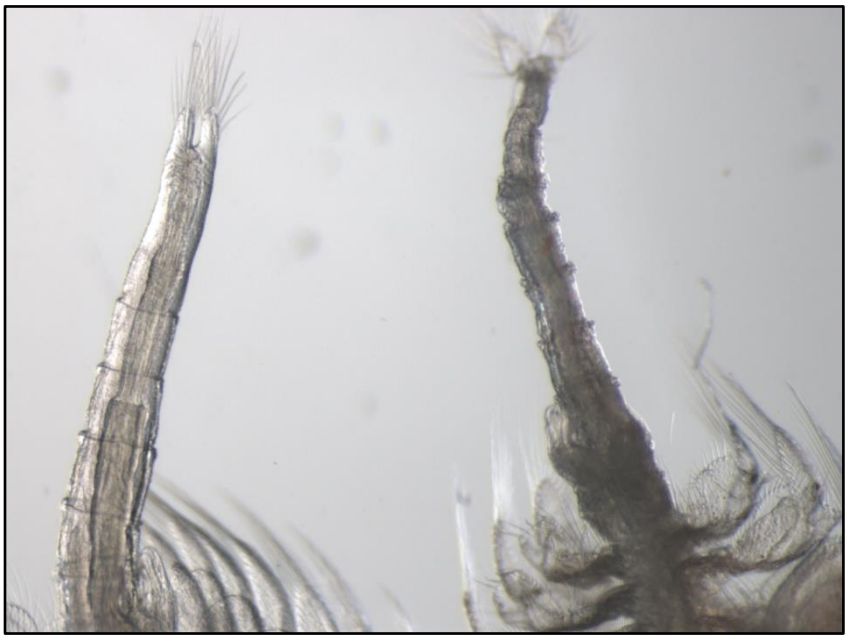

Figure 9. Comparison of water loss in brine shrimp telsons after bumetanide exposure. Left is control, right is 100μM bumetanide.

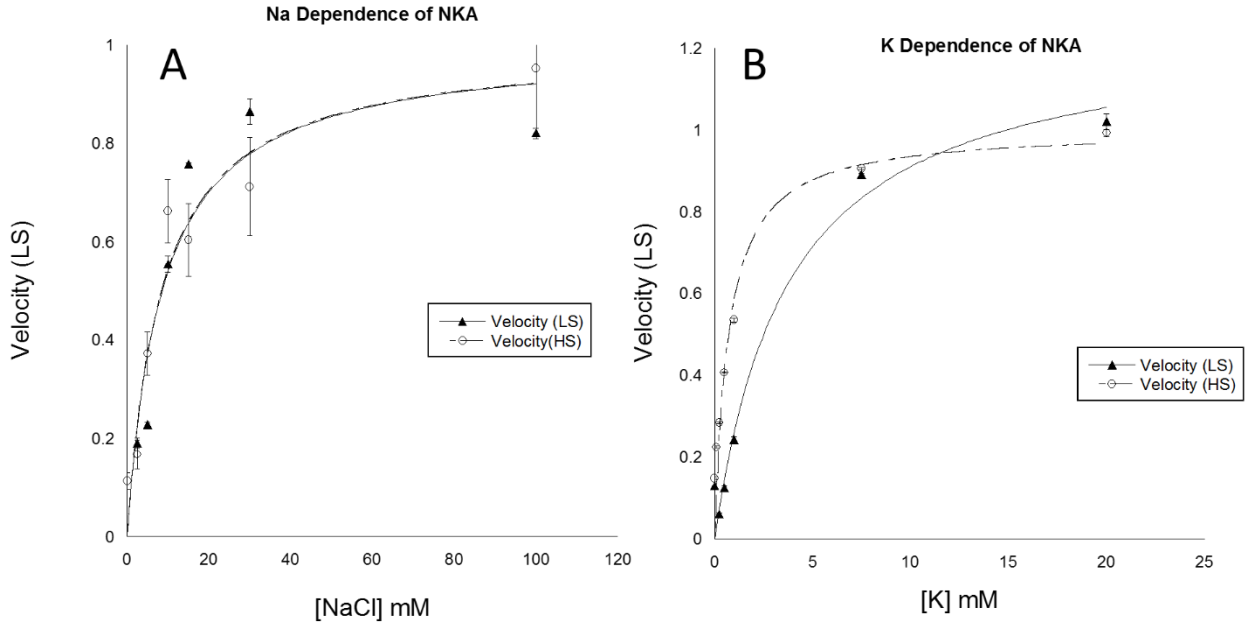

Figure 10. [Na^+] and [K^+] Dependence of NKA from brine shrimp. **A** $K_{0.5}$ for Na^+ from 2M NaCl (high salt, HS) or 250mM NaCl (low salt, LS) adapted brine shrimp. The $K_{0.5}$, from the LS group was 8.54, approximately equal to the $K_{0.5}$ of the HS group (8.4). The R^2 values were 0.913 and 0.933 for LS and HS, respectively. **B** $K_{0.5}$ for K^+ from 2M NaCl (high salt, HS) or 250mM NaCl (low salt, LS) adapted brine shrimp. The $K_{0.5}$ from the LS group was 3.78, greater than the $K_{0.5}$ of the HS group (0.7). The R^2 values were 0.975 and 0.946 in LS and HS, respectively.

Relative gene expression of NKA as a consequence of salt adaptation

Quantititive PCR was performed with primers designed for the α1(NN) cDNA, α2(KK), with an α-tubulin control using two-step RT-qPCR. Amplification plots are shown in figure 11. Increasing salinity had little effect on the amplification of both the loading control α-tubulin and the α1(NN) (Fig 11B, 11C). However, as salinity decreased from 2M to 250mM NaCl, the amplification curves showed significant shifting to the right for the low-salt (250mM) cDNAs, indicating a lower starting amount of a2(KK) mRNA in that group (Fig 11A).

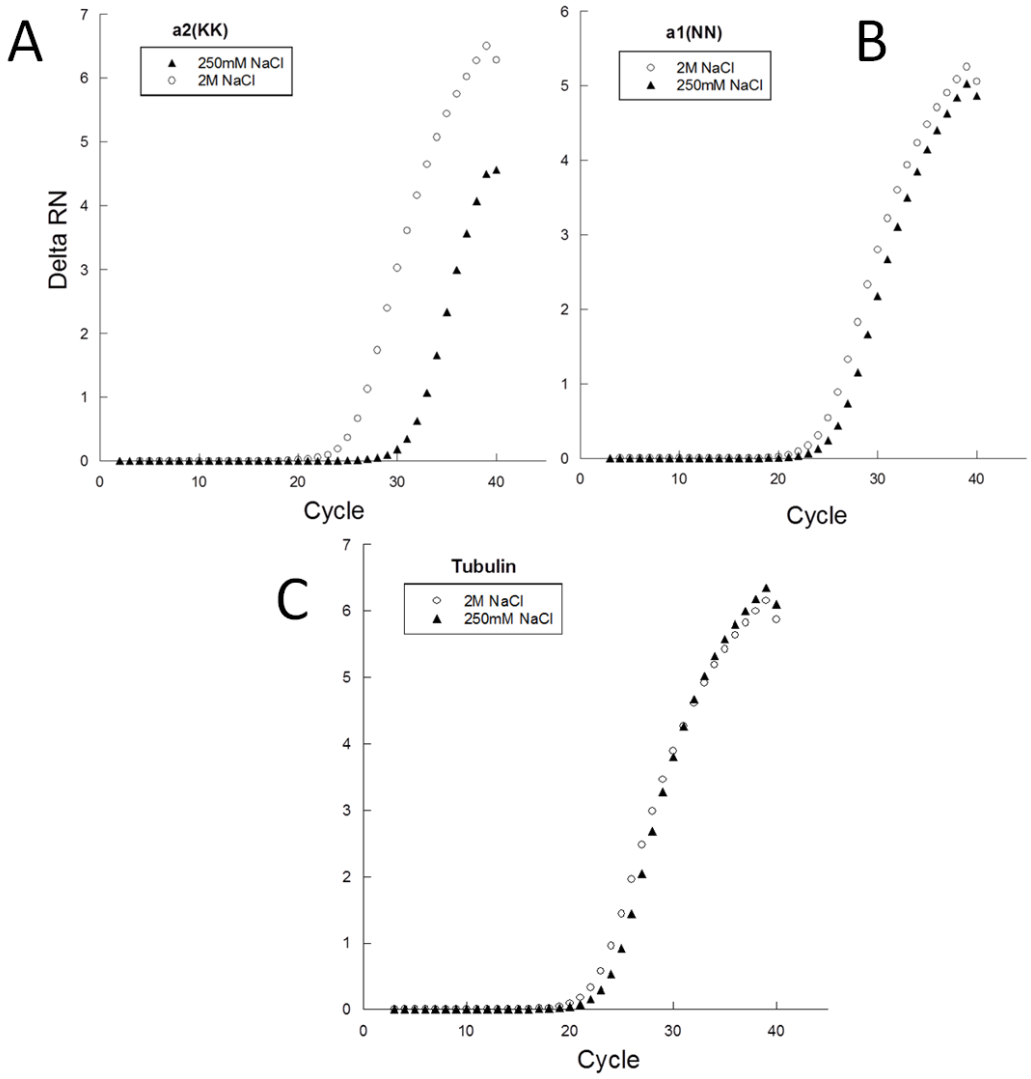

Figure 11. qPCR amplification plots of a1(NN) and a2(KK) subunits. Y axis represents the change in luminescence, or delta RN, of the reaction. The x axis represents the cycle number. There is no substantial shifting of the amplification plots between 2M and 250mM reared brine shrimp for the α1(NN) or α-tubulin transcripts, however the plot for α 2(KK) shows left shifting for the 2M NaCl group relative to the 250mM NaCl group.

CHAPTER IV: DISCUSSION

Brine shrimp are crustaceans which live in inland salt lakes and are capable of adapting to extreme salinities[11]. They possess an altered Na,K-ATPase α subunit which they upregulate in response to increased salinity[10]. It has been proposed that this subunit helps the brine shrimp to pump Na^+ across a steep concentration gradient[10], however the exact stoichiometry has not been deduced until recent work from our laboratory has suggested that the α2KK pumps have a unique stoichiometry of $2Na^+$:1:K^+:1ATP[14]. This is the first demonstration of a NKA that has a transport stoichiometry different from $3Na^+$:$2K^+$:1ATP. In this paper, I sought to determine whether the brine shrimp a2KK pump also had a unique apical expression pattern which would directly exploit its special stoichiometry for the adaptation of brine shrimp to extreme salinities. However, a2KK is expressed in the basolateral membrane like NKA in most epithelia.

Expression of the α-subunit of the Na,K ATPase as an adaptation to salinity

A. franciscana possess two NKA α-subunit isoforms which share 74% identity[10]. The defining differences between the two α-subunits are two asparagines, 324 and 776 in α1(NN), which are lysines in the second, α2(KK)[10]. These two residues (position 324 and 776) are located in a conserved cation binding region of the NKA; they are near one of the two "shared sites". Two ion binding sites are referred to as "shared" because they reciprocally bind Na^+ and K^+ on alternate sides of the membrane. A third site, the Na^+ exclusive site, only binds Na^+ ions from the cytoplasm and deposits it extracellularly. The two lysine residues occupying locations 324 and 776 have a very distinct effect in α2(KK), which in addition to altering ion affinities enables the enzyme to alter its stoichiometry. Its critical importance to brine shrimp salt tolerance is

evident by its over-expression in response to increasing salinity[10]. Although the NKA has a well-known canonical $3Na^+$ for $2K^+$ exchange, based on Jorgensen and Amat[10] and with our laboratory have compelling data to challenge this dogma and propose that brine shrimp exploit this non-canonical mode of operation in response to extreme saline environments. Their hypothesis[10] along with our findings suggest that the α2-(KK) subunit possesses a reduced stoichiometry ($2Na^+$:$1K^+$:1ATP) in the α2-(KK) compared to the well-defined canonical ($3Na^+$:$2K^+$:1ATP). This reduced stoichiometry is so unique (thus far the only example) that we contend it plays a critical role for the brine shrimp's remarkable ability to adapt and tolerate hypersaline conditions.

In order to determine mRNA expression differences between the high-salt-adapted and low-salt-adapted brine shrimp, quantitative PCR analysis was performed and revealed a significant upregulation of the α2-(KK) subunit in brine shrimp adapted to higher concentration (2M NaCl) vs. lower concentration (250mM NaCl), consistent with the observations of Jorgenson and Amat[10]. The left shift of the amplification plot for the a2(KK) subunit corresponded to an almost 2-fold increase in expression of the 2M NaCl adapted brine shrimp group over the 250mM NaCl adapted brine shrimp (Fig 11). This amplified expression was not seen with the ubiquitously expressed α1(NN) subunit.

Taken together, the reduced stoichiometry of the α2-(KK) pump[10,14] in combination with its increased expression in response to increasing salinity, suggests an adaptive function for the α2 form in extreme salinities. The reduced stoichiometry may allow the brine shrimp to overcome the increased energy requirement of pumping three Na^+ ions against the steep concentration gradients that these organisms are able to tolerate. Thus, it stands to reason that the α2-(KK) isoform of the Na,K-ATPase is advantageous in adapting to high salinity.

Metepipodites are Na^+ and Cl^- excreting organs

Initial proposals that metepipodites may function similarly to teleost gill epithelia were based on measurements of transepithelial potential differences (TEP) and ion analyses indicating active Cl^- excretion and passive Na^+ movement into the external media[11]. Additionally, morphological similarities exist between chloride cells of teleost gills and cells of the metepipodites in brine shrimp[11]. In teleost gill epithelia, the chloride cells are specialized for ion regulation using an extensive contiguous basal membrane providing a large surface area for the NKA to create a Na^+ gradient, utilized by NKCC to drive the influx of Na^+, K^+, and $2Cl^-$ ions[13]. Chloride ions are then actively pumped out of the organism at the apical membrane via a CFTR-channel, which produces the measurable differences in TEP[4]. This effect on TEP then allows the recycled extracellular Na^+ to leave the organism through a single-stranded tight junction between adjacent chloride cells which are 'leaky' to cations. These ion movements allow for the maintenance of a hypotonic internal environment of the organism relative to the concentrated external environment.

I assessed these similarities in the context of different concentrations of salt-adapted brine shrimp. Membrane staining revealed a highly convoluted architecture of the metepipodite membranes with a surplus of mitochondria in relation to the surrounding phyllopodia. Both of these features resemble teleost gill chloride cells, which are mitochondrial rich and have an extensive basolateral membrane[6]. An abundance of mitochondria indicates a subsequent increase in available ATP, which is needed by the NKA to pump Na^+ across the steep concentration gradient found between the cytoplasm of the cell and the tubular lumen of the membraneous chloride cell[13]. It is this gradient that provides the energy for NKCC, which

expressed in many secretory tissues for maintenance of cell volume and integrity[21], to pump Cl^-, along with K^+ and Na^+, into the gill epithelial cell, where Cl^- then exits down its electrochemical gradient through a CFTR-channel[6].

I tested for both the presence and function of NKCC in brine shrimp with a specific inhibitor, bumetanide, in order to assess differences in functional importance between 2M NaCl- and 250mM NaCl adapted brine shrimp. After 24 hours, little or no effect was apparent in 250mM NaCl-adapted brine shrimp, while impairment of processive swimming or death was observed in a dose-responsive manner in brine shrimp adapted to 2M NaCl (Fig. 8). The response in 2M NaCl-adapted brine shrimp and lack of a response in 250mM NaCl-adapted brine shrimp indicates that NKCC is necessary for survival in hypersaline environments. This finding is consistent with other systems, which have observed that NKCC is activated upon cell shrinkage and works in sync with other membrane ion transporters to regulate cell volume through water gain and loss[21]. Without NKCC activity, signs of water loss were observed in the brine shrimp in concentrated media (Fig. 9).

Altered ion affinity of NKA in hypersaline adapted brine shrimp

Jorgenson and Amat identified an increase in expression of the α2(KK) NKA subunit in 2M versus 250mM NaCl-adapted brine shrimp along with altered ion affinity compared to a1(NN)[10]. In order to determine the localization and morphological differences that may be representative of effects by α2(KK), I measured 2M NaCl adapted brine shrimp α2(KK) expression in combination with altered ion affinity. The $K_{0.5}$ for K^+ and Na^+ were determined by ouabain-sensitive phosphate liberation from ATP hydrolysis. My data indicate that the NKA $[K^+]$ affinity is higher in 2M NaCl adapted brine shrimp, suggesting the α2(KK) may have an

altered K^+ affinity. An increase in K^+ affinity of the α2(KK) form may be beneficial in compensating for the increased competition from Na^+ in a hypersaline environment. However, preliminary data from our lab demonstrates the α2(KK) is capable of $3Na^+/2Na^+$ exchange in the absence of K^+, thus the K^+ affinity may be skewed due to this effect[14]. An altered $K_{0.5}$ for ion binding was observed only with K^+, as the $K_{0.5}$ for Na^+ showed no differences between groups (Fig. 10A and 10B).

Proposed mechanism of salt extrusion in highly saline environments

Due to the locations of these two lysine mutations in conserved ion-binding regions of the enzyme, it was proposed, and our lab identified, a reduced stoichiometry that may be advantageous for brine shrimp survival in extreme saline environments. This reduction in stoichiometry ($2Na^+$:$1K^+$ vs. $3Na^+$:$2K^+$) per ATP means that there is more energy invested per ion transported, allowing the pump to remain active in 2M NaCl environments. To date our lab is the first to report a noncanonical stoichiometry for any NKA.

The location of α2(KK) NKA however, has yet to be identified. My first hypothesis was NKA expression may occur in the apical membrane of the gut epithelia as a mechanism for Na^+ extrusion and prevention of excess Na^+ accumulation in the hemolymph. However, this was not the case as NKA expression was only present on the basolateral membrane of the gut epithelia in both 250mM and 2M NaCl adapted animals. This illustrates that uptake of Na^+ ions likely occurs through the gut, into the hemolymph by the NKA. Once in the hemolymph, $[Na^+]$ and $[Cl^-]$ will rise throughout and eventually reach the metepipodites, where NKA expression remained on the basolateral membrane in both 2M and 250mM SW adapted brine shrimp. However, the metepipodites did have specialized morphology similar to what exists in the chloride cells of

teleost fishes. They possess increased mitochondrial content and membrane amplification compared to the rest of the phyllopodia, adaptations which likely help by increasing the surface area over which NKA maintain a constant concentration gradient. The brine shrimp also appear to have an increased expression of NKA in response to increased salinity in their environment, which it likely uses to create a localized steep Na^+ concentration gradient in the basolateral membrane, similar to teleost fish gills. The NKCC utilizes this gradient to import K^+, and $2Cl^-$ into the cell, which may be used to extrude Cl^- through a CFTR-channel at the apical membrane. The positive TEP created by exiting Cl^- ions drives Na^+ to leave passively through leaky tight junctions between chloride cells and accessory cells[7].

CHAPTER V: CONCLUSION

The Na,K-ATPase (NKA) is an essential membrane pump that helps to establish a cell's ion gradients and membrane voltage[1]. It is used by many organisms to adapt to salt in their environment, such as teleost fishes, which extrude salt across their gill epithelia[5]. Brine shrimp (*Artemia franciscana*) are extreme halophiles and are known to possess an α2 version of the pump's catalytic α subunit which has 2 key asparagines to lysine substitutions (α2(KK)) in ion binding regions[10]. Evidence for altered stoichiometry of this subunit as an adaptation to their extreme environments has been established[14], however, the precise molecular and physiological mechanisms have not previously been clarified.

To determine how this α2(KK) form can be utilized to tolerate extreme salinities, I performed immunohistochemical analysis on different tissues to examine localization and other adaptive characteristics. I determined the cellular localization of the NKA was expressed on the basolateral membrane, as is most common, in brine shrimp gut and metepipodites.

Gene expression was also characterized in brine shrimp adapted to 2 different salinities, and NKA ion affinity was measured from these groups as well. It appears that brine shrimp upregulate only the α2 form of the pump in response to increasing salinity, while the α1 form remains relatively unchanged. Additionally, I demonstrate the NKA apparent affinity for K^+ is decreased in 250mM NaCl adapted animals, while the apparent affinity for Na^+ was unaltered. As 250mM NaCl adapted animals have more of the α1-(NN) than the α2-(KK) subunit compared to 2M NaCl-adpated animals based on qPCR analysis, the altered affinity can be attributed to differential ion dependencies of α1-(NN) and α2-(KK).

Based on these results, it was determined that brine shrimp are likely utilizing a mechanism of salt adaptation similar to that found in marine fish gills, in which salt extrusion

occurs in a mitochondrial rich, membraneous cell layer in the metepipodites of the brine shrimp. There, basolateral NKA establishes a localized steep Na^+ concentration gradient, which is then utilized by another membrane transporter, NKCC, to bring in Na^+, K^+, and Cl^- in to the cell. Cl^- is actively pumped out of the apical membrane, providing a positive TEP that allows Na^+ to leave passively through leaky single-pass tight junctions between adjacent cells.

Brine shrimp appear to utilize a mechanism of ion regulation similar to the mechanism utilized by marine teleosts[11]. Their salt extruding organs, the metepipodites, appear to have many similarities with the chloride cells of teleost gills, including cells, which possess a highly amplified membrane and enriched mitochondrial content[5]. NKA is located basolaterally in the gut, where water and ions are taken in from the environment into the hemolymph[9]. NKA was also found to be expressed in the basolateral side of the metepipodites, indicating that the brine shrimp are not utilizing NKA to directly extrude Na^+ into the environment. In addition, brine shrimp express a more energetically favorable form of the NKA in response to salinity while the housekeeping form is not substantially upregulated during this process. My work suggests that brine shrimp are adapting to extreme salinities in their environment via the upregulation of an NKA with altered stoichiometry and regulate ions in a similar to the way that marine teleost gills function to allow for survival in extreme environments.

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APPENDIX A: SOLUTIONS

Sodium citrate buffer: (10mM sodium citrate, 0.05% Tween 20, pH 6.0)

Buffer HS: 25mM imidazole, 1mM EDTA, 250mM sucrose. pH=7.4

Bradford's reagent: 100mg Coomassie blue G, 50mL ethanol, 100ml 85% phosphoric acid. Bring to 1L with ddH_2O

ATPase assay Solution: 2.4mM EGTA, 96.4mM KCl, 14.5mM $MgCl_2$,16mM Na_2ATP, 239.5mM imidazole

Stopping Solution: 2ml H_2O, 2ml 1M HCl, 0.13g Absorbic acid, 200µL 10% NH_4MoO_4

ACG Solution: 2% Na-arsenite, 2% Na-citrate, 24mM Glacial acetic acid

TBS: 50mM Tris, 150mM NaCl, pH 7.6

Blocking Solution: 10% normal goat serum, 1% BSA in TBS