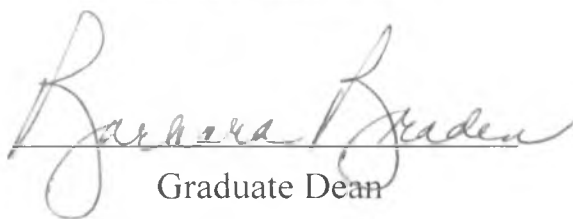


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**The Na/K-ATPase in the gills of Antarctic and New Zealand
nototheniids: The Physiologic and Molecular Effects of Warm-
acclimation.**

By

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ABSTRACT

Antarctic fish, living in -2°C waters, have a serum osmolality of $\approx 600\text{mOsm/kg}$ nearly double that of temperate teleosts. The increase in serum osmolality is due to proportional increases in the Na^+ and Cl^- concentrations as compared to temperate teleosts, including members of the same family. The sodium and potassium adenosine triphosphatase (Na/K-ATPase) in the chloride cells of the gill is responsible for maintenance of serum ion concentrations. The effects of thermal acclimation in two Nototheniid species, the stenothermal Antarctic *Trematomus bernacchii* and the eurythermal New Zealand *Notothenia angustata*, were investigated by measuring serum osmolality, gill Na/K-ATPase activity, sodium pump density and ouabain affinity. Both fishes were acclimated at their upper and lower viable thermal temperatures. Warm acclimation ($+4^{\circ}\text{C}$) of the *T. bernacchii* significantly decreased their serum osmolality from 550 mOsm/kg to 450 mOsm/kg compared to cold-acclimation (-1.5°C) and this was accompanied by a two-fold increase in gill Na/K-ATPase activity. Warm-acclimation ($+14^{\circ}\text{C}$) of the *N. angustata* did not significantly change their serum osmolality from 330 mOsm/kg or gill Na/K-ATPase activity compared to the cold-acclimated ($+4^{\circ}\text{C}$) *N. angustata*. Using [^3H]-ouabain binding techniques, the B_{max} and K_{d} values of gill Na/K-ATPase enzymes were determined. No difference in the B_{max} or K_{d} of the warm-acclimated *T. bernacchii* accounted for the increase in Na/K-ATPase activity. The change in gill Na/K-ATPase activity in the warm-acclimated *T. bernacchii* is not mediated by an increase in the number of enzyme sites and is not reflected by a change in ouabain affinity for Na/K-ATPase.

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The Na/K-ATPase is composed of two subunits; a catalytic α -subunit with 4 isoforms and a β -subunit with 3 isoforms. In previous teleost studies, several isoform mRNAs and $\alpha 1$ and $\alpha 3$ proteins have been identified in gills. As a first step towards quantifying the Na/K-ATPase α -subunit, we determined the mRNA and protein isoform composition in the gills of the Antarctic *T. bernacchii*. In other teleost studies, especially with salinity acclimation experiments, changes in Na/K-ATPase α isoform expression accompanied changes in Na/K-ATPase activity. As the first step in determining the molecular effects of warm acclimation, the gill Na/K-ATPase α isoform expression was determined. Partial cDNA sequences of *T. bernacchii* $\alpha 1$, $\alpha 2$, and $\alpha 3$ isoforms were derived from RT-PCR using total RNA from brain, muscle, kidney and gill and subsequent sequencing of sub-cloned products. There is 72-74% nucleotide identity between the three *T. bernacchii* isoform sequences and 71%, 76% and 78% compared to the rat isoforms for the $\alpha 1$, $\alpha 2$, and $\alpha 3$, respectively. The *T. bernacchii* $\alpha 1$, $\alpha 2$, and $\alpha 3$ amino acid (AA) sequences, within the 13 AA region that defines the isoforms, share 65%, 65% and 94% AA identity with the rat $\alpha 1$, $\alpha 2$, and $\alpha 3$ sequences, respectively. There are two unique and radical substitutions in the *T. bernacchii* sequences; in the $\alpha 1$ a glutamine for a histidine at position 503(rat) and in the $\alpha 2$ an alanine for an arginine at position 493(rat). Using isoform specific primers, the mRNA for all three isoforms was found in *T. bernacchii* brain, gill, heart, trunk kidney and muscle. Using isoform specific antibodies, the Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ subunit isoforms were found in the tissues of both *T. bernacchii* and *N. angustata*, a temperate nototheniid. This is the first report detecting all three isoforms at the nucleotide and protein level in teleosts. Additional

experiments determined the *T. bernacchii* gill Na/K-ATPase α isoform mRNA and protein expression during warm acclimation.

The Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms have different kinetic and physiologic properties. It was hypothesized that the increase in activity is due to a change in the gill Na/K-ATPase α isoform expression. Using western blotting and isoform specific antibodies, the band density of the Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ -subunit isoform proteins was measured in cold and warm-acclimated *T. bernacchii*. Na/K-ATPase $\alpha 3$ -subunit isoform protein decreased $56 \pm 0.1\%$ ($p < 0.05$) during warm acclimation. There were no significant changes in $\alpha 1$ or $\alpha 2$ -subunit isoform proteins. The Na/K-ATPase $\alpha 3$ -subunit isoform has a lower intracellular Na^+ affinity, increases intracellular Na^+ concentrations ($[\text{Na}^+]_{\text{IC}}$) when transfected into HeLa cells, and inhibits the activity of the $\alpha 1$ isoform. Our results suggest a relationship between the decreased serum osmolality of warm acclimated *T. bernacchii* and the expression of the $\alpha 3$ isoform, possibly due to the expected decrease in $[\text{Na}^+]_{\text{IC}}$ with decreasing serum osmolality.

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CHAPTER ONE

Discussion of Antarctic fish Osmolality and the Na/K-ATPase.

Antarctic Fish

Environment

Due to global geographical events 25 million years ago (mya), a circumpolar current formed around the Antarctic continent (Eastman, 1993; Macdonald *et al.*, 1987)(Fig. 1.1). As of 14 mya, the water temperature and climate are similar to present day conditions (Eastman, 1993), separating Antarctic and temperate nototheniids. Water temperatures in the Southern Ocean vary between 8°C at high latitudes and -2°C at the lowest latitudes. In McMurdo Sound at a latitude of 78°S, where fishes in these studies were caught, the water temperature is $-1.86^{\circ}\text{C}\pm 0.1^{\circ}\text{C}$ regardless of depth or season (Littlepage, 1965). Salinity, density and oxygen levels of McMurdo Sound do not significantly vary with depth or season either.

Fish

The types and diversity of Antarctic fishes are relatively invariable (Eastman and DeVries, 1986). The suborder Notothenioidei is highly endemic to Antarctica, comprising ~35% of all Antarctic fish species and 55% of all benthic species on the coastal continental shelf (Eastman, 1993). However, notothenioids exhibit substantial morphological and ecological differences based upon their preferred habitat within the Antarctic environment (Eastman and McCune, 2000). *Trematomus bernacchii*, a benthic scavenger living at 0-80m, is one of the most common Antarctic notothenioidei and has the anatomy and lifestyle thought to represent the most ancestral Antarctic fishes (Macdonald *et al.*, 1987). In the studies presented here, *T. bernacchii* will be compared with *Notothenia angustata*, a New Zealand benthic notothenioid of the same family (Nototheniidae) living in sub-polar waters between 2°C and 18°C.

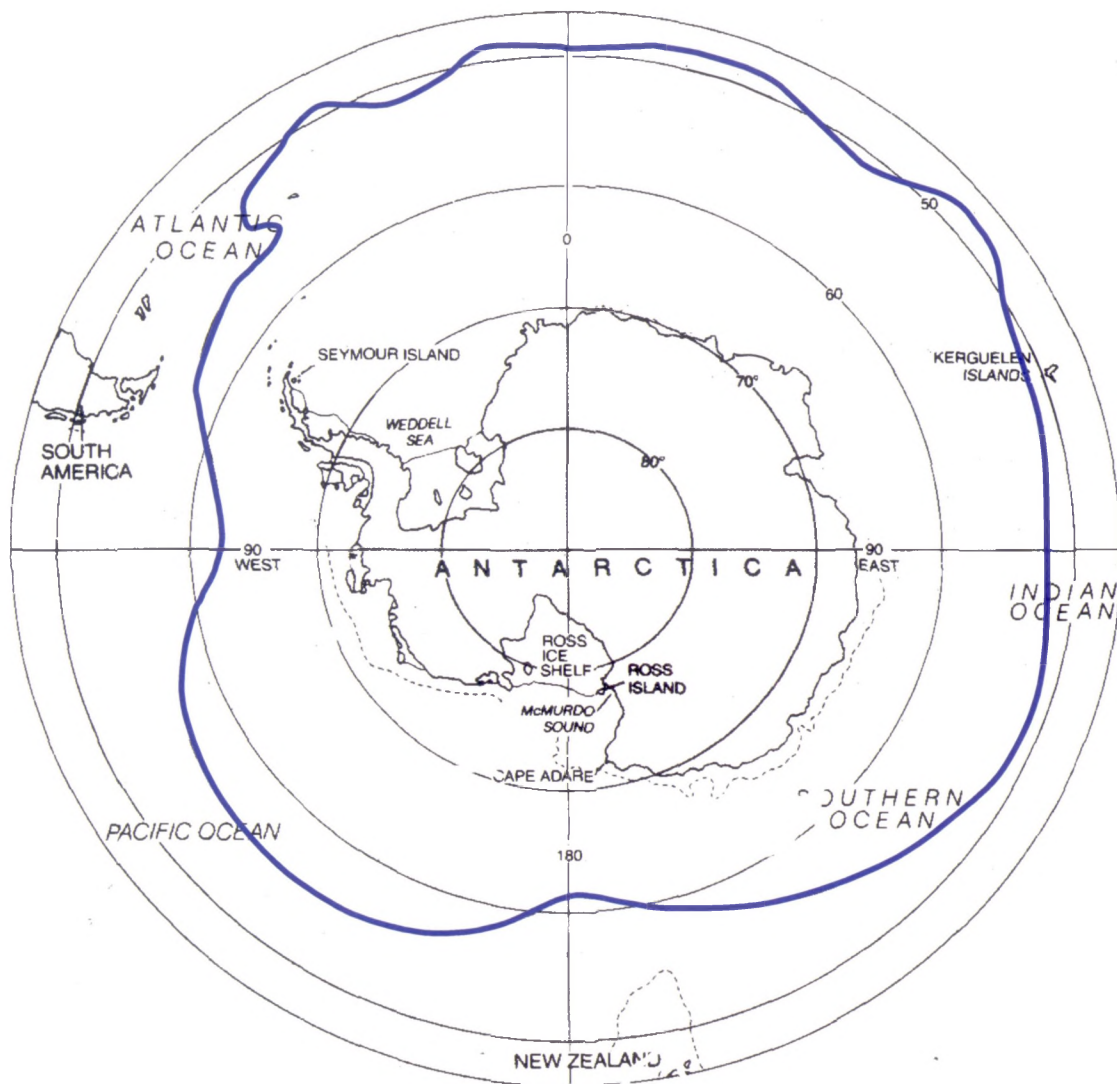


Figure 1.1 Map of Antarctica and the surrounding Southern Ocean adapted from Eastman, 1986. The heavy blue line indicates the path of the Antarctic Convergence.

Evolutionary adaptations of Antarctic fish

Antarctic notothenioides have evolved several adaptations to manage the extreme low temperatures, the presence of ice, limited coastal habitat and a lack of year long primary productivity (Eastman, 1993). The most well studied adaptation is the production of antifreeze glycoproteins (AFGP), discovered by DeVries, *et al* (1970). Antarctic fish AFGP's are 8 glycoproteins of varying sizes from 4200Da (AFGP 8) to 34000Da (AFGP 1) (Eastman and DeVries, 1986). The 8 isoforms are all composed of repeating units of the tripeptide Thr-Ala-Ala with a disaccharide attached to each Thr (DeVries, 1988). AFGPs adsorb to ice crystals via interactions between the hydroxyl groups in the sugars and carbonyl groups of the amino acids, thereby preventing the further growth and accumulation of ice crystals in the body (Eastman and DeVries, 1986). AFGPs act in a non-colligative manner lowering the serum freezing point by -1.5°C (DeVries *et al.*, 1970). They are found in all tissues in the body except the vitreous humor, endolymph and urine (DeVries, 1988; Macdonald *et al.*, 1987). Second, Antarctic fish are aglomerular, forming their urine by a process of tubular secretion only (Eastman and DeVries, 1986). AFGPs would normally pass through a glomerulus due to their small size and neutral charge, however they are not secreted into the lumen. Therefore, an aglomerular kidney retains the AFGPs, and theoretically conserves the energy required to produce more AFGPs (Eastman and DeVries, 1986). Notothenioides above the Antarctic convergence have glomeruli.

A third adaptation of Antarctic fish is an increase in the poly-unsaturated fatty acid (PUFA) composition of plasma membranes compared to marine fish in temperate waters (Morris and Schneider, 1969). It is known that with all poikilotherms, as

temperature decreases there is both short and long term re-structuring of the plasma membranes including an increase in the number of PUFA (Hazel and Prosser, 1974). The double bonds of PUFAs allow more plasma membrane “fluidity” whereas the single bonds of saturated fatty acids become rigid at low temperatures. Membrane fluidity is important for proper functioning of trans-membrane enzymes and proteins. Antarctic fish also have an increased number of lipid deposits throughout their bodies, which help maintain buoyancy as they do not have swim bladders (Eastman and DeVries, 1986).

The fourth evolutionary adaptation of Antarctic fish, which led to these studies, is an increase in the concentration of ions in the blood. The serum osmolality of Antarctic fishes is approximately 600mOsm/kg, nearly double the serum osmolality (330mOsm/kg) of temperate marine teleosts including other members of the Notothenioid family living above the Antarctic convergence (O'Grady and DeVries, 1982). The increase in serum osmolality is due to concomitant increases in both serum $[Na^+]$ and $[Cl^-]$ (Dobbs and DeVries, 1975). The increased ion concentrations colligatively decrease Antarctic fish serum freezing points by $-1.0^{\circ}C$ (DeVries, 1982). Additionally, it is hypothesized that the increased serum osmolality decreases the normally large ionic gradient between marine teleost blood and seawater, reducing the energy required to maintain the (Prosser *et al.*, 1970). The last unique thermoregulatory aspect of Antarctic teleost physiology is that they are remarkably stenothermal, having a viable thermal environmental range of $-2^{\circ}C$ to $+6^{\circ}C$, one of the narrowest ranges reported for teleosts species (Somero and DeVries, 1967).

Osmoregulation in Marine Teleosts

Ion Movement

All marine teleosts, including Antarctic teleosts, are hypotonic to their environment of ~1000mOsm/kg, requiring the excretion of ions and conservation of water to osmo- and ionoregulate. The consumption of seawater is their only source of water yet it is also the source of excess ions. Large percentages of the ingested Mg^{2+} and SO_4^{2-} , and 30% of the Ca^{2+} are not absorbed in the intestine and are excreted rectally, while 100% of the Na^+ , Cl^- and K^+ and 70% of the Ca^{2+} are absorbed in the small intestine (Evans, 1993). The divalent ions that are absorbed are excreted renally in an iso-osmotic urine, while the monovalent ions and a small percentage of Ca^{2+} are excreted via the gills (Evans, 1993). Absorption of ions through the intestinal tract and excretion of ions via the kidney and gills controls serum osmolality in marine fish. However, the gills are the most influential regulatory site of serum osmolality and are responsible for the excretion of Na^+ and Cl^- , the major ionic contributors to serum osmolality.

Gills and Chloride Cells

The gills and the accompanying branchial epithelium serve four important functions in teleosts: respiration through respiratory cells associated with the arterioarterial circulation (Girard and Payan, 1980), maintenance of acid-base balance, excretion of nitrogenous waste products and the extrarenal excretion of monovalent ions regulating the osmotic and ionic balance of the organism. In marine teleosts there are four branchial gill arches on either side of the head, with many cartilaginous gill filaments extending off each arch. The gill surface area is further increased by the secondary lamellae, which protrude off each filament and are the primary sites for

respiration, acid-base balance and nitrogenous waste excretion (Hughes, 1984). The chloride-cell or “mitochondria-rich cell” was discovered in 1932 (Keys and Willmer, 1932). It is located at the base of the secondary lamellae and within the gill filament epithelium (Girard and Payan, 1980) and is responsible for the excretion of monovalent ions and the regulation of serum osmolality. Chloride cells have been identified in *T. bernacchii* (Masini *et al.*, 2000). Chloride cells are also found within the opercular epithelium of many teleost species including killifish (*Fundulus heteroclitus*) and tilapia (*Oreochromis mossambicus*) (Karnaky *et al.*, 1976; Karnaky *et al.*, 1977; Karnaky and Kinter, 1977) however none were found in *T. bernacchii* opercular membranes (J. Eastman, personal communication). Regardless of location, chloride cells are large columnar cells extending from the basal lamina to the seawater with an apical crypt shared with accompanying accessory cells (thought to be immature chloride cells) (Karnaky and Kinter, 1977; Zadunaisky, 1984). Chloride cells are mitochondria rich, have an extensive invagination of basolateral membrane known as the tubular reticulum system and have the highest metabolic rates of any gill cell (Karnaky, 1986; Karnaky and Kinter, 1977; Perry and Walsh, 1989; Zadunaisky, 1984). The high metabolism is due to the high concentration of Na/K-ATPase (Karnaky *et al.*, 1976) on the tubular reticular system (basolateral membrane) (Hootman and Philpott, 1979).

The Na/K-ATPase establishes and maintains the electrochemical gradient responsible for the excretion of ions, mainly Na^+ and Cl^- , through the gill chloride cells (Degnan and Zadunaisky, 1980; Karnaky, 1986) (Fig. 1.2). Na/K-ATPase hydrolyzes one ATP molecule, and uses the energy to pump 2 K^+ ions into the cell and 3 Na^+ out of the cell against their concentration gradients (Crambert *et al.*, 2000). A $\text{Na}^+/\text{K}^+/\text{2Cl}^-$

cotransporter on the basolateral membrane, uses the low intracellular Na^+ gradient to bring in a Na^+ , a K^+ and 2 Cl^- . The Cl^- , because of the negative charge and increased concentration within the cell, are excreted down their electrochemical gradient via a CFTR-like Cl^- channel on the membrane of the apical crypt (Karnaky, 1986). The loss of these negative charges and a leaky tight junction between the chloride cell and its accompanying accessory cell allows the paracellular diffusion of Na^+ into the seawater (Karnaky, 1986; Zadunaisky, 1984). K^+ is recycled via an inwardly-rectifying K^+ channel on the chloride cell basolateral membrane (Suzuki *et al.*, 1999) and there is evidence that there is some K^+ secretion via an unidentified K^+ channel on the apical membrane (Marshall, 1981) (see Fig. 1.2). The primary control of chloride cell activity is serum osmolality, which induces cell swelling or shrinking which activates the transporters and exchangers within the cell (Zadunaisky, 1996).

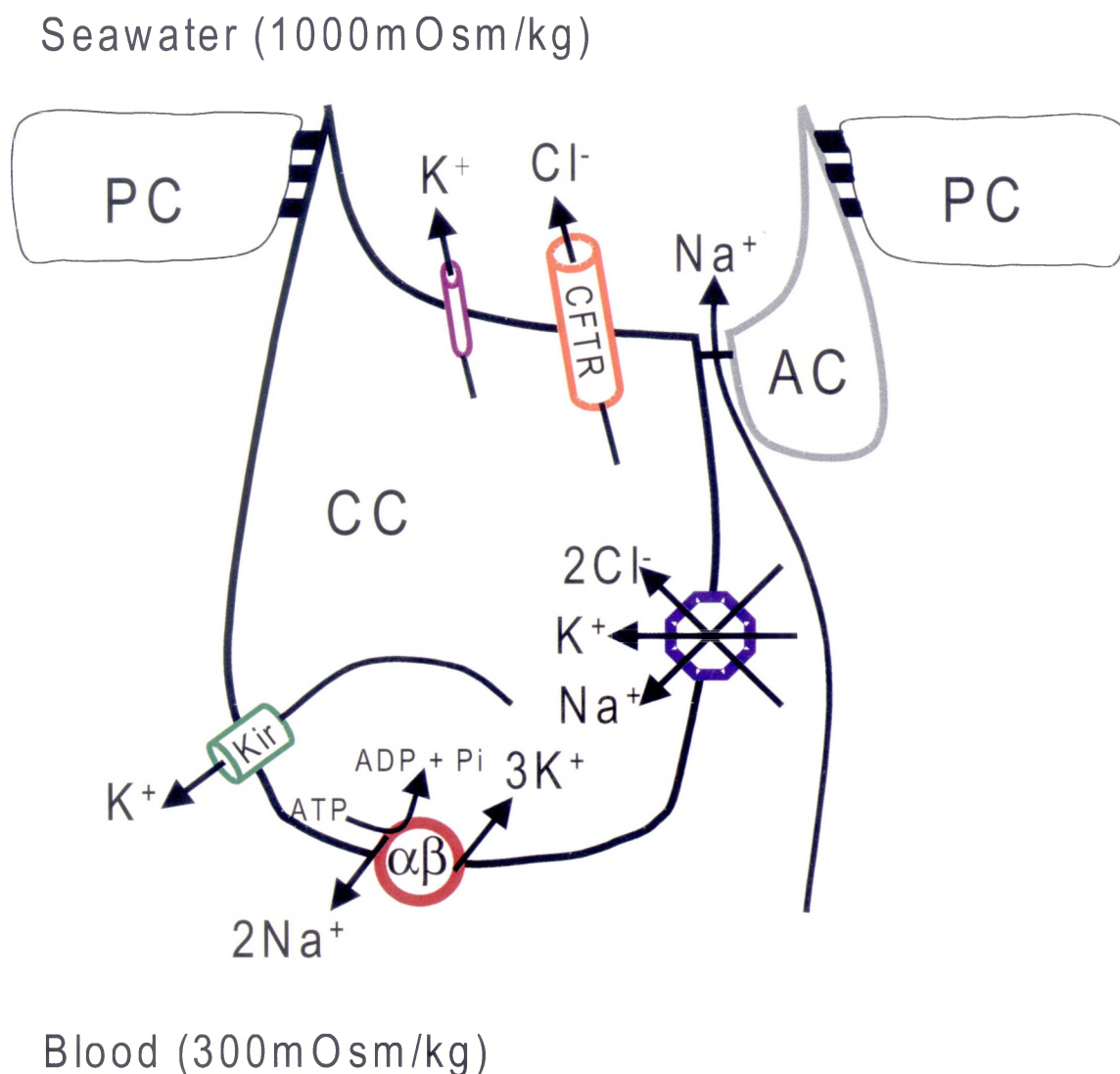


Figure 1.2 Schematic drawing of a chloride cell in a temperate marine teleost. The Na/K-ATPase (red), Na⁺/K⁺/2Cl⁻ cotransporter (blue) and inwardly-rectifying K⁺ channel (green) are shown on the basolateral membrane and a CFTR-like Cl⁻ channel (orange) and a theorized but unknown type of K⁺ channel (purple) are within the apical crypt. CC – chloride cell, AC – accessory cell, PC – pavement (epithelial) cell

Na/K-ATPase

Function

The Na/K-ATPase is an integral membrane protein, found in practically every eukaryotic cell. One third of an animal's cellular energy requirement is used to fuel the Na/K-ATPase and in cells that are electrically or transport active, it is estimated to use 70% of the available ATP (Mercer, 1993). It is an electrogenic pump and is responsible for the regulation of the electrical and chemical gradient of Na⁺ and K⁺ across cellular membranes (Horisberger *et al.*, 1991; Skou and Esmann, 1992). The asymmetric distribution of the Na/K-ATPase on basolateral membranes controls extracellular volume and osmotic balance between the intra- and extra cellular compartments, thereby regulating serum osmolality (Horisberger *et al.*, 1991). By establishing a low intracellular [Na⁺], the Na/K-ATPase drives all Na⁺-coupled secondary transport, controls free Ca²⁺ concentrations and maintains membrane potentials allowing for depolarization and repolarization activities (Skou and Esmann, 1992). The Na/K-ATPase is a member of the P-type ATPase family, which includes Ca²⁺-ATPases and H/K-ATPases (Mercer, 1993). They are all characterized by having two distinct conformational transitions and a phosphorylated protein intermediate (Lingrel and Kuntzweiler, 1994). Additionally, P-type ATPases are phosphorylated on an aspartic acid residue, an unusual residue for phosphorylation (Mercer, 1993; Skou and Esmann, 1992). The Na/K-ATPase is unique among P-type ATPases because it can be inhibited by ouabain, a cardiac glycoside.

Molecular Structure

The Na/K-ATPase is a functional heterodimer, composed of two subunits, an α -subunit of ~100kDa, and a β -subunit of ~55kDa (Lingrel *et al.*, 1990). The α -subunit is

an integral membrane protein with 10 transmembrane domains (TMD) and intracellular amino and carboxy tails (Mercer, 1993). It is the primary catalytic subunit with Na^+ , K^+ , Mg^{2+} , ATP and ouabain binding sites (Crambert *et al.*, 2000; Jorgensen, 1983). The α -subunit also contains the phosphorylated aspartic acid residue and changes its conformation causing ion turnover (Lingrel and Kuntzweiler, 1994). The β -subunit has one transmembrane domain with the extracellular carboxy tail having multiple disulfide bonds and glycosylation sites (McDonough *et al.*, 1990). The β -subunit has no known catalytic function of its own, however its presence is required for catalytic function of the heterodimeric enzyme. Also, there is evidence that the β -subunit is involved in the folding of the α -subunit, the integration of the α -subunit in the membrane and transport of the holo-enzyme to the membrane and influences cation binding affinity (Crambert *et al.*, 2000; Geering, 1991; Lutsenkko and Kaplan, 1993; Schmalzing G. and Gloor S, 1994; Therien *et al.*, 1996). There are multiple isoforms of both the α - and β -subunits (Blanco and Mercer, 1998). Additionally, there is evidence of a third subunit, γ , a small peptide that co-precipitates with the Na/K-ATPase and modulates cation activation and affinity (Béguin *et al.*, 1997), however it is not needed for function of the holoenzyme (Mercer *et al.*, 1999). Generally it is thought that the subunits associate in a 1:1(:1) stoichiometry (Lingrel and Kuntzweiler, 1994; Mercer, 1993; Skou and Esmann, 1992), however there is evidence of the enzyme functioning with two of each subunit (Blanco *et al.*, 1994).

α -subunit Isoforms

Four isoforms of the Na/K-ATPase α -subunit have been identified. In an individual species they share approximately 85% amino acid (AA) identity and 75%

nucleotide (NT) identity (Fig. 1.3) (Herrera *et al.*, 1987; Shamraj and Lingrel, 1994; Shull *et al.*, 1986; Takeyasu *et al.*, 1990). Comparing the same isoform in different species yields 90% or greater AA identity (Blanco and Mercer, 1998). Two major regions of low identity sequence define the α isoforms; thirty residues at the end of the amino-terminus and 13 residues within the large intracellular loop between TMD 5 and TMD 6 beginning with Lys-490 (of the rat $\alpha 1$ isoform) (Lingrel *et al.*, 1990; Pressley, 1992; Sweadner, 1989).

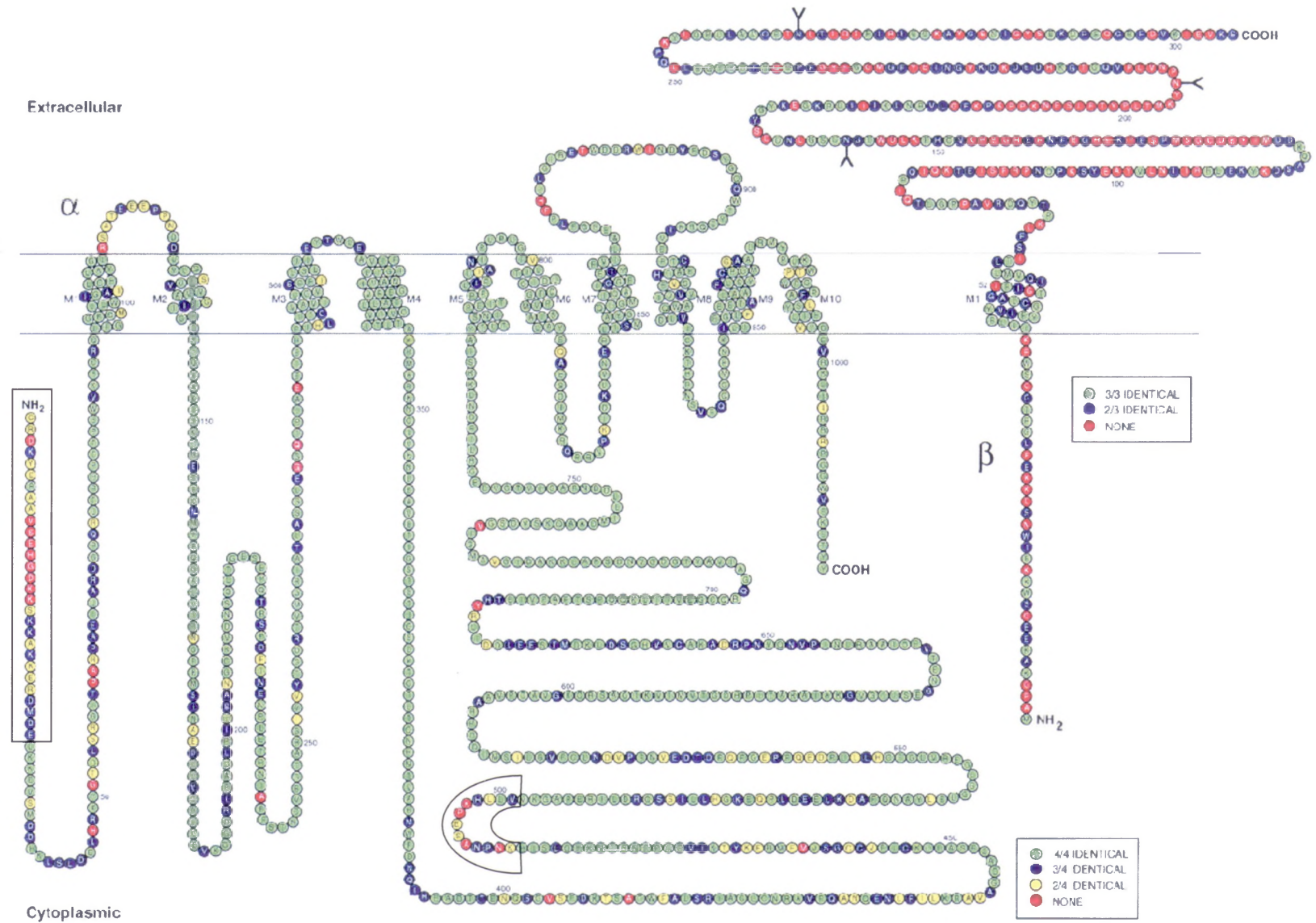


Figure 1.3 Schematic representation of the membrane topology of the rat Na/K-ATPase α and β -subunits from Blanco and Mercer (1998, copyright permission from APS).

Primary sequences shown are that of the rat α 1 and β 1 isoforms. The amino acid residues are colored to indicate the identity between the α (1, 2, 3 and 4) and the β (1, 2 and 3) isoforms. The boxed regions highlight the two isoform defining regions.

The isoforms have different tissue specificity and developmental distribution (Orlowski and Lingrel, 1988). The $\alpha 1$ isoform is the primary isoform of the kidney, however it is expressed at low levels in all tissues (Orlowski and Lingrel, 1988; Takeyasu *et al.*, 1990; Young and Lingrel, 1987). The $\alpha 2$ isoform is most abundant in skeletal and heart muscle (Herrera *et al.*, 1987; Schneider *et al.*, 1985; Takeyasu *et al.*, 1990; Young and Lingrel, 1987). The $\alpha 3$ isoform is most abundant in nervous tissues, but all three isoforms have been found in the brain (Emanuel *et al.*, 1987; Herrera *et al.*, 1987; Takeyasu *et al.*, 1990; Young and Lingrel, 1987). Finally, the $\alpha 4$ isoform has only been found in rat testis (Shamraj and Lingrel, 1994). Additionally, the α -subunit isoforms differ in their ouabain sensitivities and cation binding affinities. Ouabain binding differences are most apparent in the rat (Lucking *et al.*, 1996; Sweadner, 1985), while in many species no differences in ouabain affinity are noted (Blanco and Mercer, 1998). *In vitro* studies with transfected cells were used to determine ouabain affinities without K^+ . Under these conditions, the affinities are similar for all α -subunits, however with K^+ antagonism the K_d for ouabain increases depending upon the α isoform; $\alpha 1 > \alpha 3 > \alpha 2$ (Crambert *et al.*, 2000). In transfected Hela cells, external K^+ affinity is two times higher in $\alpha 1$ and $\alpha 2$ versus the $\alpha 3$ while internal Na^+ affinity is 3 times lower with the $\alpha 3$ (Munzer *et al.*, 1994). In all systems examined, the $\alpha 3$ isoform has the lowest affinity for Na^+ (Blanco and Mercer, 1998) and when the $\alpha 3$ is transfected individually into cells, it maintains a three times higher intracellular $[Na^+]$ than the $\alpha 1$ or $\alpha 2$ (Crambert *et al.*, 2000; Therien *et al.*, 1996; Zahler *et al.*, 1997).

The α -subunits of the Na/K-ATPase are also regulated differently by hormones and protein kinases. Insulin increases the activity of the $\alpha 1$ and $\alpha 2$ while not affecting

the $\alpha 3$ (Ewart and Klip, 1995). Thyroid hormone differentially regulates the activity of all three isoforms, especially in the heart and skeletal muscle (Horowitz *et al.*, 1990) and post-transcriptionally in the brain (Corthesy-Theulaz *et al.*, 1991). Dopamine selectively inhibits the activities of the $\alpha 2$ and $\alpha 3$ compared to the $\alpha 1$ activity (Nishi *et al.*, 1999). The α isoforms genomic DNA each have varying trans and cis acting sequences within their promoter regions; and within the coding region the isoforms are all affected differently by protein kinases. PKA stimulates the $\alpha 3$, while decreasing $\alpha 1$ and $\alpha 2$ activity, PKG decreases $\alpha 1$ and $\alpha 3$ with no change in $\alpha 2$ activity (Blanco and Sánchez, 2001) and PKC regulates $\alpha 1$ activity but does not appear to regulate $\alpha 2$ and $\alpha 3$ (Béguin *et al.*, 1996). Differential effects of hormones and protein kinases, as well as different distributions and cation affinities of the isoforms, provide mechanisms by which an organism can regulate Na/K-ATPase activity in a wide variety of cell types and physiological conditions.

Molecular Identification of the α -subunit in Marine Vertebrates

In marine species, Na/K-ATPase α -subunits have been fully cloned in the torpedo ray (*Torpedo californica*) (Kawakami *et al.*, 1985), the white sucker (*Catostomus commersoni*) (Schonrock *et al.*, 1991), the european eel (*Anguilla anguilla*) (Cutler *et al.*, 1995) the tilapia (Genbank Assesion numbers U82549 and AF109409) and the zebrafish (*Danio rerio*) (Rajarao *et al.*, 2001). In the european eel gill, the sequence was identified as the Na/K-ATPase $\alpha 1$ isoform, and in the tilapia both the $\alpha 1$ and $\alpha 3$ were identified. Analysis of the isoform identifying amino acid sequences in the large intracellular loop of both the white sucker clone and the torpedo ray clone suggest both are $\alpha 1$ isoforms. In

the zebrafish, eight clones of the Na/K-ATPase α -subunit were identified from cDNA, five of which are α 1 isoform like, one is α 2 isoform like and two are α 3 isoform like (Rajarao *et al.*, 2001). Partial Na/K-ATPase α -subunit clones have also been identified in rainbow trout (*Oncorhynchus mykiss*) (Kisen *et al.*, 1994) and spiny dogfish (α 3, *Squalus acanthias*) (Hansen, 1999). In the brown trout (*Salmo salar*) Northern blot analysis with an α 1 isoform probe showed a single band indicating the presence of the α 1 isoform in the kidney and gills (Madsen *et al.*, 1995). The presence of Na/K-ATPase α isoforms has also been determined by Western blots which have identified α 1 and α 3 proteins in tilapia (Lee *et al.*, 1998), as well as α 3 proteins in the gills of Atlantic salmon (*Salmo salar*) (D'Cotta *et al.*, 2000). In the Antarctic *T. bernacchii*, the α -subunit has only been detected in chloride cells immunohistochemically with an antibody that detects all α -subunit isoforms (Masini *et al.*, 2000).

Antarctic fish and the Na/K-ATPase

Antarctic fish - Preliminary data

Antarctic fish regulate their serum osmolality in response to a change in both environmental salinity (O'Grady and DeVries, 1982) and temperature. Upon warm acclimation from -1.5°C to 4°C for five weeks, the serum osmolality of Antarctic species decreases significantly by nearly 25% (Gonzalez-Cabrera *et al.*, 1995; Guynn *et al.*, 2002a). The decrease is due to proportional decreases in serum $[\text{Na}^+]$ and $[\text{Cl}^-]$ (Gonzalez-Cabrera *et al.*, 1995). The response is both reversible and “dose” dependent as warm acclimation to 1°C causes a smaller yet still significant decrease in serum osmolality (Gonzalez-Cabrera *et al.*, 1995). These responses are thought to be

physiological, and not pathological or stress-induced. Previous studies have shown that the stress response to warming in cold water teleosts is reflected by an increase in serum osmolality due to increases in Cl⁻ (Franklin *et al.*, 1991). (Umminger and Gist, 1972).

The decrease in serum osmolality in warm-acclimated Antarctic teleosts is accompanied by significant increases in Na/K-ATPase activity in osmoregulatory tissues (Gonzalez-Cabrera *et al.*, 1995). In the previous study, four tissues were examined for Na/K-ATPase activity, gill and trunk kidney, representing osmoregulatory tissues and muscle and liver, representing non-osmoregulatory tissues. There was a specific and significant change in the Na/K-ATPase activity in the osmoregulatory tissues and not in the other tissues tested. Therefore, it can be concluded that the increase in enzyme activity is not a direct effect of the temperature dependence of enzymes, but rather is a temperature-mediated increase in Na/K-ATPase activity specific to osmoregulatory tissues.

Regulation of Na/K-ATPase activity

Gill Na/K-ATPase activity is primarily regulated by hormones and changes in serum osmolality. Changes in serum osmolality and gill Na/K-ATPase have been seen with temperature acclimation. Serum osmolality increases in cold acclimated fish only when the temperature of acclimation is near 0°C (Burton, 1986). However, varied responses of gill Na/K-ATPase activity have been reported. Many find an increase in gill Na/K-ATPase activity with cold acclimation (Jurss *et al.*, 1987; Starnes *et al.*, 1994; Stuenkel and Hillyard, 1980), which has been explained by compensation of active transport processes with decreasing temperature, in an effort to maintain homeostasis. However, others have found in Arctic char (*Salvelinus alpinus*) (Starnes, 1993) and

rainbow trout (Robertson and Hazel, 1995), gill Na/K-ATPase activity decreases with cold acclimation. In eels, no change in serum osmolality was observed, however increases in “internal sodium space” as a result of decreased active transport of sodium in the gill was found with cold acclimation (Maetz and Evans, 1972).

Better documented are the changes in serum osmolality and gill Na/K-ATPase with adaption to different environmental salinities. Teleosts acclimated to saltwater consistently exhibit acute increases in serum osmolality and chronic increases in gill Na/K-ATPase activity versus freshwater controls (Jensen *et al.*, 1998; Kamiya and Utida, 1969; Zadunaisky *et al.*, 1995). In some fish there is a concomitant increase in chloride cell size with seawater acclimation (Langdon and Thorpe, 1984; Yoshikawa *et al.*, 1993), specifically an increase in the surface area of the basolateral membrane where the Na/K-ATPase is located. An increase in the total number of chloride cells is another response to seawater acclimation that increases the number of Na/K-ATPase (Karnaky, Jr. *et al.*, 1976; Thomson and Sargent, 1977). In *T. bernacchii* however, the increase in Na/K-ATPase activity however is not accompanied by any changes in chloride cell apical membrane linear surface which would indicate an increase in chloride cell size (Krysl *et al.*, 1999).

Increases in the mRNA expression and protein levels of the gill Na/K-ATPase during seawater adaptation and smoltification (migration from freshwater to seawater) contribute to the increased gill Na/K-ATPase activity in seawater-adapted fish. Seawater adaptation increased the gill Na/K-ATPase activity and the α -subunit mRNA in rainbow trout (Kisen *et al.*, 1994), European eel (Cutler *et al.*, 1995), European sea bass (*Dicentrarchus labrax*) (Jensen *et al.*, 1998), tilapia (Hwang *et al.*, 1998), brown trout

(Madsen *et al.*, 1995) and Atlantic salmon (D'Cotta *et al.*, 2000). Increased α -subunit proteins were found in seawater-adapted tilapia (Hwang *et al.*, 1998; Lee *et al.*, 1998) and Atlantic salmon (Madsen *et al.*, 1995). However, the increases in both mRNA and protein levels cannot fully explain the large fold increases in gill Na/K-ATPase activity. Therefore, other mechanisms besides increased transcription, which leads to increased Na/K-ATPase activity are thought to be involved.

One of the proposed mechanisms involves specific changes in isoform composition that would lead to changes in Na/K-ATPase activity at the enzymatic level due to kinetic or regulatory differences in the α isoforms. Lee *et al* (1998) observed a significant increase in the level of $\alpha 1$ isoform proteins in seawater-adapted tilapia compared to the freshwater tilapia, whereas the levels of $\alpha 3$ isoform protein did not change. In contrast, increased gill Na/K-ATPase activity during smoltification and seawater adaptation of Atlantic salmon freshwater smolts was accompanied by increases in $\alpha 3$ isoform protein specifically (D'Cotta *et al.*, 2000). Adaptation to increased salinity in temperate teleosts is accompanied by an increase in Na/K-ATPase activity analogous to warm acclimation of Antarctic teleosts.

Rationale and Objectives

The information presented above provides a strong foundation for further investigation of the Na/K-ATPase enzyme in Antarctic fish. Understanding how Antarctic fish use the Na/K-ATPase to maintain an elevated serum osmolality will provide information about the function and regulation of the Na/K-ATPase isoforms. Beyond the basic science value of understanding these unique mechanisms, defects in Na/K-ATPase activity are implicated in cardiovascular disease and hypertension,

diabetes, obesity, fetal abnormalities, neurological diseases such as Alzheimer's and manic depression, and pulmonary disease (Rose and Valdes, Jr., 1994).

Antarctic fish are especially useful because of their comparative value. "One of the tenets of comparative physiology is that the understanding of basic biological processes is enhanced by studying animals in extreme environments: the extreme variable will often dominate the process, permitting its actions to be isolated." (Macdonald *et al.*, 1987). Antarctic fish have unique thermal adaptations due to their extreme environment, the ability to change enzyme activity with temperature acclimation, and a temperate New Zealand counterpart for comparison studies. The New Zealand *Notothenia angustata* is a member of the same family as *T. bernacchii*, however it lives above the Antarctic Convergence at $\sim 8^{\circ}\text{C}$ and has a serum osmolality of approximately 350mOsm/kg. *N. angustata* was used to determine if changes in Antarctic fish physiology are a function of their phylogeny or environment. Similar studies have used temperature acclimation of polar species as a method of studying physiological processes and osmoregulation. Warm acclimated *T. bernacchii* have been used to examine mitochondrial enzyme processes in cold-adapted Antarctic teleosts (Weinstein and Somero, 1998) and the expression of heat-shock proteins (Carpenter and Hofmann, 2000). Staurnes, *et al* (1994) used the Atlantic cod (*Gadus morhua*) and Schwarzbaum, *et al* (1991) used two Arctic freshwater teleosts, the stenothermal roach (*Rutilus rutilus*) and eurythermal Arctic char, to compare their osmoregulatory responses to thermal acclimation. Thermal and metabolic comparative studies have been performed using Antarctic teleosts and a temperate counterpart within the same family to examine; critical temperature limits (van Dijk *et al.*, 1999), the temperature dependence of cytochrome-*c* oxidase activity

(Hardewig *et al.*, 1999), glycolytic capacities (Hardewig *et al.*, 1998) and the expression of heat-shock proteins (Carpenter and Hofmann, 2000). The ability to respond to the changing environmental temperature requires pre-existing mechanisms in Antarctic fish, and it is these mechanisms that may provide information about the regulation of Na/K-ATPase activity in any species. Therefore the objectives of this thesis include:

Objective 1: Determine if temperature acclimation changes the serum osmolality of the New Zealand *Nothenia angustata*, a temperate notothenioid.

Objective 2: Determine if the increased Na/K-ATPase activity of warm acclimated *T. bernacchii* is due to an increased number of Na/K-ATPase pumps or a change in the Na/K-ATPase affinity for ouabain.

One of the important aspects of Na/K-ATPase structure, physiology and regulation is the presence of multiple isoforms of each subunit. These studies focus on the α -subunit because it is the catalytic subunit with the binding sites for the cations and ouabain. Teleost Na/K-ATPase α -subunit isoforms have not been examined in detail and have never been examined in Antarctic teleosts. There is an abundance of evidence supporting the distinctness of each isoform, yet little is known about the different physiologic functions. The fact that the $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms maintain sequence identity across species supports the idea that each α isoform has a distinct function (Pressley, 1992). Examining regions of low amino acid identity between isoforms that are preserved when comparing an isoform across different species may convey information about their function. Then, by examining the molecular changes in the

Na/K-ATPase α -subunit isoform composition of warm acclimated Antarctic fish, further information about the role and regulation of the isoforms may be determined. Antarctic fish are a unique group of organisms in which to examine how the universal Na/K-ATPase α isoforms produce a very different serum osmolality than most species. The following objectives address the molecular aspects of the Na/K-ATPase in Antarctic *T. bernacchii*:

Objective 3: Determine the Na/K-ATPase α -subunit isoforms present in *T.*

***bernacchii*.**

Objective 4: Determine the tissue distribution of Na/K-ATPase α -subunit isoforms in *T. bernacchii* and *N. angustata*.

Objective 5: Determine if warm acclimation of *T. bernacchii* changes the gill Na/K-ATPase α -subunit isoform mRNA and protein composition.

CHAPTER TWO

Characterization of gill Na/K-ATPase activity and ouabain binding
in Antarctic and New Zealand Nototheniid Fishes

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Introduction

Teleosts of the sub-order Notothenioid living below the Antarctic Convergence thrive in the world's coldest seawater (Somero and DeVries, 1967). A serum osmolality of approximately 600mOsm/kg, composed of proportional increases in serum $[Na^+]$ and $[Cl^-]$, lowers the serum freezing point by 1.0°C (Dobbs and DeVries, 1975) and is one mechanism with which Antarctic fish resist freezing. The gill, using the Na/K-ATPase of the chloride cell (Karnaky, 1986), regulates the serum monovalent ion concentrations and osmolality in marine teleosts. Although gill Na/K-ATPase activity has been extensively studied in other species, its role in the regulation of serum osmolality in Antarctic teleosts acclimated to different temperatures has only been examined preliminarily (Gonzalez-Cabrera *et al.*, 1995).

Upon warm acclimation to +4°C for five weeks, the serum osmolality of Antarctic species decreased by nearly 25% and there were significant increases in Na/K-ATPase activity in osmoregulatory tissues (Gonzalez-Cabrera *et al.*, 1995). The serum and enzyme responses to temperature in Antarctic teleosts, in conjunction with their stenothermality and evolutionary adaptations, makes them a unique model system for studying the regulatory mechanisms of the Na/K-ATPase using thermal challenges.

In the present study we optimized the gill Na/K-ATPase activity assay for the Antarctic *T. bernacchii* as has been done for the gilt head bream (*Sparus auratus* L.) (Ventrella *et al.*, 1990) and the European sea bass (Trigari *et al.*, 1985). In addition, we compared the effect of thermal acclimation on the temperate New Zealand *Notothenia angustata* versus the Antarctic *T. bernacchii* and investigated two characteristics of the Na/K-ATPase which may result in increased Na/K-ATPase in warm acclimated Antarctic

notothenioids. The goal was to determine if increased Na/K-ATPase activity and decreased serum osmolality, as observed in Antarctic notothenioids, occurs in a warm-acclimated temperate notothenioid of the same family. The second goal was to study the mechanism accounting for the increased gill Na/K-ATPase activity in warm-acclimated *T. bernacchii* by examining Na/K-ATPase site number and the enzyme's affinity for ouabain. Our study examines serum osmolality, gill Na/K-ATPase activity, and the ouabain binding characteristics of both the polar *T. bernacchii* and the temperate *N. angustata*.

Methods

Fish collection

Trematomus bernacchii (100-400 g) were caught with hook and line, and traps in McMurdo Sound, Antarctica from September to November (1998 and 1999), transported to 200L laboratory aquaria at McMurdo Station, Antarctica and held in free flowing seawater tanks for 5-7 days at -1.5°C until the acclimation experiments began. McMurdo Sound has an annual water temperature of -1.87°C regardless of season or depth (Littlepage, 1965). The New Zealand *Notothenia angustata* were caught by hook and line from Portobello Bay, New Zealand and were transported by air to McMurdo within 48 hours of capture and held at 8°C in McMurdo Sound seawater for at least two weeks until the acclimation experiments began.

Acclimation

T. bernacchii were randomly separated according to size (100-250 g) and placed (6-10 each) in open mesh cages (12.5 ft^3) in 200L acclimation tanks maintained at -1.5°C or 4°C ($\pm 0.1^{\circ}\text{C}$) for five weeks. The -1.5°C tanks were free flowing using seawater

pumped from McMurdo Sound. For all tanks warmed above -1.5°C , McMurdo Sound seawater was heated and maintained within 0.1°C using a temperature control device (Cole-Parmer, Vernon Hill, IL) that switched power between a submerged heating coil and a water pump that pumped -1.5°C water into the tanks when cooling was needed. Due to the intermittent flow of fresh seawater, the water in the warm tanks was aerated and circulated. The *N. angustata* were acclimated at 4°C or 14°C in 60 L aquarium tanks. The upper and lower lethal limits of environmental temperature of the *N. angustata* are 18°C and 2°C (A.L. DeVries, personal communication). During the acclimation period, the fish were not fed nor was any effort made to control the photoperiod.

Tissue Collection

Blood was collected through a caudal vessel with a non-heparinized 30-gauge $\times \frac{1}{2}$ needle and 1 cc. plastic syringe from fish lightly anesthetized with 3-aminobenzoic acid ethyl ester methanesulfonate salt (MS222, Sigma, St. Louis, MO). The blood was stored for 4 hours at 4°C in a polyethylene tube, centrifuged at 2000g for 5 min, and the serum transferred to a clean tube for osmolality and ion concentration measurements. At the end of the acclimation periods, the fish were fully anesthetized with MS222, and placed on ice. The gills were perfused through the bulbous arteriosus with 30 ml of ice cold air-equilibrated heparinized physiologic Ringers at 0°C to remove red blood cells. The Ringers for the cold-acclimated fish was 280 mM NaCl, 5 mM KCl, 3 mM MgCl_2 , 3 mM CaCl_2 , 10 mM NaHCO_3 , 10 mM HEPES, 5 mM glucose, pH 7.8 at 0°C while the Ringers for the warm-acclimated fish was the same except for 240 mM NaCl. The gills

were excised and gently agitated in 0°C Ringers for 20 minutes. The gills were placed on a cold plate (Sigma, St. Louis, MO) at 0°C and the filament epithelia were then scraped from the cartilaginous supporting tissue with the edge of a glass microscope slide and flash frozen in liquid nitrogen and stored at -80°C. For the chloride cell enriched suspensions, chloride cells were isolated in a manner similar to that described by Perry and Walsh (1989). The gill tissue was not frozen but homogenized with 5 passes through the tip of a 6≡ Pasteur pipette and filtered through a series of sieves (250 μm, 150 μm, and 73 μm) via gentle stirring with a glass pestle. The sieves were washed multiple times with air-equilibrated Ringers as described above but without the Ca⁺² and Mg⁺² and the addition of 2 mM EDTA as described previously (Pärt and Bergstrom, 1995; Verbost *et al.*, 1994; Wong and Chan, 1999). The final filtrate was collected and spun at 200 g for 5 min. The cell pellet was gently resuspended in air-equilibrated Ca⁺² and Mg⁺² free Ringers solution with 2 mM EDTA, and counted with a hemacytometer. Following counting, the cell suspension was flash frozen in liquid nitrogen and stored at -80°C.

Determination of Serum Osmolality.

Serum osmolality was determined using a vapor pressure osmometer (Wescor Inc., Logan, UT).

Membrane Preparation

Gill epithelia scrapings and gill cell suspensions stored at -80°C were homogenized on ice in 250 mM sucrose, 5 mM β-mercaptoethanol, 1 mM EDTA, and 50 mM imidazole buffer, pH 7.8 with 10 strokes of a teflon/glass homogenizer attached to a hand held drill (600 rev/min). The homogenate was centrifuged at 1200 g for 15 min at

4°C, and the supernatant was collected and centrifuged at 12,000 g for 90 min at 4°C. The pellet was resuspended in 50 mM imidazole buffer, pH 7.8 and the protein concentration determined (Lowry *et al.*, 1951).

Na/K-ATPase Assay Optimization.

The Na/K-ATPase assay conditions were optimized using cold-acclimated Antarctic *T. bernacchii* gill tissue. The Na/K-ATPase activity was determined over a range of 25 to 100 µg of crude membrane protein using the previously described assay conditions (Gonzalez-Cabrera *et al.*, 1995) of 100 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 50 mM imidazole buffer, pH 7.4, and 5 mM disodium-ATP (Sigma, St. Louis, MO). After selecting an appropriate protein concentration, the Na⁺ and K⁺ ratio was optimized by testing Na/K-ATPase activity with six ratios of NaCl:KCl; 4:1, 6:1, 8:1, 10:1, 14:1 and 18:1 at 37°C. The optimal Na⁺:K⁺ ratio was determined at 37°C, rather than at 4°C as was the case in subsequent assays, to maximize the activity levels and therefore determine any significant differences between the six salt ratios. The other assay parameters were not altered. Crude gill membranes from 2-3 fish were pooled to obtain the protein necessary for one experiment and the data presented is the number of experiments performed with separate tissue pools. Using the optimal 4Na⁺:1K⁺, the optimal Na⁺ concentration was determined at 4°C. The assay for optimal Na⁺ concentration, within the 4Na⁺:1K⁺ ratio, was determined at 4°C because the remaining assays were to be run at 4°C. Na/K-ATPase activity was compared at 100, 150, and 200 mM Na⁺ using the same buffer and ATP concentrations as above. To optimize buffer and pH, three buffers: HEPES, imidazole, and Tris were chosen because they are commonly used in other Na/K-ATPase studies (Pagliarani *et al.*, 1988; Paxton and Umminger, 1983;

Thomson *et al.*, 1977; Trigari *et al.*, 1985). Initially, the buffers were tested at 37°C to determine which one resulted in the highest Na/K-ATPase activity over a pH range of 6.5-9.0. The optimal pH at 4°C was then determined using HEPES buffer which was previously determined to be the optimal buffer. Finally, the Na/K-ATPase activity was optimized over a range of 0.5 - 24 hours incubation time.

Na/K-ATPase Activity.

Na/K-ATPase activity in the gills of warm and cold-acclimated *T. bernacchii* and *N. angustata* was determined using optimal conditions from the above experiments. The final Na/K-ATPase activity assay conditions were 50 µg protein incubated in 100 mM NaCl, 25 mM KCl, 5 mM MgCl₂, 50 mM HEPES and 5 mM ATP pH 7.4 at 4°C for 30 min. Ouabain (2 mM) was added to duplicate tubes to determine ouabain sensitive ATPase activity (Na/K-ATPase activity). Assays were carried out at 4°C to more closely reflect *in vivo* conditions. Everything but the 5 mM ATP was added to borosilicate glass tubes and shaken at 90 rev/min for 10 minutes at 4°C. ATP was added and incubation carried out for 30 minutes at 4°C with 90 rev/min. The reactions were stopped with the addition of 100 µl ice-cold trichloroacetic acid. The protein was pelleted with a 3000 g spin for 10 min at 4°C and inorganic phosphate measured in the supernatant as previously described (Ames, 1966).

Ouabain Binding.

To determine appropriate conditions for binding, protein was tested over a range of 5-140 µg, using two different [³H] ouabain concentrations, 4 nM or 100 nM with an incubation time of three hours. 7 µg of protein was determined optimal because the

maximal binding of [³H]-ouabain/mg protein reached a plateau at 5-10 µg. Less than 5% of the total [³H]-ouabain added was utilized and non-specific binding was less than 5% of the total amount bound (data not shown). The incubation time was tested to insure equilibrium binding at 4 nM and at 100 nM [³H]. Ouabain binding was carried out at 4°C with samples taken at 15 time points ranging from 0.25-36 hours. Equilibrium was reached within 20 hours. Subsequent binding experiments were carried out for 24 hours.

Initially, to estimate the K_d of ouabain in Antarctic *T. bernacchii* gills three homologous competition binding assay (Jeffries *et al.*, 1997) were completed resulting in a K_d of 10-20 nM. Then the K_d for ouabain was determined from saturation binding experiments. Three different samples of *T. bernacchii* gill tissue were used in 3-4 saturation experiments each. [³H] ouabain concentrations from 1×10^{-11} to 1×10^{-5} M were used to determine the saturation binding profile and K_d . Non-specific binding was determined using 1000 fold concentrations of unlabeled ouabain. In initial experiments, Cymarin (Sigma, St. Louis, MO) was also used to define non-specific binding because it binds the Na/K-ATPase however is not similar in structure to [³H] ouabain. However cymarin provided the same results as unlabeled ouabain, and ouabain was routinely used for subsequent assays because of the precedent in the literature.

After determining the optimal conditions to be used in the equilibrium ouabain binding assays, a comparison study of cold- and warm-acclimated notothenioid gill tissue using [³H]-ouabain was performed. The ouabain binding assay consisted of 100 mM NaCl, 5 mM MgCl₂, 5 mM ATP, 50 mM HEPES pH 7.4 at 4°C with 12 concentrations of [³H]-ouabain from 1×10^{-10} to 1×10^{-6} M in disposable flint glass tubes with 40 µg of membrane protein in a volume of 250 µl. Non-specific binding was measured in

duplicate tubes using a concentration of unlabeled ouabain that was 1000-fold the concentration of labeled ouabain. The tubes were incubated for 24 hrs at 4°C and shaken at 100 rev/min. The reactions were stopped by diluting the reactions with 5 ml ice cold 50 mM HEPES buffer (pH 7.4) and quickly filtering the diluted suspension through Whatman GF/A glass fiber filter paper using a Brandel M48T cell harvester (Gaithersburg, MD). Tubes and filters were rinsed three times with 5 ml ice cold 50 mM HEPES buffer (pH 7.4). The filter papers were put into plastic scintillation vials with 10 ml biodegradable scintillation cocktail, shaken, and placed in the dark for 24 hrs before counting. All vials were counted with a Beckman scintillation counter (Model L5 5000TD, Fullerton, CA). Saturation binding data was analyzed with non-linear regression according to the equations for one and two site binding hyperbolas with GraphPad Prism (SanDiego, CA). The best fit equation was determined via the F test. All data reported are corrected for non-specific binding.

Statistical Analysis.

The mean results of the Na/K-ATPase activity assays and the ouabain binding experiments (B_{max} and K_d) were analyzed using an unpaired Student's t-test. The osmolality data and the results of the optimization studies were analyzed with either a one way ANOVA with Tukey post test or a two way ANOVA.

Results

Osmolality

The warm-acclimated Antarctic *T. bernacchii* experienced a significant decrease in their serum osmolality as compared to the cold-acclimated *T. bernacchii* at weeks one, two and five (Fig. 2.1). However, the New Zealand *N. angustata* experienced no

significant difference in serum osmolality in the warm-acclimated group compared to the cold-acclimated group at week five (Fig. 2.1).

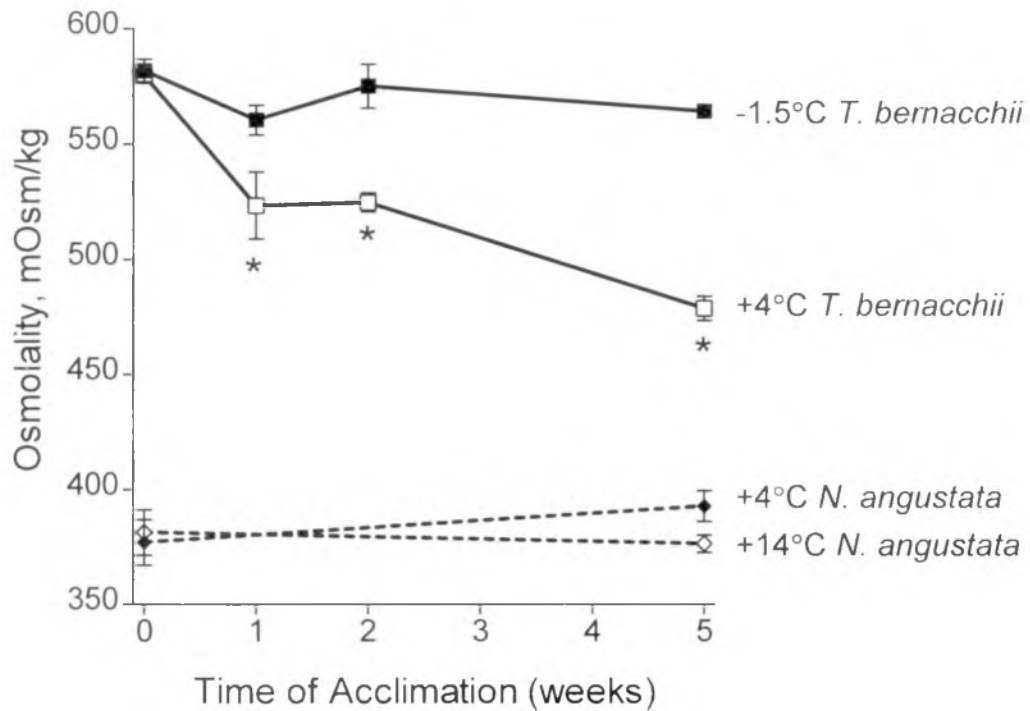


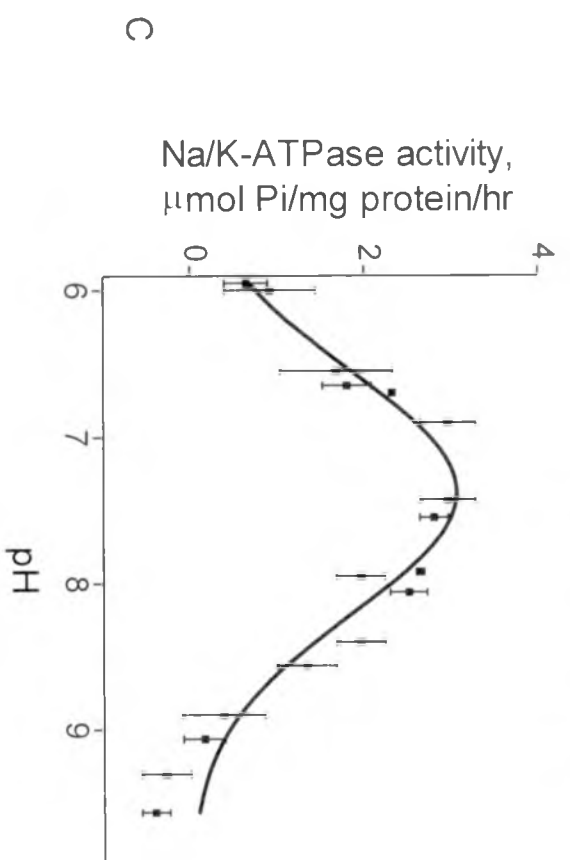
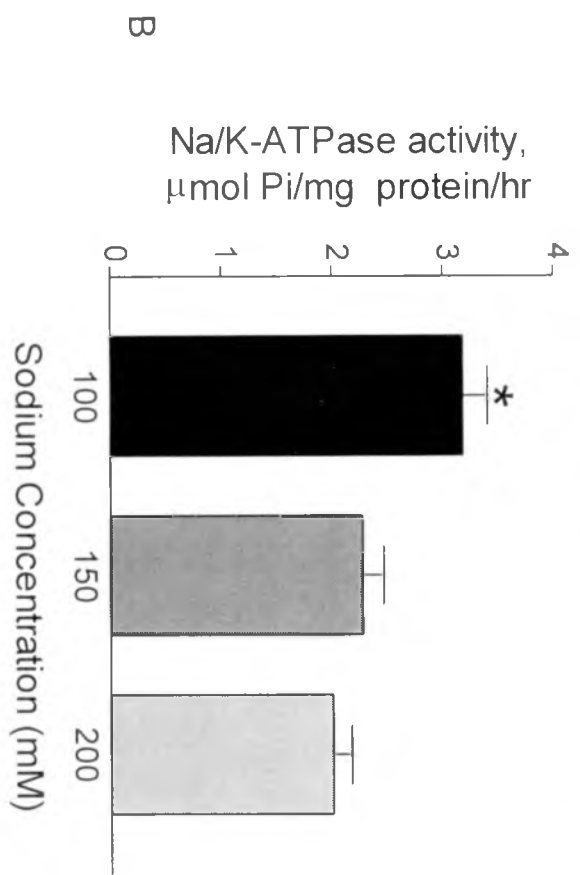
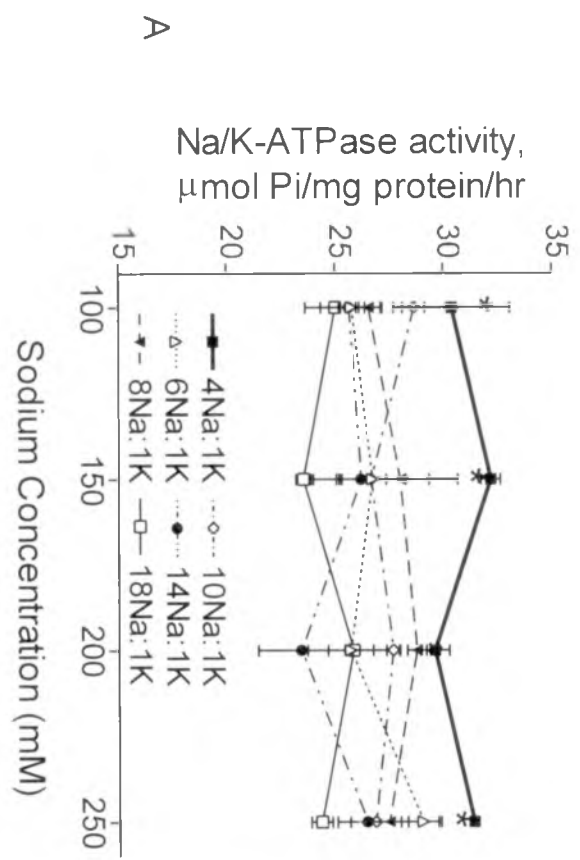
Figure 2.1 Serum osmolality (mean±SEM) of Antarctic *T. bernacchii* (solid lines) and New Zealand *N. angustata* (dashed line) acclimated to their upper and lower viable temperatures. The serum osmolalities of the *T. bernacchii* acclimated at +4°C (open squares, n=20) were significantly different at weeks one, two, and five compared to week 0, whereas *T. bernacchii* acclimated at -1.5°C (filled squares, n=20) had no significant changes in osmolality compared to week 0. Serum osmolalities of *N. angustata* acclimated at +4°C (filled diamond, n=6) and at +14°C (open diamond, n=6) were not significantly different at week five compared to week 0. * = $p < 0.0001$ using one way ANOVA analysis with a Tukey post test.

Na/K-ATPase Activity Optimization.

The amount of gill protein used in this assay was 50 μg protein because the Na/K-ATPase activity was linear with respect to protein concentration in a broad range around 50 μg protein (data not shown). Also, the total ATPase activity was at least twice the non-specific ATPase activity. The results of the optimal $\text{Na}^+:\text{K}^+$ ratio experiments assayed at 37°C showed that a ratio of $4\text{Na}^+:\text{K}^+$ gave significantly higher activity in Antarctic *T. bernacchii* gill tissue (Fig. 2.2a). Using this ratio, 100 mM NaCl (with 25 mM KCl) had the highest activity of the three Na^+ concentrations tested at 4°C (Fig. 2.2b). Although in Fig. 2.2a at 37°C, the $[\text{Na}^+]$ does not significantly change the activity within the $4\text{Na}^+:\text{K}^+$ ratio, at 4°C 100 mM $[\text{Na}^+]$ led to significantly higher activity than 150 or 200 mM (Fig. 2.2b). A Na^+ concentration of 250 mM was not examined because the osmolality of the assay solution was over 600 mOsm/kg. HEPES buffer resulted in higher activity than imidazole and Tris buffers (data not shown), and the optimum pH using HEPES at 4°C was determined to be pH 7.4 (Fig. 2.2c). No net Na/K-ATPase activity was seen at or above pH 9. Gill Na/K-ATPase activity was not significantly different from 30 minutes – 2 hour, therefore the assay incubation time was chosen was 30 minutes (Fig. 2.2d). Beyond 4 hrs, there was a general decrease in enzyme activity that could be due to a number of factors including but not limited to protein degradation, loss of protein to non-specific binding to the glass and vesicle formation.

To summarize, gill Na/K-ATPase assay optimization results indicated that the optimal assay conditions were 50 μg protein in 100 mM NaCl, 25 mM KCl, 5 mM MgCl_2 , 50 mM HEPES, 5 mM disodium-ATP, pH 7.4 with ± 2 mM ouabain, in a final

reaction volume of 0.5 ml incubated at 4°C for 30 min. The optimal ratio $\text{Na}^+:\text{K}^+$ was lower than those determined in other vertebrate species (Skou, 1979), however Pagliarani *et al* (1988) also found a $4\text{Na}^+:1\text{K}^+$ ratio optimal for assaying kidney Na/K-ATPase activity in the sea bass. The $[\text{Na}^+]$, pH, buffer type and incubation time are in agreement with conditions found in the literature for marine teleost Na/K-ATPase assays (Gonzalez-Garcia *et al.*, 1987; Ho and Chan, 1980; Pagliarani *et al.*, 1988; Schwarzbaum *et al.*, 1991; Trigari *et al.*, 1985; Ventrella *et al.*, 1990).



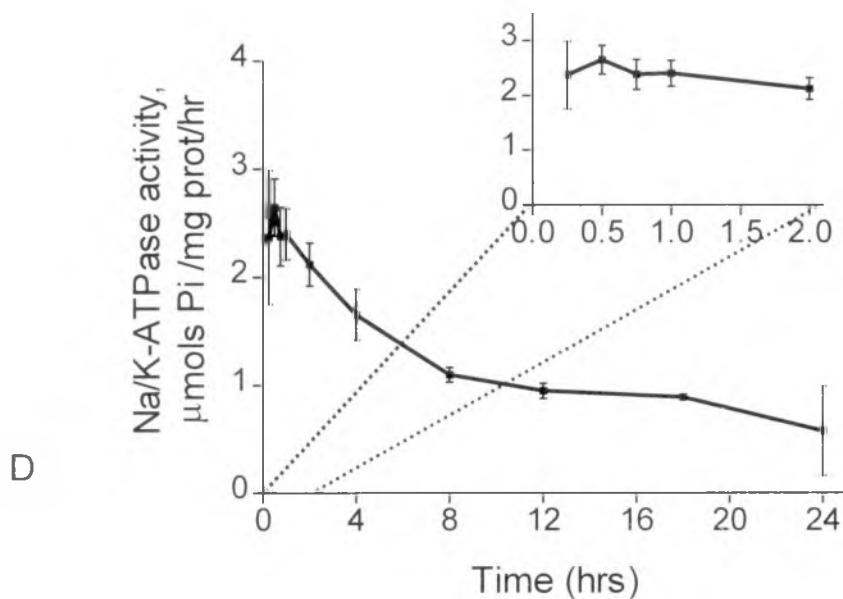


Figure 2.2 A. The effects of six Na:K ratios at given sodium concentrations on Na/K-ATPase activity in cold-acclimated *T. bernacchii* gill tissue assayed at 37°C (n=3 experiments using different pools of membranes, mean±sem). A two-way ANOVA (*=p<0.0001) was used to compare the significance of the Na⁺:K⁺ ratio at each of 4 Na⁺ concentrations. It was determined that a ratio of 4Na:1K allowed the highest activity regardless of Na concentration. B. Gill Na/K-ATPase activity of cold-acclimated *T. bernacchii* assayed at 4°C using different sodium concentrations while maintaining a 4Na:1K ratio (n=5). Analyzed by one way ANOVA with a Tukey post test, *=p<0.005. C. Gill Na/K-ATPase activity of cold-acclimated *T. bernacchii* assayed at 4°C at pH 6.0-9.5 using HEPES buffer (n=5). The highest activity was measured at pH 7.4. D. Gill Na/K-ATPase activity of *T. bernacchii* assayed over a 24 hour time period at 4°C, n=3. The inset is a magnification of enzyme activity from 15 min to 1 hour.

Gill Na/K-ATPase Activity.

Warm-acclimated, *T. bernacchii* had significantly increased gill Na/K-ATPase activity compared to cold-acclimated Antarctic fish after five weeks of acclimation (Fig. 2.3). This data is in agreement with Gonzales-Cabrera *et al* (1995) and is consistent with the decrease in serum osmolality of the warm-acclimated Antarctic fish. In contrast, the *N. angustata* exhibited no significant difference in gill Na/K-ATPase after five weeks of acclimation (Fig. 2.3). This result is consistent with results from the osmolality studies indicating no change in the osmolality in *N. angustata* upon warm acclimation. When comparing the gill Na/K-ATPase activities between Notothenioid species, the Na/K-ATPase activity of the New Zealand *N. angustata* was approximately four times that of the warm-acclimated Antarctic *T. bernacchii*.

Ouabain Binding

The saturation ouabain binding curves for warm-acclimated *T. bernacchii* at 1, 2 and 5 weeks of acclimation showed that there were no significant differences in either B_{\max} or K_d (Fig. 2.4, values summarized in Fig. 2.5 and Table 2.1). There was also no significant difference in B_{\max} values between cold- and warm-acclimated Antarctic *T. bernacchii* at one and two weeks of acclimation (Fig. 2.5). At five weeks, however, there was a significant increase in B_{\max} of the cold-acclimated fish compared to the warm-acclimated fish and compared to 1 and 2 week cold-acclimated *T. bernacchii* (Fig. 2.5). Also shown in figure 2.5, the New Zealand notothenioid *N. angustata* exhibited no significant change in B_{\max} between the control fish acclimated at +4°C compared to the fish acclimated at +12°C for five weeks (Fig. 2.5). Although there was a decrease in K_d values at five weeks for *T. bernacchii* at both acclimation temperatures, the values were

not statistically significant between temperatures or weeks of acclimation (Table 1). Additionally, there were no significant differences in the K_d of ouabain between *T. bernacchii* and *N. angustata* at any of the tested time periods (Table 1).

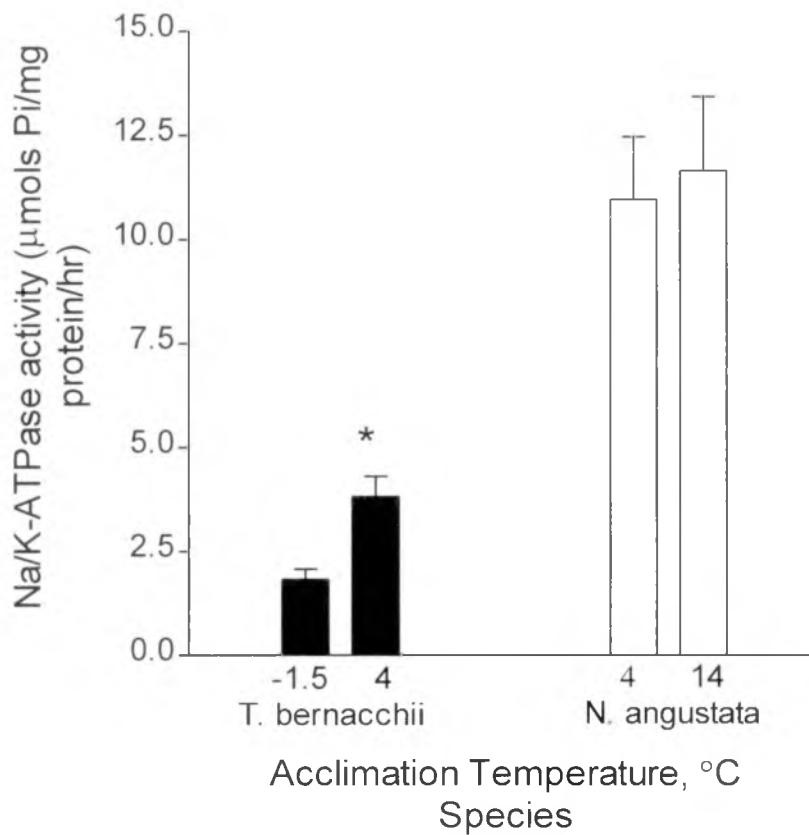


Figure 2.3 Gill Na/K-ATPase activity in five week cold- and warm-acclimated Antarctic *T. bernacchii* (n=8 at each temperature) and New Zealand *N. angustata* (n=3 at each temperature). The Na/K-ATPase activity at each acclimation temperature was compared within species with an unpaired Student's t-test, *= p<0.0001.

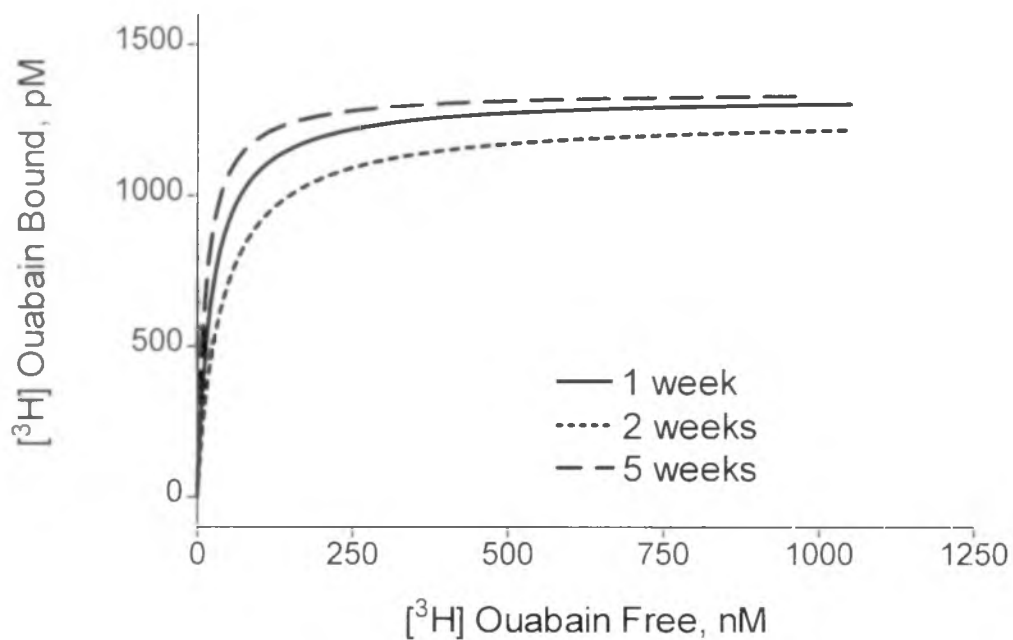


Figure 2.4 [³H]-ouabain saturation binding curves comparing warm-acclimated *T. bernacchii* at 1, 2 and 5 weeks of acclimation. (n=3 fish assayed at each temperature). The individual points have been removed to see the curves clearly. The curves are not significantly different (one way ANOVA, p>0.05). The B_{max} and K_d values are listed in figure 2.5 and table 2.1.

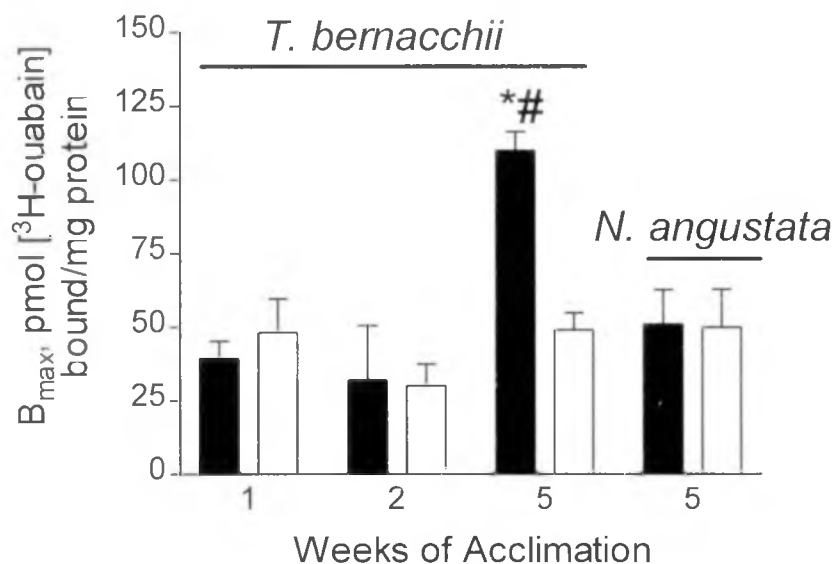


Figure 2.5 B_{max} of cold-acclimated (filled bars) and warm-acclimated (open bars) Antarctic *T. bernacchii* (-1.5°C and +4°C) and New Zealand *N. angustata* (+4°C and +14°C) as determined with saturation ouabain binding. The B_{max} of the cold acclimated *T. bernacchii* at five weeks (n=6) analyzed by Students t-test (* = $p < 0.0001$) was significantly greater than the warm acclimated *T. bernacchii* at five weeks (n=4). The B_{max} of the cold acclimated *T. bernacchii* at five weeks analyzed by ANOVA (# = $p < 0.0005$) is significantly greater than either the warm or cold acclimated *T. bernacchii* at weeks one or two (n=3 for each temperature and week). There was no significant difference between the B_{max} of the warm- and cold-acclimated *N. angustata* at five weeks (n=3 at each temperature).

Weeks of Acclimation	$K_d \pm \text{sem}$ at Cold Temperature (nM)	$K_d \pm \text{sem}$ at Warm Temperature (nM)
<i>T. bernacchii</i>		
1	24.1 ± 6.9	28.9 ± 7.3
2	30.0 ± 8.2	28.4 ± 10.3
5	17.5 ± 1.7	$13.6 \pm .4$
<i>N. angustata</i>		
5	6.4 ± 1.4	8.6 ± 1.5

Table 2.1 K_d values for ouabain in gill membranes from cold and warm-acclimated Antarctic *T. bernacchii* (-1.5°C and +4°C) and New Zealand *N. angustata* (+4°C and +12°C) as determined by saturation binding. There were no significant differences between the warm- and cold-acclimated groups in either species using Student's t-test. Additionally there were no significant differences between the *T. bernacchii* acclimated for different lengths of time using a one way ANOVA comparison.

Discussion

The present study indicates warm acclimation of the stenothermal Antarctic *T. bernacchii* results in a significant decrease in plasma osmolality, accompanied by an doubling of gill Na/K-ATPase. We report here that the optimal conditions for the Na/K-ATPase are similar to those reported in the literature in other marine teleosts (Gonzalez-Garcia *et al.*, 1987; Ho and Chan, 1980; Pagliarani *et al.*, 1988; Schwarzbaum *et al.*, 1991; Trigari *et al.*, 1985; Ventrella *et al.*, 1990). Additionally, the results indicate the increased gill Na/K-ATPase activity in warm-acclimated *T. bernacchii* is not due to an increase in the number of Na/K-ATPase pumps and is not evidenced by a change in ouabain affinity with warm acclimation. Finally, the increased gill Na/K-ATPase occurring with warming in the Antarctic notothenioid is not seen in the closely related temperate notothenioid, the New Zealand *N. angustata*. Closely related teleosts from polar and temperate regions have been used before to compare the effects of thermal acclimation on other physiologic processes (Carpenter and Hofmann, 2000; van Dijk *et al.*, 1999). Moreover, notothenioids are useful for comparing the effects of ecological differences on basic physiologic processes (Macdonald *et al.*, 1987).

Results indicated that in the polar Antarctic *T. bernacchii* there is an inverse relationship between serum osmolality and environmental temperature not observed in the temperate *N. angustata*. At environmental temperatures above 0°C, other studies have found no clear relationship between serum osmolality and environmental temperature in teleosts (Burton, 1986). However, at environmental temperatures approaching 0°C, a trend toward increased serum osmolality with decreased environmental temperature has been observed. For example, the shorthorn sculpin (*Myoxocephalus scorpius*) and the

fourhorn sculpin (*Myoxocephalus quadricornis*) were reported to have significant increases in serum $[\text{Na}^+]$ and osmolality at -0.1°C compared to 2°C (Oikari, 1975). The Arctic char increased serum osmolality and serum $[\text{Cl}^-]$ at 5°C compared to $10\text{-}12^\circ\text{C}$ (Staurnes, 1993). Umminger (1969) found increases in serum $[\text{Na}^+]$, $[\text{Cl}^-]$ and osmolality at 1.5°C compared to 4°C in killifish. In eels, no change in serum osmolality was observed, however an increase in internal sodium space was found (Maetz and Evans, 1972). These studies, though focusing on cold acclimation and increased osmolality, provide support for the decrease in serum osmolality noted in Antarctic teleosts during warm acclimation. The next focus was to determine the underlying mechanisms for these changes.

The results of gill Na/K-ATPase experiments (Gonzales-Cabrera et al, 1995 and present study) indicate a mechanism by which the Antarctic *T. bernacchii* can alter its serum osmolality during warm acclimation. The increased Na/K-ATPase activity in warm-acclimated Antarctic teleosts is compatible with the decrease in osmolality because it is anticipated that an increased pump activity would be necessary to excrete the serum Na^+ and Cl^- leading to the decreased serum osmolality. The results of the present study are supported by limited findings of decreased gill Na/K-ATPase activity in other teleosts during cold acclimation studies. Staurnes (1993) reported that the Arctic char from 5°C seawater had lower gill Na/K-ATPase activity than those from $10\text{-}12^\circ\text{C}$ seawater. Additionally, increases in gill Na/K-ATPase activity in warm-acclimated rainbow trout (from 5°C to 20°C) have been reported (Robertson and Hazel, 1995). And lastly, in the eel, a decrease in the active extrusion of sodium from the gill accompanied cold acclimation (Maetz and Evans, 1972).

However, most thermal acclimation studies in marine teleosts find an increase in Na/K-ATPase activity with cold acclimation explained by a compensation of thermally sensitive active transporters, not an increase in activity with warm acclimation as reported here. Stuenkel and Hillyard (1980) report an increase in gill Na/K-ATPase activity in the euryhaline pupfish at 15°C compared to 25°C and 30°C. Rainbow trout (*Salmo gairdneri richardson*) at 20‰ salinity were found to have higher gill Na/K-ATPase activity at 6°C than at 16°C (Jurss *et al.*, 1987). Atlantic cod at 1°C also exhibit increased gill Na/K-ATPase activity compared to those at 8°C (Staurnes *et al.*, 1994).

It has been reported that increased plasma membrane fluidity, by changes in the fatty acid composition, allows for increased activity of plasma membrane transporters such as the Na/K-ATPase (Gibbs, 1995). In the trout (Robertson and Hazel, 1995), increased membrane fluidity due to increases in cholesterol and decreases in polyunsaturated fatty acids in the plasma membranes accompanied the increased Na/K-ATPase activity in warm-acclimated teleosts. In contrast, the increased Na/K-ATPase activity in warm-acclimated *T. bernacchii* is not accompanied by significant changes in the saturated and unsaturated fatty acid composition of the gill plasma membrane (Gonzalez-Cabrera *et al.*, 1995). This result is consistent with studies in which no changes occurred in the lipid composition or fatty acid chains of cold-adapted Atlantic cod, (Staurnes *et al.*, 1994) and salt-water adapted American eel, (*Anguilla rostrata*) (Crockett, 1999) despite increased gill Na/K-ATPase activity. Therefore, it was determined that the changes in the gill Na/K-ATPase activity in warm acclimated Antarctic teleosts are mediated by differences other than changes in plasma membrane fatty acids.

In marine teleosts, a balance maintained between the active transport of ions out of the organism and passive ion fluxes from the environment (Portner *et al.*, 1998) determine serum osmolality. Active transport and passive ion fluxes are differentially sensitive to the effects of temperature. Active transport decreases with decreasing temperature and passive fluxes, occurring primarily through the gill (Motais and Isaia, 1972), are relatively temperature insensitive (Raynard and Cossins, 1991). Therefore during cold acclimation of temperate teleosts it is thought that there must be compensation made to either increase the active transport processes or decrease passive fluxes in order to maintain ionic homeostasis (Hochachka, 1988; Schwarzbaum *et al.*, 1991). Few studies have examined active versus passive fluxes, therefore the mechanisms of the increased serum osmolality at temperatures approaching 0°C in temperate teleosts is unknown. In Antarctic fish, the increased serum osmolality is necessary for survival (Eastman, 1993) and can be explained by low basal gill Na/K-ATPase activity compared to temperate teleosts (Portner *et al.*, 1998). The compensation in active transport processes hypothesized to occur in cold-acclimated temperate teleosts (Portner *et al.*, 1998) is not favorable for the temperature induced physiological challenge faced by Antarctic teleosts.

Ouabain binding experiments were performed to determine if warm-acclimated Antarctic *T. bernacchii* experienced a change in the density of Na/K-ATPase (pump sites or B_{max}) or had experienced a more subtle change exhibited by a change in ouabain affinity for Na/K-ATPase (K_d). The number of ouabain binding sites did not increase as a result of warm acclimation, thus the increase in enzyme specific activity was due to an increase in enzyme turnover. Thus other changes in the enzyme such as affinities for

substrates could be altered. The increase in B_{\max} at week five for the fish kept at -1.5°C remains unexplained. Considering there was no significant difference in the ouabain binding data at one and two weeks between warm and cold acclimation and there were no differences in the B_{\max} between weeks one and two and five in the warm-acclimated group, the fifth week B_{\max} data of the cold-acclimated group is viewed as an anomaly. Moreover it is opposite to what is expected based on an increase in enzyme activity as a result of warming and therefore could not explain the higher Na/K-ATPase activity seen in *T. bernacchii* after five weeks at 4°C .

The ouabain K_d values for the *T. bernacchii* and *N. angustata* gill were 23.75 ± 2.8 nM and 7.53 ± 1.1 nM respectively. The K_d values for both notothenioid species did not change significantly with temperature acclimation at any time point. Therefore, temperature acclimation had no effect on observed notothenioid gill Na/K-ATPase affinity for ouabain. Three ouabain binding sites have been reported in the literature for Na/K-ATPase in various species and tissues, a low affinity site with a K_d in the 0.5-2 mM range (Fricke, 1984; Hootman and Williams, 1985; Maki *et al.*, 1992; McCormick and Bern, 1989; Morrill *et al.*, 1985) an intermediate affinity site in the 20-500 nM range (Fricke, 1984; Hansen, 1999; Maki *et al.*, 1992; Morrill *et al.*, 1985) and a high affinity site in the 0.1 nM-20 nM range (Eakle *et al.*, 1992; Gonzalez-Garcia *et al.*, 1987; Hansen, 1999; Wong and Chan, 1999). The K_d for ouabain in the gills of *T. bernacchii* revealed a high affinity site. The K_d values for notothenioid gill tissue are much lower in the present study than the only other reported teleost gill Na/K-ATPase ouabain affinity (340 nM) in coho salmon (McCormick and Bern, 1989), however, the latter study involved incubation of entire gill filaments and not on isolated membranes. Despite our results it is possible

that a low affinity site for ouabain remained undetected in our binding studies because low affinity sites with a K_d above 100 nM cannot be reliably measured with vacuum filtration methodology. A change in the percentage of low affinity sites could result in changes in the activity of the enzyme. In preliminary whole cell experiments, a second low affinity ouabain site that was difficult to quantify was detected as well at the higher affinity site reported here in isolated membranes (data not shown). It is also likely that changes in pump activity reflecting changes in enzyme turnover in whole cells, as reported in the study by Hootman and Williams (1985), are not observed by ouabain affinity changes in isolated membranes.

To examine the characteristics of Na/K-ATPase that account for the increased turnover brought on by warm acclimation other parameters will need to be tested. Changes in enzyme turnover may be reflected in changes in affinities of Na^+ , K^+ , Mg^{+2} , and ATP for the Na/K-ATPase. Examples of *in vivo* conditions that are important determinants of enzyme kinetic behavior include distinct intracellular and extracellular ionic concentrations (Therien and Blostein, 1999) and phosphorylation of the α -subunit of the Na/K-ATPase (Fotis *et al.*, 1999).

A change in Na/K-ATPase α -subunit isoforms composition could provide a possible explanation for altered enzyme activity. Four distinct isoforms have been described in other species and are expressed in a tissue and developmentally-dependent manner (Blanco and Mercer, 1998). mRNA from the three most commonly-described α -subunit isoforms ($\alpha 1$, $\alpha 2$, $\alpha 3$) have been identified in the *T. bernacchii* gill (Guynn *et al.*, 2001). The α -subunit isoforms in other species have been found to have varying ouabain, Na^+ , K^+ , and ATP affinities (Blanco and Mercer, 1998). Further study will be

required to determine the role of isoform composition as an explanation for the changes in the enzyme activity.

In summary, the Antarctic teleost *T. bernacchii* has a serum osmolality that is significantly lower as a result of warm acclimation. The decrease in osmolality is accompanied by an increase in gill Na/K-ATPase enzyme activity. These two phenomenon are not observed with warm-acclimated New Zealand *N. angustata*, a notothenioid of the same family restricted to temperate waters. The increase in Na/K-ATPase activity in *T. bernacchii* was not accompanied by an increase in the number of Na/K-ATPase sites or measurable change in the enzyme's affinity for ouabain. Therefore, because activity is a function of pump density and pump turnover, the increased activity observed after warm acclimation may be due to a change in turnover of the enzyme. Future efforts will examine possible changes in Na/K-ATPase α -subunit isoforms and investigate enzyme turnover to further elucidate the cause of the increased activity of Na/K-ATPase in warm acclimated *T. bernacchii* gill.

CHAPTER THREE

Identification of mRNA and protein expression of the Na/K-ATPase α 1, α 2 and α 3 subunit isoforms in Antarctic and New Zealand Nototheniid Fishes

This chapter is *in press* ...

Guynn, S. R., Scofield, M. A. Petzel D.H. (2002) Identification of mRNA and protein expression of the Na/K-ATPase 1, 2 and 3 subunit isoforms in Antarctic and New Zealand Nototheniid Fishes. *J.Exp.Mar.Bio.Eco.*

Introduction

Teleosts of the family Nototheniidae, living below the Antarctic Convergence, thrive in the near frozen waters of the Southern Ocean including McMurdo Sound, Antarctica (Somero and DeVries, 1967). One of the physiological adaptations that allow Antarctic notothenioids to live in waters below 0°C, is a serum osmolality of ~ 600 mOsm/kg, nearly double the serum osmolality (330 mOsm/kg) of temperate marine teleosts (O'Grady and DeVries, 1982). The increased osmolality, due to higher serum [Na⁺] and [Cl⁻] (Dobbs and DeVries, 1975), lowers the freezing point of the blood by 1°C (O'Grady and DeVries, 1982). The Na/K-ATPase in the chloride cells of the gill, is the enzyme responsible for maintaining serum osmolality by providing the potential energy for Na-coupled transport of Cl⁻ thereby controlling serum [Na⁺] and [Cl⁻] (Karnaky, 1986).

The Na/K-ATPase is a functional heterodimer, composed a catalytic 110 kDa α -subunit and a 55 kDa β -subunit (Blanco and Mercer, 1998; Lingrel *et al.*, 1990). There is evidence of a third co-precipitating protein (γ) however its presence is not necessary for function. The α -subunit contains the binding sites for Na⁺, K⁺, Mg⁺², ATP and cardiotonic steroids such as ouabain (Blanco and Mercer, 1998; Lingrel *et al.*, 1990; Skou and Esmann, 1992) and the β -subunit is important for holo-enzyme packaging and insertion (Geering, 1991; Schmalzing G. and Gloor S, 1994). Both the α and β -subunits have multiple isoforms in vertebrates.

Sequence variations have identified four isoforms of the Na/K-ATPase α -subunit (Pressley, 1992; Takeyasu *et al.*, 1990). Within the same species the four paralogous isoforms have approximately 85% amino acid identity and 75% nucleotide identity

(Herrera *et al.*, 1987; Shamraj and Lingrel, 1994; Shull *et al.*, 1986; Takeyasu *et al.*, 1990). Comparing the sequences of one orthologous isoform from many different species yields an amino acid identity of 90% or greater (Blanco and Mercer, 1998). The Na/K-ATPase α -subunit isoform mRNA and protein composition and distribution has not been examined in great detail in any teleost and has never been examined in Antarctic teleosts.

Antarctic notothenioids provide an interesting model for the study of Na/K-ATPase because of their unusual osmoregulatory physiology of Antarctic notothenioids in relation to environmental temperature. The Na/K-ATPase is highly conserved in sequence and function across different species and information gained in studying notothenioid osmoregulation can be applied to the isoforms in all vertebrates. Previous results indicated the increase in serum osmolality and decrease in gill Na/K-ATPase activity of warm-acclimated *T. bernacchii* is not caused by a change in enzyme number or affinity for ouabain (Guynn *et al.*, 2002a). Based upon these results, changes in the Na/K-ATPase at the molecular level, such as a change in the Na/K-ATPase α -subunit isoform composition, may be responsible for the increase in Na/K-ATPase activity warm-acclimated *T. bernacchii*. However, in order to propose such a hypothesis, the Na/K-ATPase α -subunit isoforms present in Antarctic nototheniids needed to be determined.

Therefore, the purpose of this study was to determine the Na/K-ATPase α -subunit isoform identities, composition and tissue distribution in Antarctica *T. bernacchii* gill, brain, heart, trunk kidney and skeletal muscle. The expression of α -subunit isoform transcripts was examined using reverse transcriptase – polymerase chain reaction (RT-PCR) and verified the identity of the α -subunit isoforms by subcloning and sequencing.

Na/K-ATPase α -subunit isoform specific antibodies were used to determine the tissue distribution of the isoforms at the protein level. To determine if all nototheniids had the same isoform composition and distribution, the Na/K-ATPase α -subunit isoform protein expression in *N. angustata*, a nototheniid in the same family as *T. bernacchii*, but from temperate New Zealand waters, was examined.

Methods

Fish Collection

T. bernacchii (100-400 g) were caught with hook and line, and traps in McMurdo Sound, Antarctica from September to November (1998 and 1999), transported to 200L laboratory aquaria at McMurdo Station, Antarctica and held in free flowing seawater tanks for 5-7 days at -1.5°C . McMurdo Sound has an annual water temperature of -1.87°C regardless of season or depth (Littlepage, 1965). The New Zealand *N. angustata* were caught by hook and line from Portobello Bay, New Zealand and were transported by air to McMurdo Station within 48 hours of capture and held at 8°C in McMurdo Sound seawater for at least two weeks until the tissues were collected.

Tissue Collection

The fish were fully anesthetized with MS222 (1g/8L sea water) and placed on ice. The gills were perfused through the bulbous arteriosus with 30 ml of ice cold air-equilibrated heparinized physiologic Ringers solution at 0°C to remove red blood cells. The Ringers solution was 280 mM NaCl (*T. bernacchii*) or 150 mM NaCl (*N. angustata*), 5 mM KCl, 3 mM MgCl_2 , 3 mM CaCl_2 , 10 mM NaHCO_3 , 10 mM HEPES, 5 mM glucose, pH 7.8 at 0°C . Following perfusion; the gill arches, the heart ventricles, the

trunk kidney (containing the nephrons), a piece of skeletal muscle and the brain were all removed and flash frozen in liquid nitrogen and stored at -80°C until RNA and protein isolation.

RNA Isolation

Total RNA was isolated from *T. bernacchii* brains, gill filaments, heart, trunk kidney and muscle with TRIzol® (GibcoBRL) according to manufacturer's protocol. The integrity of the RNA was checked by gel electrophoresis in a 2% agarose gel by determining the presence of 28S, 18S and 5S ribosomal RNA bands. An A_{260}/A_{280} ratio was used to determine purity and quantity of the RNA where a ratio of 1.8 was considered to be a pure RNA preparation. After isolation of total cellular RNA, contaminating DNA was digested with amplification grade RNase-free DNase I (GibcoBRL) according to the manufacturer's protocol, with the exception that the reaction was carried out in $\frac{1}{2}$ the volume.

Primers

(All primers and *T. bernacchii* sequences in this paper are numbered according to comparison with the coding sequence of the Na/K-ATPase $\alpha 1$ isoform of *Rattus norvegicus* where the first nucleotide of the start codon is numbered 1.)

To amplify the Na/K-ATPase α -subunit isoform mRNA present in *T. bernacchii* tissue, degenerate consensus primers that were non-isoform specific were designed using published sequences from rat (Herrera *et al.*, 1987), chicken (Takeyasu *et al.*, 1990), eel (Cutler *et al.*, 1995), white sucker (Schonrock *et al.*, 1991) and electric ray (Kawakami *et al.*, 1985) (Table 1, "Consensus Generic"). Sequence analysis of the resultant PCR

product allowed us to design another sense primer specific to the *T. bernacchii* sequence upstream of the heterologous region in the large intracellular loop. This sense primer was designed using *T. bernacchii* sequence within a homologous region so that the primer was generic to all isoforms, but *T. bernacchii* specific (Table 1, “*T. bernacchii* Generic”). The anti-sense primer of this pair was further downstream (DS) than the previous consensus generic anti-sense primer, but was still a consensus isoform generic primer modeled after the other species sequences (Table 1, “Consensus Generic (DS)). After determining 500-600 bases of the three *T. bernacchii* isoform sequences, primer sets were designed to amplify 250-300 nucleotides specific to each *T. bernacchii* isoform (Table 1). For detecting low levels of the $\alpha 2$ isoform RNA, nested primers specific to the $\alpha 2$ isoform were used for a second round of amplification of cDNA from *T. bernacchii* heart and trunk kidney (Table 1).

Table 3.1. Primer pairs used to generate *T. bernacchii* Na/K-ATPase α -subunit isoform RT-PCR products.

	Sense Primer, 5' -3' (bases numbered according to rat α 1)	Anti-Sense Primer, 5' -3' (bases numbered according to rat α 1)	Product Size
Consensus Generic	GAAGAACCT(G/T)GA(G/A)GC(G/T)GTGGAG 1075 1096	GATCCT(C/T)TCTGGAGC(T/A)CC(C/T)TTCAT 1542 1519	444
<i>T. bernacchii</i> Generic	GT(G/T)GC(C/T)GAGATCCC(G/C/A)TTCAAC 1429 1450		
Consensus Generic (DS)		T(G/C)CC(C/T)GATGC(C/T)GT(G/T)CCCGAAATG 1817 1795	388
<i>T. bernacchii</i> α 1 isoform	CACAAGAATGCAACACCTGGAG 1475 1495	TGGACATGAGGCCGATGAA 1770 1752	295
<i>T. bernacchii</i> α 2 isoform	TGAGGACCAGACGGGTTTAGG 1209 1230	AGCAGAAGACAATCCCTCTGGT 1501 1480	292
<i>T. b. nested</i> α 2 isoform	GGGCTATGCAACAGAGCGGAT 1278 1299	GAAGACAATCCCTCTGGTCATATT 1512 1489	234
<i>T. bernacchii</i> α 3 isoform	CTGGTGCCTCTTTCGACAAGA 1219 1239	ACAGCTGGTATTTGTTGGTGGA 1471 1450	252

RT-PCR

Reverse transcription reactions were performed with 5 mM MgCl₂, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1 mM dNTP, 1U RNase inhibitor (ABI), 25 pmols anti-sense primer and 2.5 U of MuLV Reverse Transcriptase (ABI) in 10 ul total volume. A control with no template and a control without reverse transcriptase were included for each sample to determine the presence of contamination or genomic DNA, respectively. The RT reaction conditions were 50 min at 42°C, 5 min at 95°C and 5 min at 4°C for 1 cycle. Polymerase chain reactions were performed with 3 mM MgCl₂, 20 mM Tris-HCl, pH 8.4, 25 pmols of sense primer, 2.5 U of *Taq* DNA Polymerase (GibcoBRL) and the 10 ul RT reaction in a total volume of 50 ul. The samples were denatured for one cycle at 95°C for 5 min; amplified for 30-40 cycles of 95°C for 1 min, 55-65°C for 1 min, 72°C for 1 min; and extended for 1 cycle at 72°C for 7 min. To amplify the $\alpha 2$ isoform from heart and trunk kidney samples, the PCR product solutions originally amplified with *T. bernacchii* $\alpha 2$ isoform primers were subjected to a second PCR reaction, using primers nested within the original PCR product. 2 μ l of the initial RT-PCR amplified with the first pair of $\alpha 2$ primers was added to another PCR with 3 mM MgCl₂, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1 mM dNTP, 25 pmols sense and anti-sense nested primers and 2.5 U of *Taq* DNA Polymerase (GibcoBRL) in a 50 μ l reaction. The PCR conditions were the same in the reamplification reaction as for the initial reaction. Controls for the PCR reactions included samples with no template. PCR products were horizontally electrophoresed in 2% agarose gels and visualized with EtBr.

Cloning

Putative Na/K-ATPase α -subunit isoform PCR products were cloned using the TA® Cloning kit (Invitrogen) according to manufacturer's protocols. After verifying the size of the insert with an *Eco*R1 digestion, the isolated plasmids were quantified and purity determined using A_{260} and A_{280} . The inserts were sequenced with vector specific primers and an Applied Biosystems model 373 DNA sequencer. Sequences were compared to other sequences in Genbank with the BLAST program and the "PILE-UP" and "BEST-FIT" comparisons in the Wisconsin Package Version 10.2 Genetics Computer Group (Madison, WI).

Protein Preparation

Microsomal membrane preparations of *T. bernacchii* (brain, gill, heart, trunk kidney and muscle), *N. angustata* (brain, gill, heart, kidney and muscle), and *Rattus norvegicus* (brain, which served as positive control for all of the isoforms) were prepared. The tissues were homogenized on ice using teflon/glass homogenization tubes in 8 ml of 4°C 250 mM sucrose, 5 mM β -mercaptoethanol, 1 mM EDTA, and 50 mM imidazole buffer with 20 passes of a Wheaton PTFE pestle driven by a Con'Torque motor driver (Eberbach Corp., Ann Arbor MI). All of the preparative steps were performed at 4°C. The homogenate was centrifuged at 10000g for 10 minutes. The supernatant was saved in a separate tube and the pellet was re-homogenized in 8mls of EIS buffers. The 2nd homogenate was centrifuged at 10000g for 30 minutes. The second supernatant was combined with the first supernatant and then centrifuged at 100000g for 90 minutes. The final pellet was resuspended in 0.2-0.5 ml 50mM imidazole buffer, pH 7.8. Protein

content was determined by the methods of Lowry *et al* (1951) using bovine serum albumin as a standard.

Western Blotting

Microsomal proteins from *T. bernacchii* tissues, *N. angustata* tissues, and rat brain (10-20ug) were denatured in loading buffer (12% SDS, 40% glycerol, 0.2 M Tris-HCl pH 7, 0.0004% bromophenol blue, 178mM 2-mercaptoethanol) at 37°C for 20 min. The sample proteins and prestained protein standards (BioRad) were separated on a 4-15% gradient Tris-HCl SDS-polyacrylamide mini-gel at 200 mV for 45-60 min in 25 mM Tris base, 0.2 M glycine, 0.1% SDS running buffer. The proteins were transferred to nitrocellulose membranes (NitroPure, Osmonics, Westborough MA) using a semi-dry electroblotting apparatus (Sigma) with transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol) at 1.0 mA per cm² for 1 hour. The membranes were cut in half horizontally at 50 kDa. The higher molecular weight half of the membranes, to be treated with polyclonal anti-Na⁺/K⁺ ATPase α -subunit isoform antibodies, was blocked with 10% non-fat dry milk and 0.1% goat serum in Tris-buffered saline with Tween (TBS-T; 135 mM NaCl, 20 mM Tris, pH 7.6, and 0.1% Tween 20) for 1 hour at 23°C. The lower molecular weight half of the membranes, to be treated with monoclonal anti- β -actin antibody, was blocked in 5% non-fat dry milk in TBS-T for 1 hour at 23°C. Subsequently, the membranes were incubated at 4°C overnight in either an anti-Na⁺/K⁺ ATPase α -subunit antibody at 1/1000 dilution in the polyclonal blocking buffer or anti- β -actin antibody at 1/25000 in the monoclonal blocking buffer. The anti-Na/K-ATPase α 1, α 2 and α 3 isoform polyclonal antibodies were purchased from Upstate Biotechnology

(Lake Placid, NY) and the anti- β -actin monoclonal antibody was purchased from ICN (Aurora, OH). After the primary antibody incubation, all membranes were washed with TBS-T 3 times for 5 min, and 1 time for 15 min. The anti-Na/K-ATPase α -subunit membranes were incubated with goat anti-rabbit IgG-HRP linked secondary antibody (1/5000) diluted in polyclonal blocking buffer for 1 hour at 23°C. The anti- β -actin membranes were incubated with rabbit anti-mouse IgG-HRP linked secondary antibody (1/5000) diluted in monoclonal blocking buffer for 1 hour at 23°C. After the secondary antibody, all membranes were washed with TBS-T three times for 5 min, and one time for 15 min. The bands were visualized via enhanced chemiluminescence with Lumi-Glo reagent (Cell Signaling Technology, Beverly MA) and exposure to Kodak BioMax film.

Results

Identification of Na/K-ATPase α -subunit isoforms via RT-PCR, Cloning and Sequencing

Three partial *T. bernacchii* Na/K-ATPase α -subunit isoforms sequences (not including primer sequences), corresponding to the $\alpha 1$ (597 bp), $\alpha 2$ (666 bp) and $\alpha 3$ (630 bp) isoforms have been submitted to GenBank with the accession numbers AY081863, AY081864 and AY081865 respectively (Fig 3.1a, 3.1b and 3.1c). Analysis of the sequence identities revealed that the nucleotide sequence and deduced amino acid sequence of each isoform shares high identity with other Na/K-ATPase α -subunit isoform sequences (Table 2). In figure 3.2, a comparison of sequence similarities indicates that the *T. bernacchii* $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms are most similar to other vertebrate $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms, respectively. Additionally, the heterogeneous isoform defining region, AA 494-504 as discussed by Cutler et al (1995) and Pressley (1992) has been used to determine isoform identity. The boxed region in each of the

three *T. bernacchii* partial amino acid sequences (Fig 3.1) indicates this heterologous region in the large intracellular loop between trans-membrane domains 4 and 5.

Comparing the *T. bernacchii* sequences within this region to the Na/K-ATPase α -subunit vertebrate isoform consensus sequence clearly identified each of the sequences as the Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms (Fig. 3.3).

```

          400                                410
    Q I H E A D T T E N Q S G A T F D R
1185 CAGATCCACGAGGCCGACACCACGGAGAACCAGAGCGGTGCCACCTTCGACAGG
          418                                428
    S S A T W G A L A R I A G L C N R A
1239 AGCTCCGCCACATGGGGTGCCCTCGCCAGGATCGCCGGCCTCTGCAACCGCGCC
          436                                446
    V F L A E Q S G V P I L K (R) D V A G
1293 GTCTTCCTGGCAGAGCAGTCCGGTGTCCCCATCCTGAAGAGAGATGTAGCCGGT
          454                                464
    D A S E A A L L K C I E L C C G P V
1347 GACGCCTCAGAAGCTGCCTTGCTGAAGTGCATTGAGCTGTGCTGTGGACCCGTT
          472                                482
    G D M R D K Y Y K V A E I P F N S T
1401 GGCGACATGAGAGACAAATACTACAAGGTTGCTGAGATCCCATTCAACTCCACC
          490                                500
    N K Y Q L S I H K N A T P G E S K Q
1455 AACAAATACCAGCTTTCATCCACAAGAATGCAACACCTGGAGAGTCCAAACAG
          508                                518
L L V M K G A P E R I L D R C S T I
1509 CTGCTGGTGATGAAGGGCGCCCCAGAGAGGATTTTGGACCGCTGCTCCACCATC
          526                                536
    M M N G K E Q P L D D E M K D S F Q
1563 ATGATGAACGGCAAAGAGCAGCCTCTGGACGACGAGATGAAAGATTCTTCCAG
          544                                554
    N A Y V E L G G L G E R V L G F C Q
1617 AACGCCTACGTTGAGCTGGGAGGACTAGGAGAGAGAGTGCTAGGTTTCTGCCAG
          562                                572
    F N L P D D Q F P E G F A F D T E E
1671 TTCAACCTGCCCAGCACCAGTTCCAGAAGGCTTTGCTTTTGACACAGAGGAG
          580                                590
    T N F P T E N L C F I G L M S M I D
1725 ACAAACTTCCCCACTGAGAACCTCTGCTTCATCGGCCTCATGTCCATGATCGAC
594
    P
1779 CCT

```

Figure 3.1A *T. bernacchii* partial Na/K-ATPase α -subunit isoform mRNA nucleotide sequences (numbered on the left) and deduced amino acid sequences, single letter code above nucleotides (numbered above). The conserved arginine (R445) at the tryptic site of E₂ conformation of the enzyme is circled, the conserved FITC-reactive site of P-type ATPases is underlined and the heterogeneous isoform defining region is boxed in each sequence. A. α 1. B. α 2 and C. α 3.

370 380
 T L G S T S T I C S D K T G T L T Q
 1095 ACTCTGGGCTCCACCTCCACCATCTGCTCCGACAAGACGGGCACGCTGACCCAG
 388 398
 N R M T V A H M W F D N Q I H E A D
 1149 AACCGCATGACCGTGGCCCACATGTGGTTCGACAACCAGATCCACGAGGCGGAC
 406 416
 T T E D Q T G L G F D K S S A T W V
 1203 ACCACTGAGGACCAGACGGGTTTAGGATTTGACAAGAGCTCCGCTACTTGGGTT
 424 434
 A L S R V A G L C N R A D F K A G Q
 1257 GCTCTGTCTCGCGTGGCCGGGCTATGCAACAGAGCGGATTTCAAAGCCGGACAG
 442 452
 E N F P I L M (R) E T A G D A S E S A
 1311 GAGAACTTCCCATCCTGATGAGGGAGACAGCGGGGACGCTTCAGAGTCCGCT
 460 470
 L F K C I E L C C G S V R E M R A R
 1365 CTGTTCAAATGCATCGAGCTGTGCTGTGGGAGTGTTTCGTGAGATGAGAGCCAGA
 478 488
 N T K V A E I P F N S T N K Y Q L S
 1419 AACACCAAAGTGGCCGAGATCCCGTTCAACTCCACCAACAAATACCAGCTCTCT
 496 506
 V H E A E D N P S G H I L V M K G A
 1473 GTCCATGAAGCAGAAGACAATCCCTCTGGTCATATTCTGGTCATGAAGGGAGCG
 514 524
 P E R I L D R C S S I M I H G E E Q
 1527 CCAGAGAGAATCTTGACAGGTGCAGCAGCATCATGATCCACGGCGAGGAGCAG
 532 542
 P L D E D L T D A F Q S A Y M E L G
 1581 CCACTGGATGAAGACTTGACGGATGCTTTCCAGAGTGCCTACATGGAGCTGGGA
 550 560
 G L G E R V L G F C H L N L S S S Q
 1635 GGACTGGGAGAGAGAGTGCTGGGTTTCTGCCACTTGAATCTGTCTTCATCCCAGT
 568 578
 F P R G F T F D S E E T N F P T E Q
 1689 TCCCTCGAGGGTTCACCTTCGACAGCGAAGAAACCAACTTCCCCACCGAGCAGC
 582
 L C F L G
 1743 TCTGCTTCTGGGC

Figure 1B.

400 410
 Q I H E A D T T E D Q S G A S F D K
 1185 CAGATCCACGAAGGCCGACACCACGGAGGACCAGTCTGGTGCCTCTTTTCGACAA
 418 428
 S S V T W L S L A R V A G L C N R A
 1239 GAGCTCCGTGACGTGGCTGAGTTTGGCTCGTGTGCTGGTCTGTGTAACCGCGC
 436 446
 Q F K A G Q D A L (P) I L K R D V A G
 1293 TCAGTTCAAAGCGGGACAGGACGCCCTGCCCATCCTGAAGCGTGACGTGGCCGG
 454 464
 D A S E S A L L K C I E L C C G S V
 1347 AGACGCCTCAGAGTCTGCGCTGCTGAAGTGCATCGAGCTGTGCTGCGGCTCCGT
 472 482
 R A N R D R N K K V A E I P F N S T
 1401 GAGAGCCAACAGGGACAGGAATAAGAAGGTGGCCGAGATCCCCTTCAACTCCAC
 490 500
 N K Y Q L S I H E T E D T N D N R Y
 1455 CAACAAATACCAGCTGTCAATACACGAGACGGAGGATAACCAGAGAGTCCAAACA
 508 518
 L L V M K G A P E R I L D C C S T I
 1509 GCTGCTGGTGAAGGGCGCCCCAGAGAGGATTTTGGACTGCTGCTCCACCAT
 526 536
 M V Q G K E Q P M D E E L K E S F Q
 1563 CATGGTGCAGGGCAAGGAGCAGCCCATGGACGAAGAGCTGAAGTGAGCTTTCCA
 544 554
 N A Y M E L G G L G E R V L G F C H
 1617 GAACGCCTACATGGAGCTTGGAGGCCTGGGAGAGAGAGTGCTGGGTTTCTGCCA
 562 570
 T M L A E D Q Y P K G F A F D T D D
 1671 CACGATGCTTGCAGAGGATCAATACCCCAAGGGCTTCGCCTTCGACACGGATGA
 578 588
 V N F Q L C F L C F I G L M S M I D
 1725 TGTCAACTTCCAGACAGACCAGCTTTGCTTCATCGGCCTCATGTCCATGATCGA
 596
 P P R A A V P D A V P K
 1779 CCCTCCCCGTGCTGCTGTGCCTGATGCTGTGCCCAA

Figure 1C.

Table 3.2 Percent nucleotide and deduced amino acid identity of *T. bernacchii* partial sequences to NaK-ATPase α -subunit isoforms from other species. Number in parentheses by zebrafish isoforms indicate the isoform family designated by Rajarao (2001).

	Isoform	<i>T. bernacchii</i> α 1	<i>T. bernacchii</i> α 2	<i>T. bernacchii</i> α 3
		% Nucleotide Identity / % Amino Acid Identity		
<i>T. bernacchii</i>	α 1		72.3 / 70.7	75.2 / 73.9
"	α 2			73.0 / 72.8
"	α 3			
<i>T. mossambica</i> (tilapia)	α 1	81.4 / 83.4	74.1 / 72.9	69.8 / 71.4
"	α 3	72.4 / 72.9	74.4 / 73.8	83.3 / 87.6
<i>C. commersoni</i> (sucker)	α 1	73.7 / 81.4	69.0 / 73.8	68.9 / 75.2
<i>Anguilla anguilla</i> (eel)	α 1	80.0 / 88.4	74.0 / 76.5	74.3 / 74.3
<i>T. californica</i> (ray)	α ?	69.0 / 72.2	67.7 / 73.3	66.7 / 72.7
<i>Ratus norvegicus</i> (rat)	α 1	72.7 / 76.9	71.0 / 76.5	71.7 / 72.4
"	α 2	71.2 / 74.7	74.3 / 79.2	73.0 / 75.1
"	α 3	73.9 / 72.4	73.2 / 75.1	78.3 / 82.4
<i>Gallus gallus</i> (chicken)	α 1	73.7 / 79.9	71.9 / 78.3	70.6 / 74.3
"	α 2	70.9 / 70.7	73.7 / 76.0	72.6 / 72.7
"	α 3	73.5 / 74.4	73.7 / 76.5	78.1 / 83.3
<i>Danio rerio</i> (zebrafish)	α 1	77.2 / 83.9	72.6 / 76.9	73.5 / 73.8
"	α 2	64.6 / 65.7	75.0 / 78.3	67.6 / 71.8
"	α 3 (1)	77.2 / 80.9	70.8 / 74.2	69.0 / 72.4
"	α 4 (1)	75.7 / 79.9	69.5 / 72.9	69.7 / 71.4
"	α 5 (3)	72.9 / 73.9	73.8 / 74.7	79.7 / 84.3
"	α 6 (3)	72.2 / 74.4	73.7 / 74.7	80.2 / 88.6
"	α 7 (1)	75.9 / 83.4	69.5 / 76.1	70.0 / 74.3
"	α 8 (1)	76.9 / 84.4	69.9 / 76.0	70.2 / 74.3

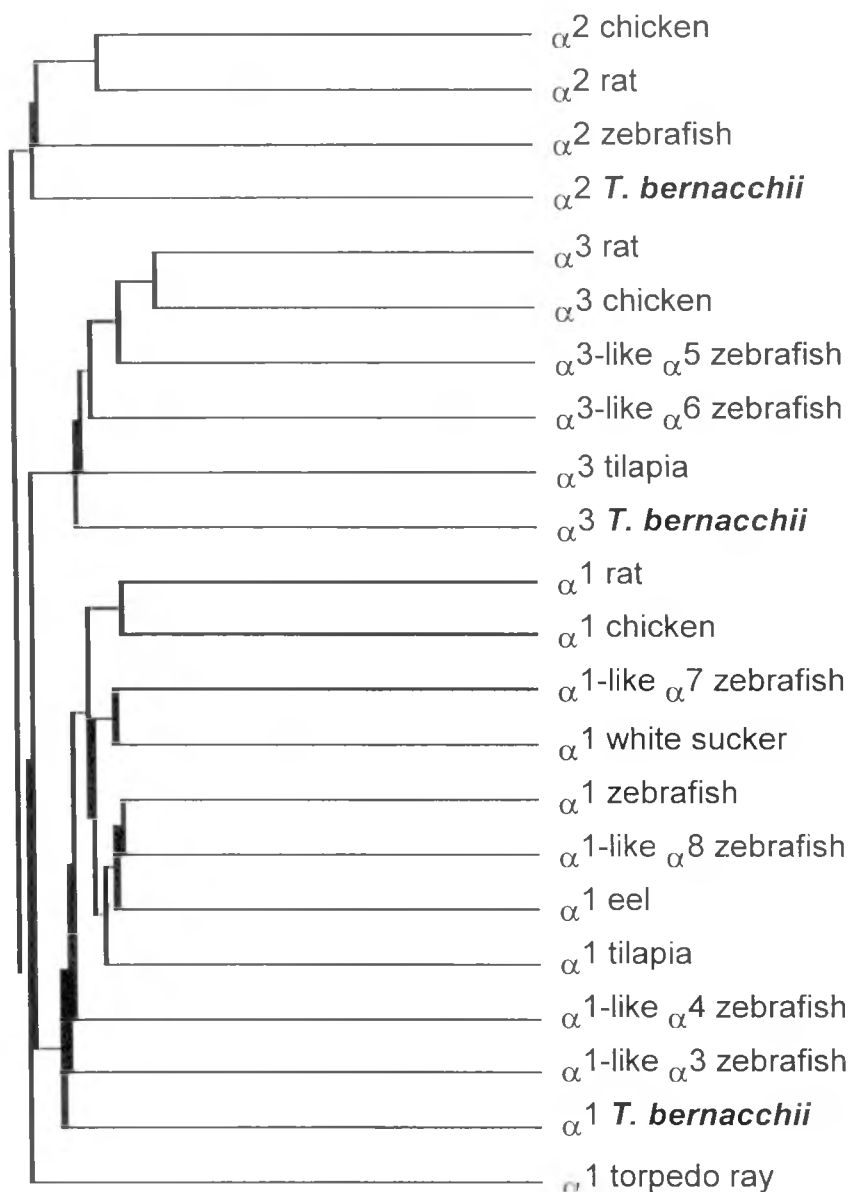


Figure 3.2 Sequence relatedness dendrogram of Na/K-ATPase α -subunits amino acid sequences constructed using the PILEUP program of the Wisconsin Package Version 10.2 Genetics Computer Group (Madison, WI). Sequences were obtained from GenBank and figure 3.1. Zebrafish isoforms are identified by both their given isoform number and their orthologous relationship to the standard Na/K-ATPase α 1, α 2 and α 3 isoforms as determined by Rajarao (2001).

Vertebrate Na/K-ATPase α 1 consensus sequence	K N A N P G E S K H L P A S P Q Y T R
<i>T. bernacchii</i> Na/K-ATPase α 1 sequence	K N A T P G E S K Q L
Vertebrate Na/K-ATPase α 2 consensus sequence	E R E D D P Q - G H I E S S V
<i>T. bernacchii</i> Na/K-ATPase α 2 sequence	E A E D N P S - G H I
Vertebrate Na/K-ATPase α 3 consensus sequence	E T E D P N D N R Y L
<i>T. bernacchii</i> Na/K-ATPase α 3 sequence	E T E D T N D N R Y L

Vertebrate Consensus	<i>T. bernacchii</i> α 1	<i>T. bernacchii</i> α 2	<i>T. bernacchii</i> α 3
α 1	9/11	1/11	4/11
α 2	0/11	8/11	3/11
α 3	2/11	3/11	10/11

Figure 3.3 Comparison of the Na/K-ATPase α -subunit isoform consensus sequences AA 494-504 (Cutler *et al.*, 1995) and experimentally determined *T. bernacchii* deduced amino acid sequences in the same region. Other possible amino acid substitutions in the consensus sequences are listed below each substituted amino acid. Amino acids in the *T. bernacchii* sequences that match the consensus sequences are bolded. (-) represents a space in the sequence. Within each column the table shows the number of *T. bernacchii* bases of an assigned isoform that are identical to the 11 base consensus sequence of each isoform from the literature.

RT-PCR Identification of Na/K-ATPase α -subunit isoforms in tissues

All three Na/K-ATPase α -subunit isoforms mRNA were found in *T. bernacchii* brain, gill, heart, trunk kidney and muscle tissues using RT-PCR and isoform specific primers (Fig. 3.4). The results of subsequent TA cloning and sequencing results indicated that each α -subunit isoform primer set amplified only one specific α -subunit isoform. In over 100 Na/K-ATPase α -subunit isoform cDNA sequences from *T. bernacchii* tissues, only three isoform sequences (shown in Fig. 3.1a-c) have been detected.

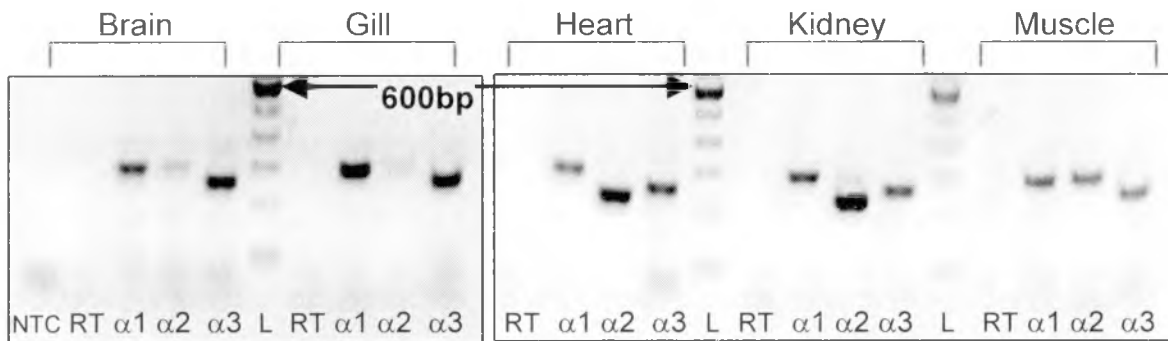


Figure 3.4 RT-PCR products of total RNA from *T. bernacchii* brain, gill, heart, trunk kidney and muscle using *T. bernacchii* Na/K-ATPase α -subunit isoform specific primers shown in Table 1. The light diffuse bands at the bottom are “primer-dimer”. NTC – no template control; L – 100 bp DNA ladder of 100bp; RT –no reverse transcriptase control; $\alpha 1$ – 292 bp, $\alpha 2$ – 295 bp in brain, gill and muscle, 234 bp in heart and kidney reamplified from the original reaction using nested primers; $\alpha 3$ – 252bp.

*Na/K-ATPase α -subunit isoform proteins in *T. bernacchii* and *N. angustata* tissues*

We probed *T. bernacchii* and *N. angustata* brain, gill, heart, trunk kidney and muscle and rat brain microsomal proteins with three polyclonal anti-Na/K-ATPase α subunit antibodies using Western blot analysis. The polyclonal Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoform specific antibodies from UBI have been proven to be isoform specific and detect proteins of sizes 110, 105 and 105 kDa in rats, respectively (Shyjan and Levenson, 1989). A ~98 kDa protein was detected in rat brain and *T. bernacchii* brain, gill and trunk kidney with the anti-Na/K-ATPase $\alpha 1$ antibody (Fig 3.5a). In the muscle, there are two distinct bands detected with the $\alpha 1$ antibody that do not correspond to the size of the Na/K-ATPase α -subunit; one at ~70 kDa and a darker one at ~150 kDa. The muscle results are similar to another polyclonal α isoform antibody which recognized the Na/K-ATPase heterodimer of both the α and β subunit at 150kDa and proteolytic fragments of the α subunit at 65kDa, while not recognizing the α subunit alone (Ura *et al.*, 1996). The anti- Na/K-ATPase $\alpha 2$ antibody detected a ~100 kDa protein in rat brain and *T. bernacchii* brain, gill, heart, trunk kidney and muscle (Fig. 3.5b). A ~100 kDa protein was detected using the anti-Na/K-ATPase $\alpha 3$ antibody in rat brain and *T. bernacchii* brain, gill, heart and kidney (Fig 3.5c). Additionally, all five tissues were probed with anti- β -actin antibodies and all *T. bernacchii* tissues and the rat brain had a band at ~42 kDa, indicating the presence of β -actin.

In the *N. angustata*, the anti-Na/K-ATPase $\alpha 1$ antibody detected the expected ~95 kDa protein band in the brain, gill, heart, trunk kidney and muscle (Fig. 3.6a). In the muscle, again there are two bands at 70 kDa and 175 kDa, however in contrast to the *T. bernacchii* a band at 95 kDa is also detected. The anti-Na/K-ATPase $\alpha 2$ detected

appropriate sized bands protein in all *N. angustata* tissues tested (Fig 3.6b). The anti-Na/K-ATPase $\alpha 3$ antibody detected appropriate sized bands in the brain, gill, heart and kidney (Fig 3.6c).

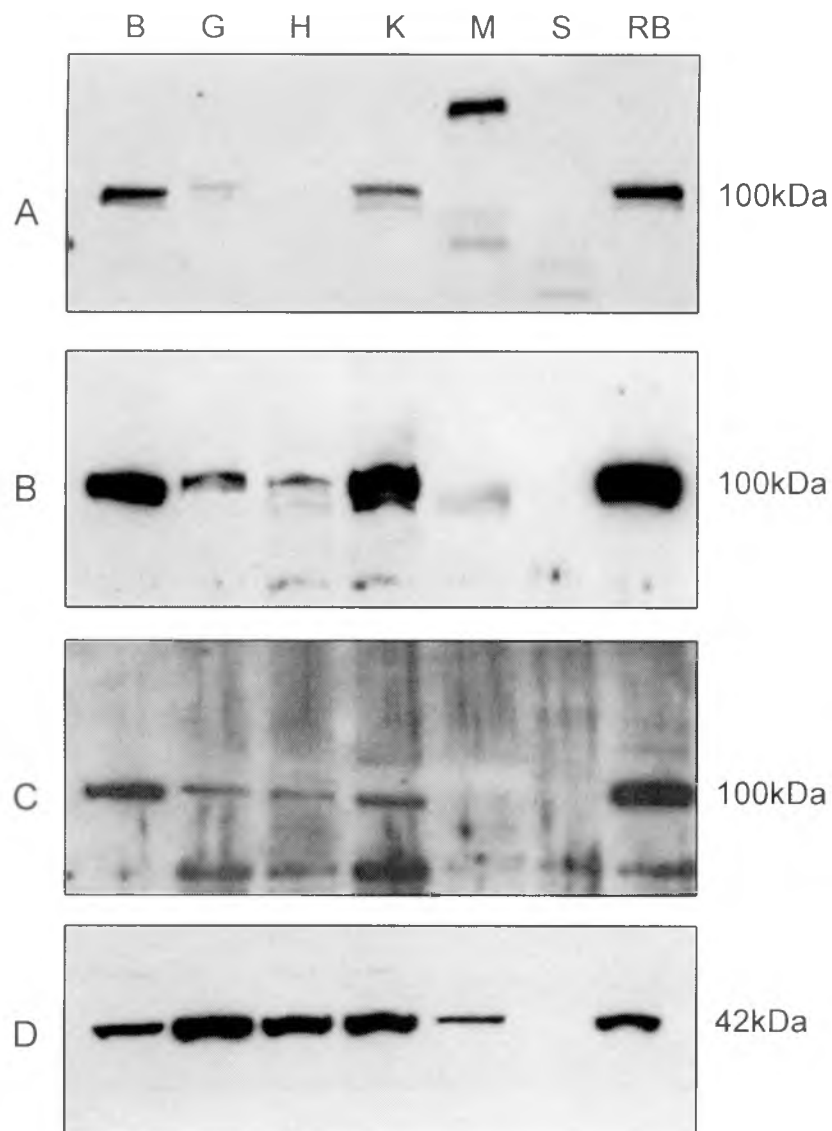


Figure 3.5 Tissue distribution of antigens in *T. bernacchii* brain (B at top), gill (G), heart (H), trunk kidney (K), muscle (M) and rat brain (RB). Protein Standard (S). A. blot with $\alpha 1$ isoform antibody. B. blot with $\alpha 2$ isoform antibody. C. blot with $\alpha 3$ isoform antibody. D. blot with anti- β -actin antibody.

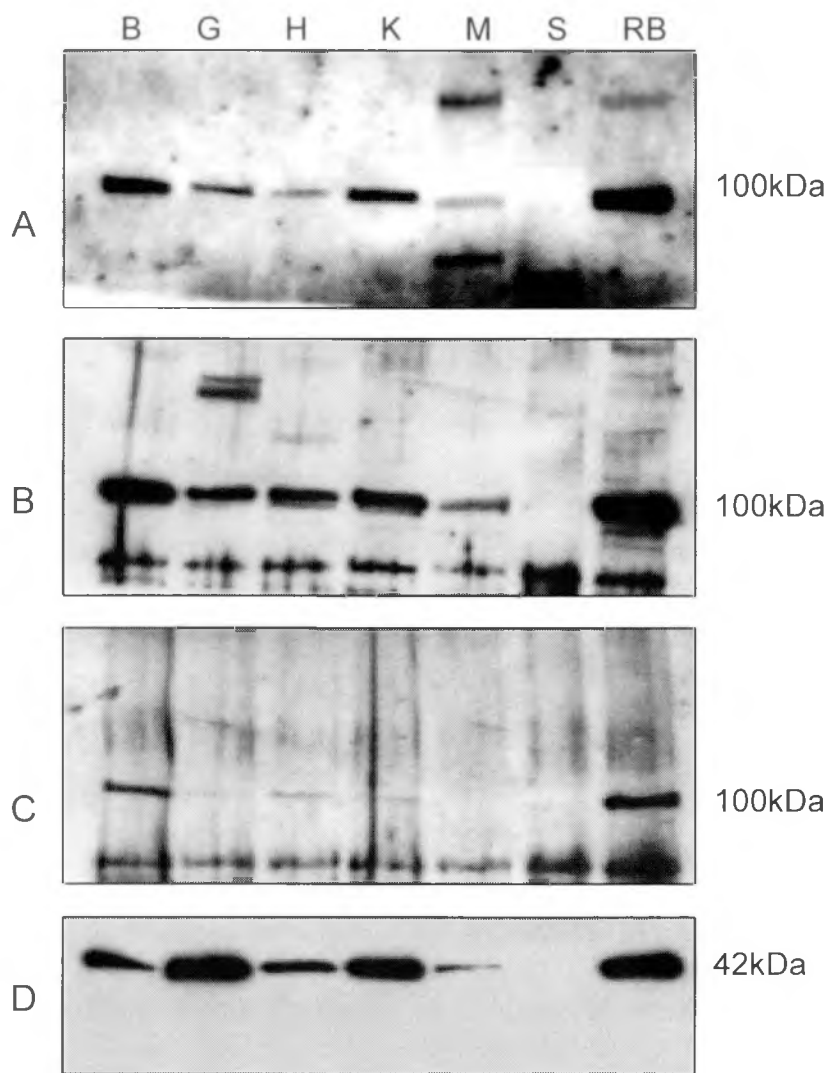


Figure 3.6 Tissue distribution of antigens in *N. angustata* brain (B at top), gill (G), heart (H), trunk kidney (K), muscle (M) and rat brain (RB). Protein Standard (S). A. blot with $\alpha 1$ isoform antibody. B. blot with $\alpha 2$ isoform antibody. C. blot with $\alpha 3$ isoform antibody. D. blot with anti- β -actin antibody.

Discussion

The present study provides the first evidence of mRNA and protein of the Na/K-ATPase $\alpha 1$, $\alpha 2$, and $\alpha 3$ isoforms in the teleost fish *T. bernacchii*. The presence of the three isoforms in the evolutionarily divergent Antarctic fish may provide a useful tool for examining the role and specific osmoregulatory functions of the three isoforms in all species.

In marine species, Na/K-ATPase α -subunits have been fully cloned in the torpedo ray (Kawakami *et al.*, 1985), the white sucker (Schonrock *et al.*, 1991), the european eel (Cutler *et al.*, 1995) the tilapia (Genbank accession numbers U82549 and AF109409) and the zebrafish (Rajarao *et al.*, 2001). In the eel gill, the sequence was identified as the Na/K-ATPase $\alpha 1$ isoform, and in the tilapia both the $\alpha 1$ and $\alpha 3$ were identified. In the zebrafish, eight clones of the Na/K-ATPase α -subunit were identified from cDNA, five of which are $\alpha 1$ isoform like, one is $\alpha 2$ isoform like and two are $\alpha 3$ isoform like (Rajarao *et al.*, 2001). Partial Na/K-ATPase α subunit clones have also been identified in rainbow trout (Kisen *et al.*, 1994) and spiny dogfish ($\alpha 3$) (Hansen, 1999). The presence of the Na/K-ATPase α -subunit is supported not only by the isolation of mRNA transcripts but also by Western blots which have identified $\alpha 1$ and $\alpha 3$ proteins in tilapia (Lee *et al.*, 1998), as well as $\alpha 3$ proteins in the gills of Atlantic salmon (D'Cotta *et al.*, 2000). In the brown trout Northern blot analysis with an $\alpha 1$ probe showed a single band indicating the presence of the $\alpha 1$ isoform in the kidney and gills (Madsen *et al.*, 1995).

This is the first study to identify the $\alpha 2$ isoform protein in teleosts. In the zebrafish, one of the eight cDNA clones was identified as $\alpha 2$, however the accompanying

sequence analysis does not conclusively define it as a vertebrate $\alpha 2$ (Rajarao *et al.*, 2001). Our sequence analysis data shows that the *T. bernacchii* $\alpha 2$ sequence shares the highest identity with the zebrafish $\alpha 2$ sequence, compared to the $\alpha 1$ or $\alpha 3$ isoform sequences (Table 2). However, there is no zebrafish protein data to confirm the presence of $\alpha 2$ isoform proteins in zebrafish. Additionally, unlike the previous studies, mRNA coding for the Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms was found to be in all *T. bernacchii* tissues examined. In comparing this study to previous studies using Northern blots (D'Cotta *et al.*, 2000; Hwang *et al.*, 1998; Schonrock *et al.*, 1991), we have used RT-PCR which is a more sensitive technique, that allowed us to precisely identify the isoform type by cloning and sequencing the products.

Additionally, all three Na/K-ATPase α isoform proteins were identified in *T. bernacchii* and *N. angustata* brain, gill and trunk kidney with isoform specific antibodies using Western blot analysis. All three isoform cDNAs have been identified in the brain of all vertebrate species examined (Blanco and Mercer, 1998; Emanuel *et al.*, 1987; Herrera *et al.*, 1987; Lingrel *et al.*, 1988; Pressley, 1992; Rajarao *et al.*, 2001). Previously, only the $\alpha 1$ and $\alpha 3$ isoform proteins have been found in teleost gills (D'Cotta *et al.*, 2000; Kisen *et al.*, 1994; Madsen *et al.*, 1995). Typically, the kidney expresses primarily the $\alpha 1$ isoform, however the $\alpha 2$ and $\alpha 3$ isoforms have also been identified as contributing less than 1% of all the kidney isoforms (Emanuel *et al.*, 1987; Herrera *et al.*, 1987; Lingrel *et al.*, 1988; Lucking *et al.*, 1996). The absence of the $\alpha 1$ isoform in the heart of *T. bernacchii* is not surprising, as others have found the $\alpha 2$ and $\alpha 3$ to be predominant in the heart (Herrera *et al.*, 1987). The $\alpha 2$ and $\alpha 3$ isoforms have been identified in skeletal muscle from other species (Herrera *et al.*, 1987), however the $\alpha 2$

isoform was found to be the predominant isoform in rat muscle (Lingrel *et al.*, 1988), similar to the results detecting only the $\alpha 2$ isoform in *T. bernacchii* muscle presented here. In the *T. bernacchii* muscle and heart, the $\alpha 1$ and $\alpha 3$ mRNA does not translate to protein implying some post-transcriptional or translational regulation.

The Na/K-ATPase α -subunit isoforms have different tissue distribution (Blanco and Mercer, 1998; Orłowski and Lingrel, 1988), different affinities for ouabain and substrate ions (Blanco and Mercer, 1998; Lucking *et al.*, 1996) and differential regulation via hormones and protein kinases (Therien and Blostein, 2000). Additionally, the α -subunits are a multi-gene family, with each α isoform coming from a separate genomic location (Kent *et al.*, 1987; Yang-Feng *et al.*, 1988). The individual isoform characteristics and chromosomal dispersion of the genes suggests they are not functionally redundant proteins but rather have distinct functions selected in response to different physiological demands (Levenson, 1994). When comparing an individual isoform across species the sequence identities are higher than when comparing different isoforms within one species which also supports the idea that the isoforms have separate functions (Broude *et al.*, 1989; Pressley, 1992). Yet, little is known about the different physiologic functions of the isoforms.

In the present study we have established the presence of Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoform mRNA and protein in the gills of *T. bernacchii*, an Antarctic notothenioid and Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoform proteins in the gills of *N. angustata*, a temperate notothenioid. In future studies, the goal will be to determine if changes in the Na/K-ATPase α -subunit isoform mRNA or protein in the gill occur with the changes in osmolality and gill Na/K-ATPase activity seen in warm-acclimated *T. bernacchii*. These

results may provide evidence as to the roles of the separate Na/K-ATPase α -subunit isoforms in all species.

CHAPTER FOUR

Changes in mRNA and protein expression of the Na/K-ATPase
 $\alpha 1$, $\alpha 2$ and $\alpha 3$ subunit isoforms in the warm acclimated Antarctic
nototheniid fish *Trematomus bernacchii*.

Introduction

In marine teleosts, the Na/K-ATPase in the chloride cell of the gill establishes the ion gradient used to excrete Na^+ and Cl^- , thereby maintaining serum osmolality. The Na/K-ATPase is a functional heterodimer, composed of two subunits, an α -subunit of ~100kDa, and a β -subunit of ~55kDa (Lingrel *et al.*, 1990). There is evidence of a third co-precipitating protein (γ) however its presence is not necessary for function. The α -subunit is the primary catalytic subunit with the Na^+ , K^+ , Mg^{2+} , ATP and ouabain binding sites (Crambert *et al.*, 2000; Jorgensen, 1983). Four isoforms of the Na/K-ATPase α -subunit have been identified, with approximately 85% amino acid (AA) identity and 75% nucleotide (NT) identity between the four isoforms within the same species (see Fig. 3.3) (Herrera *et al.*, 1987; Shamraj and Lingrel, 1994; Shull *et al.*, 1986; Takeyasu *et al.*, 1990). Comparing the same isoform in different species yields 90% or greater AA identity (Blanco and Mercer, 1998). The isoforms have different tissue specificity and developmental distribution (Orlowski and Lingrel, 1988). Additionally, the α -subunit isoforms differ in their ouabain sensitivities and cation binding affinities (Sweadner, 1989). Hormones and protein kinases (Blanco and Sánchez, 2001) also regulate the expression of the α isoform subunits of the Na/K-ATPase. Differential effects of hormones and protein kinases, as well as different distributions and cation affinities of the isoforms, provide mechanisms by which an organism can regulate Na/K-ATPase activity in a wide variety of cell types and physiological conditions.

Na/K-ATPase α isoform proteins in marine teleost gills were identified in Antarctic *T. bernacchii* ($\alpha 1$, $\alpha 2$ and $\alpha 3$) (Guynn *et al.*, 2002b), tilapia ($\alpha 1$ and $\alpha 3$) (Lee *et al.*, 1998) and Atlantic salmon ($\alpha 3$) (D'Cotta *et al.*, 2000). mRNA transcripts of the

Na/K-ATPase α 1 and α 3 isoforms have been detected in many marine teleosts (Cutler *et al.*, 1995; Kawakami *et al.*, 1985; Kisen *et al.*, 1994; Madsen *et al.*, 1995; Schonrock *et al.*, 1991), including the Antarctic *T. bernacchii* (Guynn *et al.*, 2002b). The α 2 isoform protein has only been found in *T. bernacchii* (Guynn *et al.*, 2002b) while α 2 mRNA has also been tentatively identified in zebrafish (Rajarao *et al.*, 2001). In the zebrafish, eight clones of the Na/K-ATPase α -subunit were identified from cDNA, five of which are α 1 isoform like, one is α 2 isoform like and two are α 3 isoform like (Rajarao *et al.*, 2001). The zebrafish is the only report of more than four Na/K-ATPase α -subunit isoforms in any organism.

Antarctic *T. bernacchii* can regulate their serum osmolality in response to changes in both environmental salinity (O'Grady and DeVries, 1982) and temperature (Gonzalez-Cabrera *et al.*, 1995). Upon warm acclimation from -1.5°C to 4°C for five weeks, the serum osmolality of Antarctic species decreases significantly by nearly 25% accompanied by proportional decreases in serum $[\text{Na}^+]$ and $[\text{Cl}^-]$ (Gonzalez-Cabrera *et al.*, 1995; Guynn *et al.*, 2002a). The decrease in serum osmolality in warm-acclimated Antarctic teleosts is accompanied by significant increases in gill Na/K-ATPase activity (Gonzalez-Cabrera *et al.*, 1995; Guynn *et al.*, 2002a). The increase in Na/K-ATPase activity however is not accompanied by any changes in enzyme number or ouabain affinity in *T. bernacchii* as determined by ouabain binding experiments (Guynn *et al.*, 2002a). The differential expression and regulation of the Na/K-ATPase α isoforms provide another level of Na/K-ATPase regulation. A change at the molecular level in the Na/K-ATPase α -subunit isoform expression of warm acclimated *T. bernacchii* may account for the changes in Na/K-ATPase activity while not significantly affecting total

enzyme number. The purpose of the following studies was to determine if warm-acclimation caused a change in isoform expression in the gills of the *T. bernacchii*. Immunoblots were used to examine protein expression and real time RT-PCR was used to examine mRNA levels. Examining both mRNA and protein levels may indicate whether the Na/K-ATPase α -subunit isoforms levels in warm-acclimated *T. bernacchii* are regulated prior to transcription or translation.

Methods

Fish Collection

T. bernacchii (100-400 g) were caught with hook and line, and traps in McMurdo Sound, Antarctica from September to November in 1998 and 1999, transported to 200L laboratory aquaria at McMurdo Station, Antarctica and held in free flowing seawater tanks for 5-7 days at -1.5°C . McMurdo Sound has an annual water temperature of -1.87°C regardless of season or depth (Littlepage, 1965).

Acclimation.

T. bernacchii were randomly separated according to size (100-250 g) and placed (6-10 each) in open mesh cages (12.5 ft³) in 200L acclimation tanks maintained at -1.5°C or 4°C ($\pm 0.1^{\circ}\text{C}$) for five weeks. The -1.5°C tanks were free flowing using seawater pumped from McMurdo Sound. For all tanks warmed above -1.5°C , McMurdo Sound seawater was heated and maintained within 0.1°C using a temperature control device (Cole-Parmer, Vernon Hill, IL) that switched power between a submerged heating coil and a water pump that pumped -1.5°C water into the tanks when cooling was needed. Due to the intermittent flow of fresh seawater, the water in the warm tanks was aerated

and circulated. During the acclimation period, the fish were not fed nor was any effort made to control the photoperiod.

Tissue Collection

The fish were fully anesthetized with MS222 (1g/8L sea water) and placed on ice. Blood was collected through a caudal vessel with a non-heparinized 30-gauge x $\frac{1}{2}$ inch needle and 1 cc. plastic syringe. The gills were perfused through the bulbous arteriosus with 30 ml of ice-cold air-equilibrated heparinized physiologic Ringers solution at 0°C to remove red blood cells. The Ringers solution was 280 mM NaCl (*T. bernacchii*) or 150 mM NaCl (*N. angustata*), 5 mM KCl, 3 mM MgCl₂, 3 mM CaCl₂, 10 mM NaHCO₃, 10 mM HEPES, 5 mM glucose, pH 7.8 at 0°C. Following perfusion, the gill arches were removed and flash frozen in liquid nitrogen, and then stored at -80°C until RNA and protein isolation.

Serum Osmolality

The blood was stored for 4 hours at 4°C in a polyethylene tube, centrifuged at 2000g for 5 min and the serum transferred to a clean tube. Serum osmolality was determined using a vapor pressure osmometer (Wescor Inc., Logan, UT).

RNA Isolation

Total RNA was isolated from *T. bernacchii* gill filaments with TRIzol® (GibcoBRL) according to manufacturer's protocol. The integrity of the RNA was checked by gel electrophoresis in a 2% agarose gel by determining the presence of 28S, 18S and 5S ribosomal RNA bands. An A₂₆₀/A₂₈₀ ratio was used to determine purity and quantity of the RNA where a ratio of 1.8 considered being a pure RNA preparation.

Real Time RT-PCR Theory

Real time PCR measurements are taken at the point in the amplification cycle where PCR product is first detected, rather than the traditional endpoint measurement of product accumulation following cycling (Medhurst *et al.*, 2000). Besides traditional oligonucleotide primers, a third oligonucleotide is required with the fluorescent quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) attached at the 5' end and a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), attached at the 3' end. During the DNA amplification phase, the oligonucleotide probe is degraded by the 5' to 3' exonuclease activity of DNA polymerase. This removes the quencher dye from the spatial proximity of the reporter dye, thereby allowing the reporter dye to fluoresce. The increase in reporter fluorescence is measured and is a direct consequence of target amplification during PCR. Results are expressed as a threshold cycle (C_t), which is the fractional cycle number at which the reporter dye exceeds its background fluorescence plus 10 standard deviations. It is plotted against the reporter dye fluorescence, which has been normalized to a passive reference dye, and has the fluorescence of the "no template control" (ΔR_n).

Real time Primer and Probe Design

Partial sequences of *T. bernacchii* Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms (Genbank Accession No. AY081863, AY081864 and AY081865, respectively) were evaluated using the Primer Express software (Applied Biosystems, Inc-ABI). The position of each probe was limited to regions of low identity between the isoforms in order to maximize probe specificity. The software generated probe and primers sets that were optimal in melting temperature, % G-C composition and lacking in secondary structures. Primers were of regular oligonucleotide chemistry. Attaching a FAM to the

5' end and TAMRA to the 3' end modified the probes. The nucleotide sequences for the *T. bernacchii* Na/K-ATPase α -subunit isoforms primer and probe sets are listed in Table 4.1.

α Isoform	Oligo	Oligonucleotide Sequence (5'-3')	Location	Size, bp
1	Sense primer	GTTGCTGAGATCCCATTCAACTC	244-266	128
	Probe	FAM-AGAATGCAACACCTGGAGAGTCCAAACAG-TAMRA	296-324	
	Anti-sense primer	GAGCAGCGGTCCAAAATCC	353-371	
2	Sense primer	CACCAACAAATACCAGCTCTCTGT	357-380	101
	Probe	FAM-CATGAAGCAGAAGACAATCCCTCTGGTCA-TAMRA	382-410	
	Anti-sense primer	TGCACCTGTCCAAGATTCTCTCT	435-457	
3	Sense primer	CCCCTTCAACTCCACCAACA	255-274	138
	Probe	FAM-CGGAGGATACCAATGATAACCGCTACCTG-TAMRA	299-327	
	Anti-sense primer	TTGCCCTGCACCATGATG	275-392	

Table 4.1. Na/K-ATPase α -subunit isoform primer and probe sets used in real time RT-PCR studies. FAM and TAMRA are the fluorescent dyes attached to each probe. For each primer and probe the location is based upon the nucleotide numbering of each *T. bernacchii* Na/K-ATPase α isoform sequence in GenBank (Guynn *et al.*, 2002b). The size of the expected amplicon is included in the last column.

Real Time RT-PCR

A no template control was included for all probe and primer sets within a reaction run. Additionally each set of reactions included a control human GAPDH standard curve to verify equipment function. The GAPDH total RNA, probe and primers were purchased from Applied Biosystems Inc. but the master mix was the same as used for the gill tissue reactions. This provided a control for reagent malfunction. PCR.II plasmids containing *T. bernacchii* $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoform sequences were used as both positive and negative controls for each probe and primer set. Probe and primers sets were chosen if amplification of the intended sequence was linearly proportional to the plasmid concentration, yet exhibited no amplification of the other two isoform sequences.

First strand cDNA synthesis was performed from total gill RNA with MuLV Reverse Transcriptase (RT) (Applied Biosystems, Inc.). The concentration of reagents was 5.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM dNTPs, 0.4 U RNase inhibitor, 1 μ M anti-sense primer and 1.25 U of RT in a 10 μ l total volume. Random hexamers, oligo dT and gene-specific anti-sense primers were tested as the RT primer. Gene specific anti-sense primers were chosen because they gave the highest C_t and R_n values. Controls with no template and without reverse transcriptase were included for each sample to determine the presence of contamination or genomic DNA, respectively. The reaction conditions were 30 min at 48°C and 5 min at 95°C, according to the manufacturer's protocol (Applied Biosystems Inc.).

All PCR reactions were performed using an ABI 7700 Sequence Detection System (Applied Biosystems, Inc.). Reagent concentrations were 5.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01 mM EDTA, 60 nM Passive Reference, 200 μ M

dATP, 200 μ M dCTP, 200 μ M dGTP, 400 μ M dUTP, 250 nM probe, 1000nM sense primer, 1000 nM anti-sense primer, 0.5 U AmpErase UNG (ABI) and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems Inc.) in a 50 μ l reaction tube. Probe and primer concentrations were optimized between 50-250 nM and 50-100nM respectively. The samples were incubated for 2 min at 50°C, denatured at 95°C for 5 min and amplified for 40 cycles of 95°C for 15 sec, 60°C for 1 min.

Each gill tissue sample was run with each probe and primer set in triplicate. A cold acclimated and warm acclimated sample were paired and run together to normalize reaction set differences.

Protein Preparation

Microsomal membrane preparations of *T. bernacchii* gill and *Rattus norvegicus* brain (a positive control for all of the isoforms) were prepared. The tissues were homogenized on ice using teflon/glass homogenization tubes in 8 ml of 4°C 250 mM sucrose, 5 mM β -mercaptoethanol, 1 mM EDTA, and 50 mM imidazole buffer with 20 passes of a Wheaton PTFE pestle driven by a Con'Torque motor driver (Eberbach Corp., Ann Arbor MI). All of the preparative steps were performed at 4°C. The homogenate was centrifuged at 10000g for 10 minutes. The supernatant was saved in a separate tube and the pellet was re-homogenized in 8mls of EIS buffers. The 2nd homogenate was centrifuged at 10000g for 30 minutes. The second supernatant was combined with the first supernatant and then centrifuged at 100000g for 90 minutes. The final pellet was resuspended in 0.2-0.5 ml 50mM imidazole buffer, pH 7.8. Protein content was determined by the methods of Lowry *et al* (1951) using bovine serum albumin as a standard.

Western Blotting

Microsomal protein (12.5 μ g) from *T. bernacchii* gill tissue was denatured in loading buffer (12% SDS, 40% glycerol, 0.2 M Tris-HCl pH 7, 0.0004% bromophenol blue, 178mM 2-mercaptoethanol) at 37°C for 20 min. The sample proteins and prestained protein standards (BioRad) were separated on a 4-15% gradient Tris-HCl SDS-polyacrylamide mini-gel at 200 mV for 45-60 min in 25 mM Tris base, 0.2 M glycine, 0.1% SDS running buffer. The proteins were transferred to nitrocellulose membranes (NitroPure, Osmonics, Westborough MA) using a semi-dry electroblotting apparatus (Sigma) with transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol) at 1.0 mA per cm² for 1 hour. Five antibodies were used to probe the gill proteins; polyclonal Na/K-ATPase α 1, α 2 and α 3 specific antibodies (Upstate Biotechnology, Lake Placid, NY), a monoclonal Na/K-ATPase all α -subunit antibody and a monoclonal β -actin antibody (ICN, Aurora, OH). The Na/K-ATPase all α -subunit antibody is isoform generic and recognizes all isoforms of the α -subunit. It was developed by D.M. Fambrough and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Dept. of Biological Sciences, Ames, IA, 52242. After transferring the proteins, the membranes were blocked for 1 hour at 23°C. The blocking buffer for the polyclonal antibodies was 10% non-fat dry milk and 0.1% goat serum in Tris-buffered saline with Tween (TBS-T; 135 mM NaCl, 20 mM Tris, pH 7.6, and 0.1% Tween 20) and for the monoclonal antibodies 5% non-fat dry milk in TBS-T was used. Subsequently, the membranes were incubated at 4°C overnight in their primary antibody diluted with blocking buffer. Primary antibodies were used at the following dilutions: α 1 at 1:2000, α 2 at 1:1000, α 3

at 1:2000, α generic at 1:2000 and the β -actin at 1:20000. After the primary antibody incubation, all membranes were washed with TBS-T 3 times for 5 min, and 1 time for 15 min. The membranes were then incubated with the secondary antibodies diluted with the appropriate blocking buffer (1:5000) for 1 hour at 23°C. The polyclonal Na/K-ATPase α isoform antibodies used a goat anti-rabbit IgG-HRP linked secondary antibody (1/5000). The monoclonal α generic antibody and the β -actin antibody used a rabbit anti-mouse IgG-HRP linked secondary antibody (1/5000). After the secondary antibody incubation, all membranes were washed with TBS-T three times for 5 min, and one time for 15 min. The bands were visualized via enhanced chemiluminescence with Lumi-Glo reagent (Cell Signaling Technology, Beverly MA) and exposure to Kodak BioMax film.

The results were quantified by measuring band intensity using the densitometry program Molecular Analyst (BioRad). The background was measured as the signal in the scanning area directly below the band. Blot to blot variations were normalized by probing both a cold and warm-acclimated *T. bernacchii* gill sample on each blot.

Statistical Analysis

Serum osmolalities were compared using Student's *t* test for unpaired observations. For the real time RT-PCR and immunoblots, C_t and densitometry values are both arbitrary units. Therefore, signals from the cold and warm-acclimated gills were normalized to the mean signal of the cold samples within a given reaction or blot, defined as 1.0. The normalized values of these measurements were used for analysis of the data and statistical significance was determined with Student's *t* test for unpaired observations.

Results

Serum Osmolality

The warm-acclimated Antarctic *T. bernacchii* experienced a significant decrease in serum osmolality as compared to the cold-acclimated *T. bernacchii* after five weeks of acclimation at 4°C compared to -1.5°C (n= 8 fish at each temperature, Fig. 4.1).

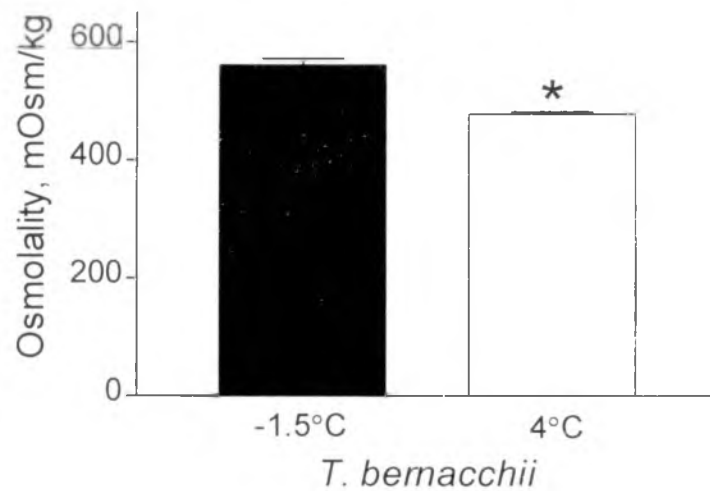
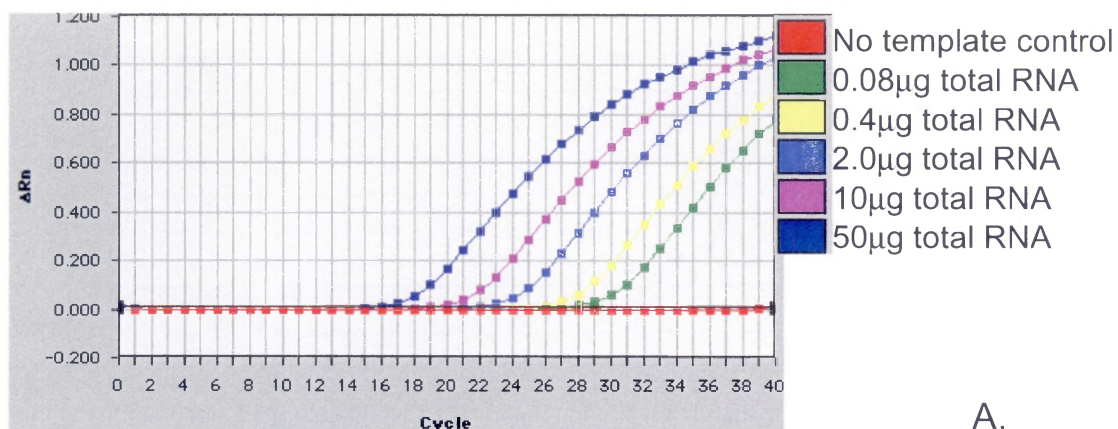


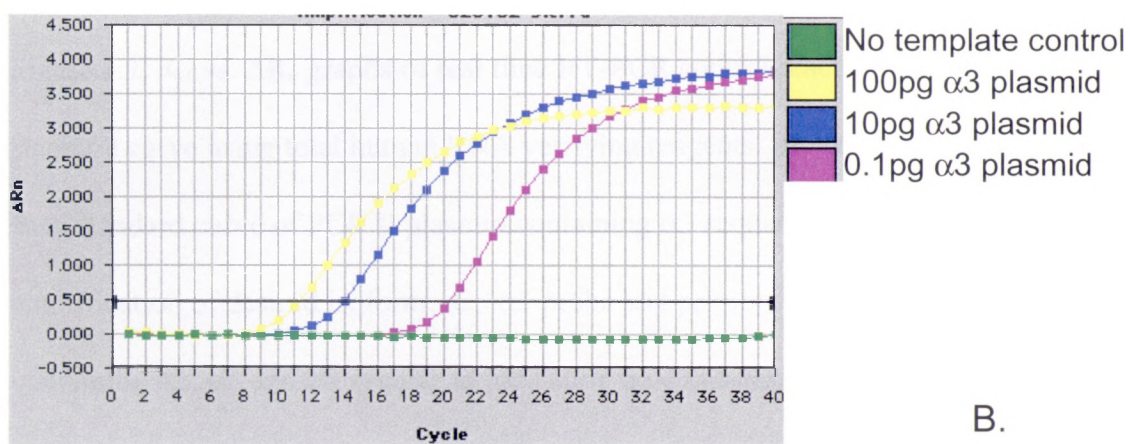
Figure 4.1. Serum osmolality of cold and warm -acclimated *T. bernacchii* after five weeks of acclimation. (* = $p < 0.001$, $n = 8$)

Real Time RT-PCR Controls

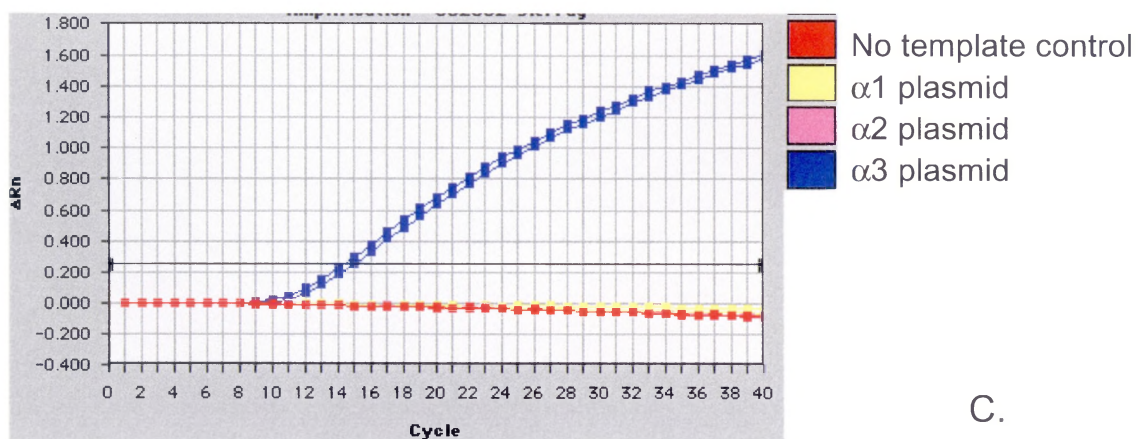
T. bernacchii Na/K-ATPase α isoform probe and primer sets specific to each isoform were determined based upon the results of several control reactions. Figure 4.2 shows the graphic results of representative control reactions. The first three graphs have a linear scale of ΔR_n on the y-axis, indicating each probes reporter signal, and the C_t value on the x-axis, indicating the cycle number at which the reporter signal crossed the threshold. The human GAPDH standard curve was run in conjunction with each set of reactions verifying reagent and thermocycler function (Fig. 4.2A). Plotting the C_t values against concentration yields a line with an R^2 value of 0.99 (Fig. 4.3). The $\alpha 3$ probe and primer set is shown to amplify a standard curve of PCR.II plasmid containing *T. bernacchii* $\alpha 3$ sequence (Fig. 4.2B). The C_t values were plotted against plasmid concentration and analyzed with linear regression yielding an R^2 value of 0.99 (Fig.4.3). The $\alpha 1$ and $\alpha 2$ probe and primer sets amplified plasmids containing the respective sequences in a linearly proportional manner also. The $\alpha 3$ negative control reaction shows that PCR.II plasmid containing $\alpha 1$ and $\alpha 2$ (pink line under red baseline) sequence were not amplified as indicated by no fluorescence detected above baseline (Fig. 4.2C). Plasmid containing $\alpha 3$ sequence however was amplified, as indicated by the blue amplification curve. The probe and primer sets chosen for the $\alpha 1$ and $\alpha 2$ isoforms did not cross-amplify the inappropriate sequences either (data not shown). Using *T. bernacchii* total RNA from the gill in a series of dilutions, the $\alpha 3$ probe and primer amplified product that when analyzed with linear regression generated a r^2 value of 0.99 (Fig. 4.2D and Fig. 4.3).



A.



B.



C.

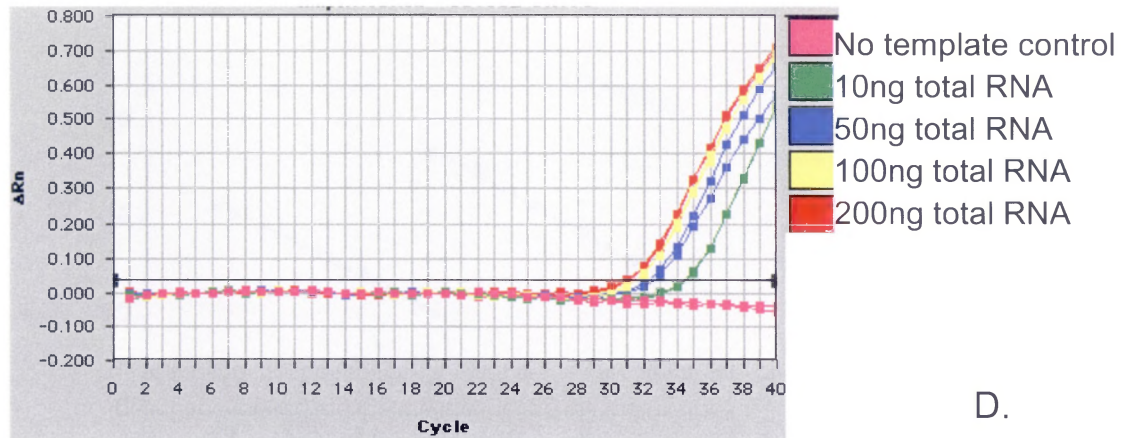


Figure 4.2. C_t vs. ΔR_n graphs of real time RT-PCR control reactions. A. GAPDH standard curve using total human RNA verifying reagents. B. $\alpha 3$ probe and primer set with standard curve of PCR.II plasmid containing $\alpha 3$ sequence, indicating proportional amplification of desired isoform. C. $\alpha 3$ probe and primer set with PCR.II plasmids containing $\alpha 1$, $\alpha 2$ and $\alpha 3$ sequence, indicating no cross-amplification of the inappropriate isoform. D. $\alpha 3$ probe and primer set with standard curve of *T. bernacchii* total gill RNA.

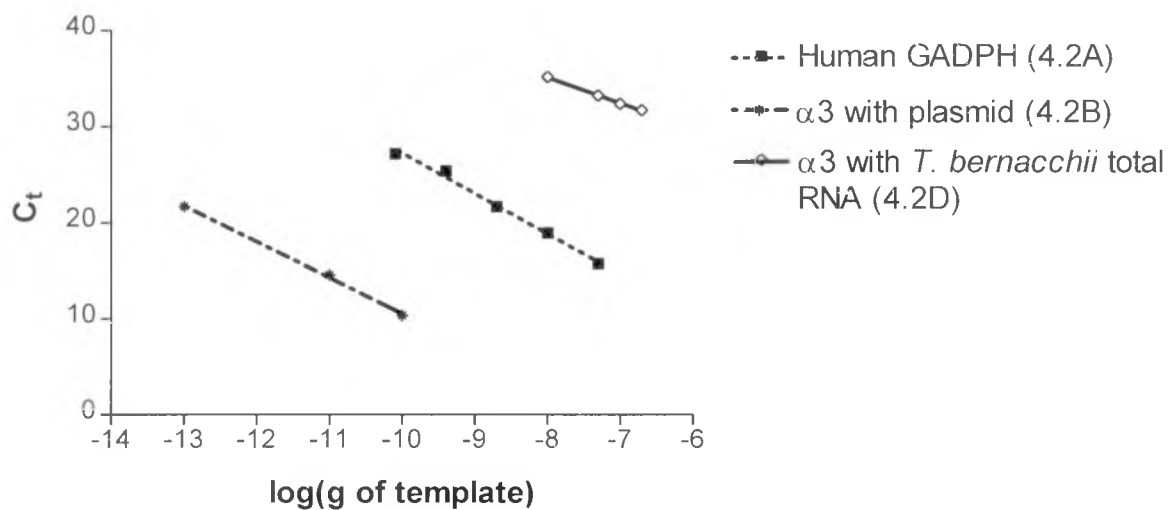


Figure 4.3. Linear regression of real time RT-PCR results shown in figure 4.2. Human GADPH is the line generated from figure 4.2A, $\alpha 3$ with plasmid is the line generated from figure 4.2B and $\alpha 3$ with *T. bernacchii* total RNA is the line generated from figure 4.2D. The r^2 value for all three linear regressions is 0.99.

Real Time RT-PCR Data

The change in mRNA expression of Na/K-ATPase α isoforms with warm acclimation in *T. bernacchii* gill was determined using 3 sets of real time RT-PCR isoform specific probe and primers. Total RNA from both a cold and warm acclimated fish were used with each set of probe and primer in each set of reactions. The C_t value for each isoform from the warm acclimated fish was compared to the corresponding isoform C_t value in the cold acclimated fish to determine if warm acclimation caused a change from the cold acclimated value set as 1.0. There was no change in *T. bernacchii* gill Na/K-ATPase $\alpha 1$, $\alpha 2$ or $\alpha 3$ isoform mRNA expression with warm acclimation as determined by real time RT-PCR (Fig. 4.4).

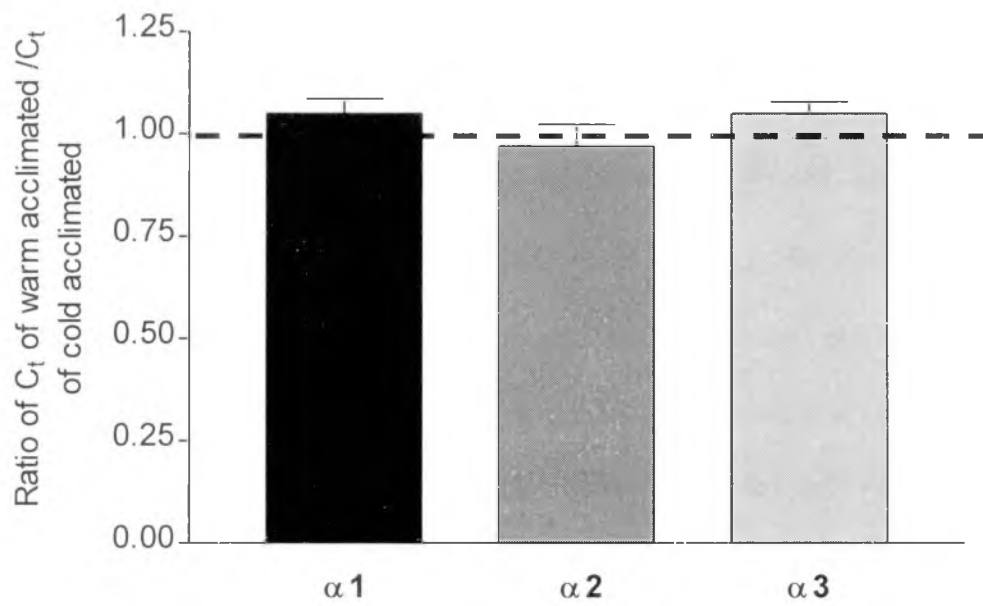


Figure 4.4 Graph of relative changes in Na/K-ATPase α isoform mRNA expression in *T. bernacchii* gill with warm acclimation. There is no significant change in $\alpha 1$, $\alpha 2$ or $\alpha 3$ isoform expression with warm acclimation. $n=6$ pairs of cold and warm acclimated fish.

Western Blotting

The presence of the Na/K-ATPase α isoforms proteins was determined in gill tissue from cold and warm acclimated Antarctic *T. bernacchii*. Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoform specific antibodies and a Na/K-ATPase α -subunit isoform generic antibody were used. Additionally, the gill tissues were probed with a β -actin antibody originally to be used to compare the response of a “housekeeping” gene on warm acclimation.

Representative Western blots for each antibody with cold and warm acclimated gill tissue is shown in figure 4.5. Each blot included the proteins of one cold and one warm acclimated fish and was performed in triplicate. The results from all of the blots (n=5 fish at each temperature), normalized to each cold-acclimated fish and quantified via densitometry, are summarized in figure 4.6. The results are calculated as the difference from unity (1.0); $\alpha 1 - 1.1 \pm .2$, $\alpha 2 - 1.2 \pm .1$, $\alpha 3 - .45 \pm .01$, all $\alpha - .36 \pm .1$ and β -actin $- 2.8 \pm .4$. Most important is the significant decrease in $\alpha 3$ band density in warm acclimated *T. bernacchii* compared to cold acclimated *T. bernacchii*. This coupled with a significant decrease with the all isoform antibody indicates that the expression of the Na/K-ATPase α -subunits, specifically the $\alpha 3$ isoform, changes with warm acclimation. No significant changes were seen in the $\alpha 1$ or $\alpha 2$ isoform as determined by western blotting. Lastly, β -actin cannot be defined as a “housekeeping gene” in this system because there was a 2.5 fold increase in β -actin that was unexpected and as of now, unexplainable.

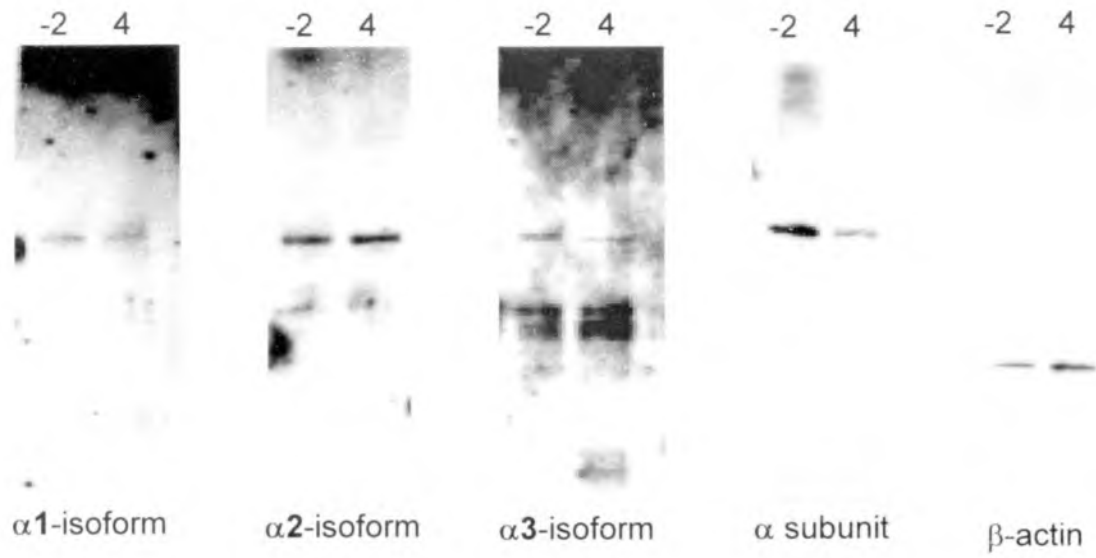


Figure 4.5. Representative immunoblots of cold (-1.5°C) and warm (4°C) acclimated *T. bernacchii* gill tissue.

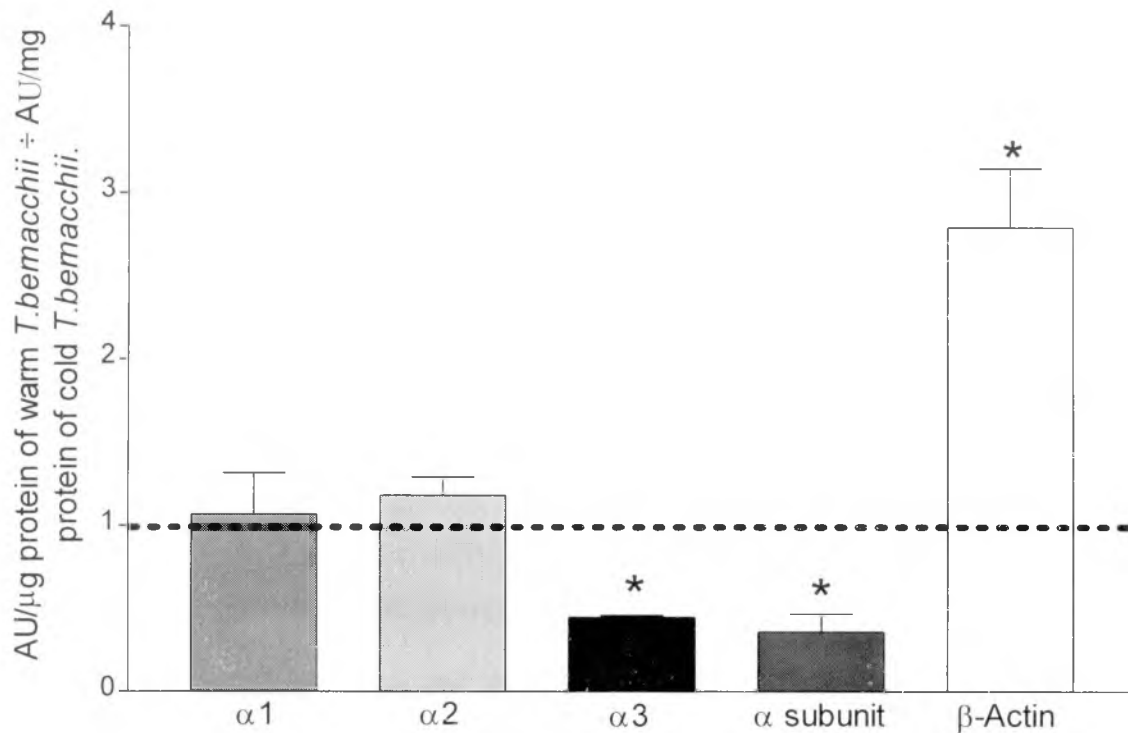


Figure 4.6. Summary of the immunoblotting results probing *T. bernacchii* gill tissue for Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms, all Na/K-ATPase α -subunits and β -actin. The $\alpha 3$ isoform and proteins detected by the generic isoform antibody decrease significantly with warm acclimation ($p^* < 0.05$, $n=5$). A significant increase in β -actin is also seen.

Discussion

Data presented in this study indicate that warm acclimation of *T. bernacchii* decreases the gill Na/K-ATPase $\alpha 3$ isoform protein expression, but does not change mRNA expression of any isoform. The results from the Western blotting of the $\alpha 3$ isoform is supported by a significant decrease as determined by the Na/K-ATPase generic α isoform antibody. The lack of any change in isoform mRNA expression indicates that with warm acclimation of *T. bernacchii*, regulation is occurring at the translational or post-translational level.

The $\alpha 3$ isoform exhibits several kinetic characteristics that are consistent with Antarctic fish physiology. In several studies, the internal Na^+ affinity of HeLa cells, transfected with the $\alpha 3$ isoform only, is significantly higher than that of cells transfected with the $\alpha 1$ or $\alpha 2$ isoform (Jewell and Lingrel, 1991; Munzer *et al.*, 1994; Therien *et al.*, 1996; Zahler *et al.*, 1997). External K^+ affinity was also found to be lower with $\alpha 3$ transfected cells versus $\alpha 1$ and $\alpha 2$ (Zahler *et al.*, 1997). Additionally, cells transfected with only the $\alpha 3$ isoform have the highest intracellular Na^+ concentrations ($[\text{Na}^+]_{\text{IC}}$) compared to cells transfected with only the $\alpha 1$ or $\alpha 2$ isoforms (Crambert *et al.*, 2000; Therien *et al.*, 1996; Zahler *et al.*, 1997). It is hypothesized that the $\alpha 3$ isoform may be activated to deal with an excess of intracellular Na^+ because the low affinity for Na^+ intracellularly gives them a large reserve capacity for activity (Munzer *et al.*, 1994). Lastly, it was found that the expression of the $\alpha 3$ isoform via transfection caused a compensatory decrease in cells with established $\alpha 1$ isoform activity without decreasing $\alpha 1$ isoform numbers (Zahler *et al.*, 1997).

T. bernacchii gill cells in their native -1.5°C environment, have a higher $[\text{Na}^+]_{\text{IC}}$ (65mM) than other vertebrates because they have a significantly higher serum osmolality (Dobbs and DeVries, 1975). A high $[\text{Na}^+]_{\text{IC}}$ environment is best suited for the $\alpha 3$ isoform, with a low intracellular Na^+ affinity. Upon warm acclimation, the serum osmolality decreases, theoretically causing a decrease in the $[\text{Na}^+]_{\text{IC}}$. The new cation concentrations may change the $\alpha 3$ isoform expression. A decrease in the numbers of $\alpha 3$ isoform could allow an increase in the activity of the $\alpha 1$ isoforms already existing within the cell.

In marine teleosts, gill Na/K-ATPase activity is primarily regulated by changes in serum osmolality. Changes in environmental salinity affecting serum osmolality and gill Na/K-ATPase are well documented. Teleosts acclimated to saltwater consistently exhibit acute increases in serum osmolality and chronic increases in gill Na/K-ATPase activity versus freshwater controls (Jensen *et al.*, 1998; Kamiya and Utida, 1969; Zadunaisky *et al.*, 1995). Increases in the mRNA expression and protein levels of the gill Na/K-ATPase during seawater adaptation and smoltification (migration from freshwater to seawater) contribute to the increased gill Na/K-ATPase activity. Seawater adaptation increased the gill Na/K-ATPase activity and the α -subunit mRNA in rainbow trout (Kisen *et al.*, 1994), European eel (Cutler *et al.*, 1995), European sea bass (Jensen *et al.*, 1998), tilapia (Hwang *et al.*, 1998), brown trout (Madsen *et al.*, 1995) and Atlantic salmon (D'Cotta *et al.*, 2000). Increased α -subunit proteins were found in seawater adapted tilapia (Hwang *et al.*, 1998; Lee *et al.*, 1998) and Atlantic salmon (Madsen *et al.*, 1995).

However, in temperate teleost studies the increases in both mRNA and protein levels cannot fully explain the large fold increases in gill Na/K-ATPase activity.

Therefore, other mechanisms besides increased transcription, including but not limited to changes in translation or post-translational modifications or regulatory processes, leading to increased Na/K-ATPase activity are thought to be involved. One of the proposed mechanisms involves specific changes in isoform expression, causing kinetic and regulatory differences that would lead to changes in Na/K-ATPase activity. Lee *et al* (1998) observed a significant increase in the level of $\alpha 1$ isoform proteins in seawater-adapted tilapia compared to the freshwater tilapia, whereas the levels of $\alpha 3$ isoform protein did not change. However, during smoltification of Atlantic salmon and seawater adaptation of freshwater Atlantic salmon smolts the increased gill Na/K-ATPase activity was accompanied by increases in $\alpha 3$ isoform protein specifically (D'Cotta *et al.*, 2000). In tilapia, a 2.6 fold increase in mRNA was accompanied by a 5.4 fold increase in protein, an inconsistency suggested to be compensated with either changes in translation or post-translational kinetics that may be dependent on isoform, cell type, species or physiological conditions (Hwang *et al.*, 1998). Seawater adaptation in temperate teleosts is analogous to the changes that occur with warm-acclimation in the Antarctic teleost. Changes in temperature cause a change in serum osmolality in Antarctic fish necessitating the need for Na/K-ATPase regulation.

Changes in isoform expression or activity mediated by the actions of hormones, neurotransmitters or disease status is well documented (reviewed in Therien and Blostein, 2000). Thyroid hormone differentially regulates the activity of all three isoforms in the heart, skeletal muscle (Horowitz *et al.*, 1990) and post-transcription ally in the brain (Corthesy-Theulaz *et al.*, 1991). Insulin increases the activity of the $\alpha 1$ and $\alpha 2$ while not affecting the $\alpha 3$ (Ewart and Klip, 1995). Corticosteroids from the adrenal gland

preferentially regulate $\alpha 2$ isoform expression in rat aorta (Michea *et al.*, 1998).

Dopamine selectively inhibits the activities of the $\alpha 2$ and $\alpha 3$ isoforms compared to the $\alpha 1$ activity (Nishi *et al.*, 1999). Hypothyroidism caused a marked increase in $\alpha 1$ isoforms in the thyroid gland (LeGrow *et al.*, 1999), where chronic renal failure changes the composition of α isoforms in muscle tissue (Bofill *et al.*, 1994). Additional evidence of isoform specific regulation is the varying effect of protein kinases. PKA stimulates the $\alpha 3$, while decreasing $\alpha 1$ and $\alpha 2$ activity, PKG decreases $\alpha 1$ and $\alpha 3$ with no change in $\alpha 2$ activity (Blanco and Sánchez, 2001) and PKC regulates $\alpha 1$ activity but does not appear to regulate $\alpha 2$ and $\alpha 3$ (Béguin *et al.*, 1996). Increases in Na/K-ATPase activity in the anterior gills of the blue crab (*Callinectes sapidus*) were not accompanied by increases in either mRNA or protein, in comparison to the posterior gill where increases in both mRNA and protein were noted, indicate post-translational regulatory processes causing the increase in Na/K-ATPase activity (Towle *et al.*, 2001). These studies provide evidence for the reorganization of the Na/K-ATPase α -subunit isoform expression or activation as a physiologic method to regulate Na/K-ATPase activity.

A decrease in Na/K-ATPase $\alpha 3$ isoforms in the gills of *T. bernacchii* is an effect of warm acclimation, as is decreased serum osmolality and increased Na/K-ATPase activity. As discussed, several mechanisms including hormones, neurotransmitters and protein kinases may be involved. There are many other aspects of protein regulation that could account for a change in isoform protein expression and activity. One component is changes in the degradation of Na/K-ATPase α -subunits (Pollack *et al.*, 1981) leading to either the $\alpha 3$ mRNA or protein degrading faster in warm-acclimated fish. An example of this is in rat kidney with hypokalemia causing an 82% decrease in $\alpha 2$ protein, but only a

35% decrease in mRNA compared to the $\alpha 3$, implicating decreased stability of $\alpha 2$ mRNA or increased degradation of $\alpha 2$ pumps (McDonough *et al.*, 1992). In another study of hyperthyroid status in rat hearts, the increase in $\alpha 2$ isoform proteins far exceeded the increase in $\alpha 2$ isoform mRNA (Hensley *et al.*, 1992). By analyzing the time course, the data suggested that the turnover times for the $\alpha 1$ versus the $\alpha 2$ isoform mRNA were quite different implying varying rates of mRNA translation or degradation. There is also evidence that glucocorticoids directly regulate Na/K-ATPase α -subunit isoform translation differentially via a modulatory element in the 5' untranslated region of the mRNA (Devarajan and Benz, Jr., 2000). No change in mRNA stability was noted in this study, however the rate of specific isoform translation could be regulated via glucocorticoids in a manner thought to be related to the initiation of the peptide chain, the rate limiting step in translation. However, the relationship between an increase in environmental temperature and modifications of mRNA stability, translation or regulation of activity that leads to increased ion excretion in Antarctic teleost gills is unknown.

CHAPTER FIVE

Conclusions

Summary

These studies were initiated with the simple observation that warm-acclimated Antarctic teleosts experience a decrease in serum osmolality. In marine teleosts, serum osmolality is controlled primarily by the Na/K-ATPase in the gill chloride cells. The subsequent finding of increased gill Na/K-ATPase activity in Antarctic *T. bernacchii* is consistent, as active transport is required to excrete the ions thereby lowering serum osmolality. The goals of the experiments presented here were to further the understanding of the mechanisms by which the Na/K-ATPase is regulated.

Antarctic teleosts provide a unique opportunity to study the ubiquitous Na/K-ATPase in an environment where the same enzyme is present in other vertebrates, is used to facilitate different results. As shown in Chapter II, the ability to osmoregulate due to temperature is not a function of all nototheniids, but is specific to Antarctic nototheniids. The nature of the increased Na/K-ATPase activity was then further examined to determine if a change in enzyme number was responsible for the increase in activity. According to ouabain binding experiments however, there is no significant difference in cold versus warm acclimated Na/K-ATPase numbers. Given that the number of enzymes and the turnover rate of those enzymes define enzyme activity, an increase in Na/K-ATPase activity with no increase in pump number indicates an increase in the Na/K-ATPase turnover rate in the gills of warm acclimated *T. bernacchii*. Additionally, no change in ouabain affinity between cold and warm acclimated gill tissue was found. In other studies, most remarkably in the rat, changes in ouabain affinity have accompanied changes in activity and indicated Na/K-ATPase α -subunit isoform differences.

The presence of Na/K-ATPase α -subunit isoforms with varying kinetic and regulatory properties provides mechanisms for increased adjustment of Na/K-ATPase activity. In Chapter III, the three main Na/K-ATPase α -subunit isoform proteins and mRNA were found in *T. bernacchii* gill and other tissues and the α isoform proteins were found in *N. angustata* tissue, including the gills. The cDNA sequence identity to the previously described $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms in other vertebrates is remarkable given the evolutionary pressure from the harsh Antarctic environment. *T. bernacchii* is the first teleost to be reported to have all three α isoform proteins in the gill.

Differential regulation of the isoforms may allow changes in Na/K-ATPase activity that cannot be measured with ouabain binding studies. In Chapter IV, it is reported that warm-acclimation causes a significant decrease in Na/K-ATPase $\alpha 3$ isoforms while not affecting the $\alpha 1$ and $\alpha 2$ isoform protein expression. The change in protein expression however is not accompanied by any changes in α isoform mRNA expression. This suggests that warm acclimation of *T. bernacchii* regulates gill Na/K-ATPase α -subunit expression at the translational or post-translational level. The regulatory process, from gene to active protein, consists of several steps including transcription, translation and holoenzyme processing. With warm acclimation of *T. bernacchii*, gene transcription to mRNA appears to be unregulated as there is no change in mRNA expression. However, in the translation or post-translational processing, including holoenzyme formation and membrane insertion, the $\alpha 3$ isoform protein expression is being down-regulated with warm acclimation.

The $\alpha 3$ isoform is especially suited to cold *T. bernacchii* physiology because it has a low intracellular affinity for Na^+ and *T. bernacchii* have the highest $[\text{Na}^+]_{\text{IC}}$ of any

marine teleost (65mM) (O'Grady and DeVries, 1982). Upon warm acclimation however, the serum osmolality decreases which should cause a decrease in $[Na^+]_{IC}$, thereby necessitating an isoform with a higher intracellular Na^+ affinity. A theoretical model for the processes occurring in the *T. bernacchii* gill and chloride cell with warm acclimation is shown in figure 5.1.

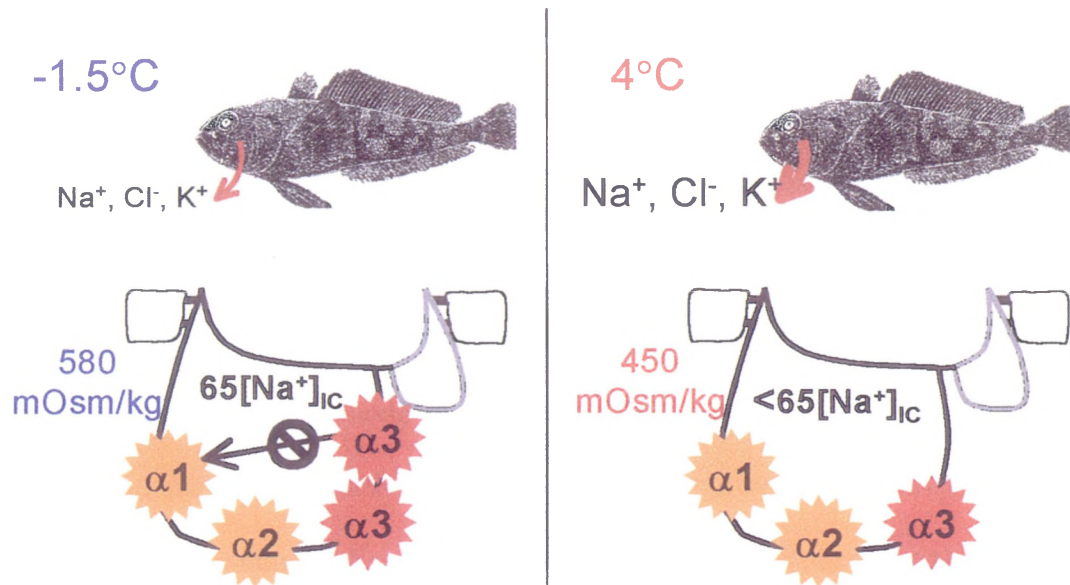


Figure 5.1 Theoretical model of changes in *T. bernacchii* gill Na/K-ATPase isoforms in the chloride cells. On the right, the serum osmolality of *T. bernacchii* is ~ 580 mOsm/kg with an $[\text{Na}^+]_{\text{IC}}$ of 65 mM. The $\alpha 3$ isoforms present in the chloride cell are equipped for activity because of their low intracellular Na^+ affinity. Additionally, the $\alpha 3$ may inhibit the activity of the existing $\alpha 1$ isoforms, similar to a study in transfected cells by Zahler *et al* (1997). With warm acclimation (on the left), the $\alpha 3$ isoform protein in the gill decreases by 50% as indicated by the Western blot results. This decreases the amount of $\alpha 1$ inhibition and with a lower $[\text{Na}^+]_{\text{IC}}$, the conditions are appropriate for an increase in $\alpha 1$ and $\alpha 2$ (both having a higher intracellular Na^+ affinity) activity leading to the increase in Na/K-ATPase activity found with warm acclimation. The question that remains however, is which event, either the decrease in $[\text{Na}^+]_{\text{IC}}$ or the decrease in $\alpha 3$ isoforms, occurs first?

Indications

Antarctic organisms provide distinct tools for comparative studies of basic physiologic processes, due to the added constraints of the harsh Antarctic environment. Examining the activity and molecular biology of the Na/K-ATPase, a ubiquitous protein essential to vertebrate physiology, in an organism with pressures different from most vertebrates can provide information about the proteins regulation that cannot be obtained by looking at organisms that exist under “normal” conditions. The Na/K-ATPase is implicated in many disease states such as cardiovascular disease, diabetes, obesity, fetal abnormalities, neurological diseases, and pulmonary disease (Rose and Valdes, Jr., 1994). The regulation of the Na/K-ATPase in Antarctic teleosts may elucidate regulatory pathways that exist in disease states.

The presence of the Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms in Antarctic *T. bernacchii* may have important implications when examining the physiologic significance of the multiple isoforms. The three isoforms are theorized to have specific functions because the identity of the isoforms is conserved across vertebrate taxa and they have developmental and tissue specific distribution (Blanco and Mercer, 1998; Pressley, 1992). Chromosomal dispersion of the Na/K-ATPase α isoform genes also imply that the isoforms are not functionally redundant, instead have different characteristics used to respond to different physiologic needs (Levenson, 1994). The Na/K-ATPase α -subunit isoforms in *T. bernacchii* support the theory that these particular three isoforms confer some selective advantage (Pressley, 1992) because they have high sequence identities also. Isoform identities are maintained in *T. bernacchii*, even with the substantial morphological and ecological diversification occurring within Antarctic

nototheniids due to the restricted polar environment (Eastman and McCune, 2000). Examining isoform regions of amino acid sequence identity that are highly conserved between species may convey information about function, as this suggests strong selective pressure discouraging changes in the protein structure which could alter isoform function (Pressley, 1992). . Antarctic fish use the same Na/K-ATPase α -subunit isoforms to regulate their serum osmolality at a significantly different level than all other marine teleosts and most vertebrate species. Examining the molecular changes in the Na/K-ATPase α -subunit isoform expression of warm acclimated Antarctic fish may reveal further information about the role and regulation of the isoforms in all species. Also, temperature acclimation provides a tool by which one can alter the molecular aspects of the Na/K-ATPase and thereby examine the roles of the isoforms under different physiologic demands.

Though not the focus of this study, the presence of the Na/K-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms in Antarctic *T. bernacchii* may provide evidence in the study of the isoforms evolution. The conservation of protein structure and similar exon and intron patterning of the isoforms indicate a common ancestor gene, which then duplicated and diverged into the present gene family (Broude *et al.*, 1989; Levenson, 1994). Pressley (1992) stated that the presence of amino acid sequences from Na/K-ATPase α -subunit isoforms in teleosts which “correspond to mammalian and avian isoforms might suggest that all vertebrate isoforms are derived from the same initial gene duplication”. Gene duplication events arise from the unequal crossing over or accidents of replication where a length of DNA is repeated within the gametes genome and then continued in the next generation with two copies of the same sequence. The sequences are then modified by mutation into

different proteins. Gene duplication is one method leading to the introduction of new genes. The high degree of identity among the *T. bernacchii* isoforms to the general α isoform consensus sequences and the presence of $\alpha 1$, $\alpha 2$ and $\alpha 3$ proteins in *N. angustata*, suggests that the three distinct isoforms evolved from a gene duplication event prior to the evolution of bony fish. This supports Serluca *et al* (2001) stating that the three vertebrate isoforms originated by gene duplication prior to the last common ancestor of the jawed vertebrates, or approximately 500 mya and Broude *et al* (1989) dating it to 650 mya. Further large scale gene duplication events are proposed in bony fish which lead to the numerous zebrafish isoforms (Rajarao *et al.*, 2001; Serluca *et al.*, 2001) however evidence of such has not been demonstrated in this study with *T. bernacchii* sequences.

Future Directions

The results presented here lead to several new questions about Antarctic *T. bernacchii* physiology and the molecular biology of the Na/K-ATPase α -subunit isoforms. One of the hardest questions to answer is how to determine the percent each isoform contributes to the total gill Na/K-ATPase activity. This is the only true way to determine what isoforms are responsible for the increase in Na/K-ATPase activity found in warm-acclimated *T. bernacchii*. One group of researchers accomplished this by measuring the percent of phosphoenzyme formed at varying ouabain concentrations in rat axolemma. The data was fit with a non-linear regression allowing for multiple affinity sites thereby determining the individual percentage of isoform activity compared to the total amount of Na/K-ATPase activity (Munzer *et al.*, 1994). However, this approach is difficult especially in species where the isoforms do not have ouabain affinities that greatly differ from another.

To examine the kinetic and regulatory properties of the *T. bernacchii* isoforms more clearly, I would determine the all three full *T. bernacchii* sequences, including untranslated regions, and express them in transfected cells. The full sequences would allow further examination of isoform defining regions as well as different regulatory regions such as phosphorylation sites. This would also allow one to examine the possible transcription factor sites, which would indicate regulation at that translational level. Expression would allow each isoform to be dissected with regard to its cation and ouabain affinities and inherent turnover rates. *Xenopus* oocytes, HeLa cells and yeast have all been used in other Na/K-ATPase α isoform studies to examine ouabain binding properties, turnover rates, Na⁺, K⁺ and ATP activation constants (Crambert *et al.*, 2000; Jewell and Lingrel, 1991; Muller-Ehmsen *et al.*, 2001; Munzer *et al.*, 1994; Therien *et al.*, 1996; Zahler *et al.*, 1997).

In *T. bernacchii*, immunohistochemistry, using isoform specific antibodies to localize the isoforms within the gills and chloride cells and compare the localization of the different isoforms (membrane and cytoplasm) during warm acclimation would indicate what type of reorganization of the isoforms is occurring in the cells. Studies like this have been done in rat motor neurons (Dolapchieva, 1998) and ocular ciliary epithelium (Ghosh *et al.*, 1991) with α isoform specific antibodies. In marine species, a Na/K-ATPase generic α isoform antibody has been used in rainbow trout (Witters *et al.*, 1996) and stingray (Piermarini and Evans, 2001) gill epithelium. Additional studies that could be done in the *T. bernacchii* include determining Na/K-ATPase α isoform mRNA and protein degradation rates as a function of activity. And lastly, there are the β and γ subunits. There is evidence that Na/K-ATPase β -subunit does contribute to the overall

number of holoenzymes that are inserted into the membrane and regulation can occur at the β -subunit production level (McDonough *et al.*, 1992). This indicates that the β -subunit may play a larger role in regulation of the Na/K-ATPase than originally thought. The presence of the γ -subunit in *T. bernacchii* gill has yet to be determined, however it's expression could play a role in the holoenzymes Na^+ affinity during warm acclimation.

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