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Expression and Localization of SLC9A2 and SLC9A3 Sodium Hydrogen Exchangers and Their Possible Role in Acid-Base Regulation in Freshwater Rainbow Trout (Oncorhynchus mykiss)

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Goran Ivanis

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ABSTRACT

Teleost fish, like other vertebrates maintain blood pH at a constant value, with the gills and kidney being major sites of regulation. This thesis examines the involvement of two members of the Na^+/H^+ exchange (NHE) gene family, NHE2 and NHE3 in branchial and renal acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*) Using homology cloning techniques and mining of genomic databases, genes orthologous to NHE2 (trout NHE2) and NHE3 (trout NHE3a and NHE3b) were described and characterized. Applying a variety of techniques including immunocytochemistry, in situ hybridization, Western blotting and real-time PCR, I demonstrated that NHE2 and NHE3 are the predominant gill and kidney isoforms, respectively. In the gill, NHE2 and NHE3 are expressed in a subset of mitochondria rich cells (MRCs) termed PNA^+ (peanut lectin agglutinin) MRCs. NHE2 mRNA levels were significantly increased during ambient hypercapnia, responses that may have been linked to elevated levels of plasma cortisol.

In the kidney, NHE3 appeared to be localized to the apical membrane of proximal tubule cells where it is presumed to play an important role in HCO_3^- reabsorption. In support of its role in HCO_3^- reabsorption, renal NHE3 mRNA expression and protein levels were significantly increased during hypercapnia, responses that may have been cued by elevated plasma cortisol.

RÉSUMÉ

Le poisson Téléostéen, comme d'autres vertébrés maintiennent le sang Ph à une valeur constante, avec les branches et le rein servent comme un d'emplacements principaux du règlement. Cette thèse examine la participation de deux membres de la famille de gène de l'échange : le Na^+/H^+ (NHE), NHE_2 et NHE_3 dans le règlement branchial et rénal d'acido-basique en truite arc-en-ciel d'eau douce (*Oncorhynchus mykiss*) utilisant des techniques de clonage d'homologie, le cherche de les bases de donnes en génomique et de gènes orthologous à NHE_2 (truite NHE_2) et NHE_3 (truite NHE_3a et NHE_3b) ont été décrits et caractérisés. Appliquant une variété de techniques comprenant immunohistochimie, l'hybridation *in situ*, Western blot et le PCR en temps réel, j'ai démontré que NHE_2 et NHE_3 sont les isoforms prédominants de branchie et de rein, respectivement. En branchie, NHE_2 et NHE_3 sont exprimés dans sous-ensemble de cellules riches de mitochondries (MRCs), nommée PNA^+ (positive d'agglutinine de lectin d'arachide) MRCs. Des niveaux du mRNA NHE_2 ont été augmentés signifiant pendant l'hypercapnie ambiante, les réponses qui ont pu liées aux niveaux élevés du cortisol de plasma.

Dans le rein, NHE_3 semble d'être localisé à la membrane apicale des cellules de tubule proximales, où on le présume de jouer un rôle important dans la réabsorption de HCO_3^-. À soutien de son rôle dans la réabsorption de HCO_3^-, l'expression rénale du mRNA NHE_3 et les niveaux de protéine ont été augmentées signifiant pendant l'hypercapnie, les réponses qui peux été positionnées par le cortisol élevé de plasma.

AKNOWLEDGEMENTS

This is perhaps the easiest and hardest chapter that I have to write. It will be simple to name all the people that helped to get this done, but it will be tough to thank them enough.

First of all, I must send thanks to all the members of Perry and Gilmour labs (special thanks to Andrew Esbaugh for the northern blot analysis), without their support this project would have never reached the great result it did. A special gratitude is due to Dr Steve Perry for the quest for improvement and knowledge. The discussions, encouragement and critiques made by him were of essence to the progress of this work. I will remember one comment to the rest of my life, "You know Goran, this is very **encouraging**, but…". I have also to thank Dr. Perry for guiding me through the writing of the thesis, and for all the corrections and revisions made to the text that is about to be read. It became a lighter and more concise thesis after his suggested improvements (however not even him could remove all the verboseness I have put in this thesis).

I also must thank my family, they have supported me and helped me in many ways; more then they will ever know.

My final words go to my special girl Saska for putting up with my late hours, numerous hobbies, for paying my bills for the last two years, (I know, I am marrying her) but most of all for staying with me and surviving the ordeal.

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LIST OF ABBREVIATIONS

CA – carbonic anhydrase
FW – freshwater
MRCs – mitochondria rich cells
NBC – sodium bicarbonate co-transporter
NHEs – sodium hydrogen exchangers
NKA – sodium potassium ATPase
PNA – peanut lectin agglutinin
PNA^- MRCs – PNA negative mitochondria rich cells
PNA^+ MRCs – PNA positive mitochondria rich cells
PVC – pavement cells
SW – seawater
V-ATPase – vacuolar type hydrogen ATPase

CHAPTER 1

GENERAL INTRODUCTION

Most vertebrates maintain blood pH at a constant value. In contrast to air-breathing animals that can use respiratory adjustments for acid-base regulation, water-breathing animals rely mainly on metabolic regulation which involves direct transfer of acidic (H^+) and/or basic (HCO_3^-) molecules between the body and the environment (Heisler, 1984; Claiborne, 1998). In fish, acid-base regulation is linked to ion uptake through a specific set of ion transport proteins located in the gill. Generally, it is believed that the uptake of Na^+ is linked to H^+ excretion and the uptake of Cl^- is linked to HCO_3^- excretion (Claiborne et al., 2002b; Perry et al., 2003a; Perry et al., 2003c). The mechanisms underlying the changes in net acid excretion during acid-base disturbances are not fully understood and might differ among the species. One common acid-base disturbance is exposure of fish to hypercapnia (high water CO_2) resulting in hypercapnia induced respiratory acidosis. During hypercapnia, fish are unable to effectively get rid off CO_2 whish accumulates in the blood; eventually resulting in reduction of bood pH value (Hirata et al., 2003; Edwards et al., 2005).

The SLC9 gene family of Na^+/H^+ exchangers (NHE gene family) has been implicated in piscine acid-base regulation (Claiborne et al., 1999; Edwards et al., 2001; 2002; Choe et al., 2002; 2005; 2007; Hirata et al., 2003; Scott et al., 2005; Edwards et al., 2005; Catches et al., 2006; Tresguerres et al., 2005; 2006). The Na^+/H^+ exchangers are plasma membrane transport proteins; in mammalian cells they facilitate electroneutral trans-membrane exchange of 1 Na^+ for 1 H^+ according to concentration gradients. In eukaryotic cells, the plasma membrane Na^+/H^+ exchangers have multiple functions including pH homeostasis, volume regulation, cell proliferation, and transcellular Na^+ absorption. To date, nine Na^+/H^+ isoforms have been identified in mammals (NHE1-9;

Brett et al., 2005). NHE1-5 are localized to the plasma membrane while NHE6-9 typically exist as intracellular transmembrane proteins (Ritter et al., 2001, Brett et al., 2005). This thesis focuses on the NHE2 and NHE3 genes, apical isoforms that have already been characterized to some extent in fish but never in rainbow trout.

In mammals, NHE2 is expressed primarily in skeletal muscle, kidney and gastrointestinal tract, while NHE3 is highly expressed in the kidney, intestine and stomach (Yun et al., 1995; Malakooti et al., 1999). In the mammalian kidney, NHE3 is localized to the apical membrane of the proximal convoluted tubule where it is involved in Na^+ and HCO_3^- reabsorption (Biemesderfer et al., 1993, 1997; Amemiya et al., 1995). NHE3 null mice have decreased renal HCO_3^- and fluid absorption, systemic acidosis, hypotension, and elevated plasma aldosterone levels (Ledoussa et al., 2001; Woo et al., 2003). Although NHE2 is found in mammalian cortical thick ascending limb and distal convoluted tubule (Chambrey et al., 2001), its role in the kidney is not clear because NHE2 null mice do not exhibit any phenotypes that would suggest impaired renal function (Ledoussal et al., 2001). It was documented previously that cortisol induced transcriptional regulation of Na^+/H^+ exchange activity in the mammalian proximal nephron (Baum and Quigley, 1993). Therefore, it is possible that under acid/base disturbances, cortisol (a major stress hormone in fish) induces transcriptional regulation of NHEs in the gill and the kidney of rainbow trout (Hirata et al., 2003; Edwards et al., 2005).

Distribution and function of branchial NHE

The gill epithelium consists of different specialized cell types including pavement cells (PVC) for gas exchange and mitochondria rich cells (MRC) that are believed to be the predominant sites of acid-base and ion regulation. Earlier models for ion uptake and acid-base balance in the gill included single trans-membrane exchanger proteins located in the MRCs that passively exchange H^+/NH_4^+ for Na^+ at the apical membrane (Krogh, (1938). This continues to be the favoured model for Na^+ uptake and acid base balance at the gills of seawater (SW) fish (reviewed by Claiborne et al., 2002). In both SW and freshwater (FW), the MRC is thought to express an electroneutral apical anion exchange protein that excretes HCO_3^- in exchange for Cl^- (Claiborne, 2002). Because the chemical gradient in FW does not favour Na^+ uptake (nor can it be overcome by a potentially small favourable H^+ gradient), the feasibility of electroneutral Na^+/H^+ exchange as a viable mechanism for Na^+ uptake in FW fish has been challenged (Avella and Bornancin, 1989). Thus, current models (Fig. 1-1) for branchial Na^+ uptake and H^+ excretion often incorporate an apical membrane Na^+ channel that is in close proximity to a vacuolar-type H^+-ATPase (V-ATPase) that uses ATP to secrete H^+ into the water (see reviews by Goss et al., 1992; Evans et al., 1999; Marshall, 2002; Evans, 2002; Claiborne et al., 2002b; Hirose et al., 2003; Perry et al., 2003c; Evans et al., 2005a). H^+ secretion in the vicinity of the Na^+ channel establishes an electrochemical potential that drives the uptake of Na^+ through Na^+ channels and into the cell.

Interestingly, the mechanism of ion uptake and acid-base regulation in trout may be more complex than previously thought because relatively recent studies provided evidence for the existence of specialized MRCs that are further subdivided based on the capacity of the apical membrane to bind peanut lectin agglutinin (PNA). Thus, the cells

have been termed PNA^- MRCs (previous literature referred to these cells as mitochondria rich pavement cells) and PNA^+ MRCs (often referred to as chloride cells in earlier literature) (Goss et al., 2001; Galvez et al., 2002). Theses lectins were originally used to separate acid- and base-secreting intercalated cells of the mammalian kidney collecting duct; the α- and β-intercalated cells, respectively (Reid et al., 2003).

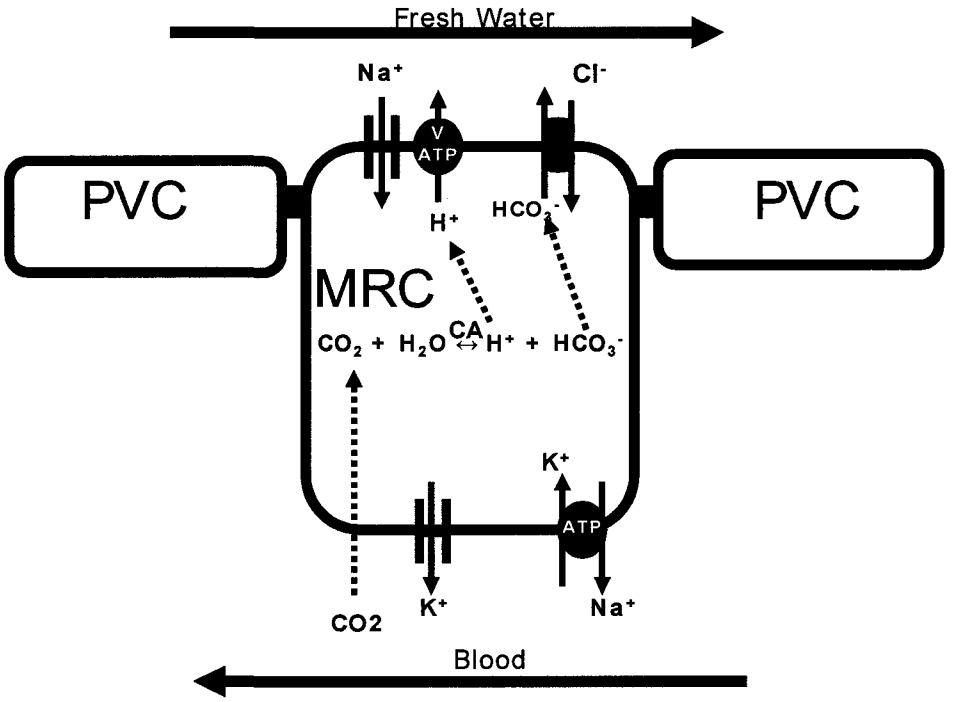


Figure 1-1: A model for acid-base balance and ion uptake in the freshwater rainbow trout gill showing the proposed role of V-type H-ATPase and Na^+ channel. Cytoplasmic carbonic anhydrase (CA) in the mitochondria rich cells (MRC) catalyses the hydration of intracellular CO_2 generating HCO_3^- for Cl^-/HCO_3^- exchange and H^+ for pumping across the apical membrane to energize the conductance of Na^+ through a Na^+ channel. PVC refers to pavement cells; modified from Perry (2003a).

Distribution and function of renal NHE

Although the gill is the major site of net acid-base excretion in fish, the kidney also plays an essential role in pH regulation by modulating HCO_3^- reabsorption (Wood et al., 1999). In mammals, the reabsorption of HCO_3^- at the proximal tubule involves movement of H^+ ions across the apical membrane and into the lumen therefore acidifying the filtrate. This is accomplished through H^+ pumping by the V-ATPase and Na^+/H^+ exchange via NHE3 (Nakhoul and Hamm, 2002; Mello-Aires and Malnic, 2002). Filtrate H^+ reacts with HCO_3^- in the presence of apical membrane bound carbonic anhydrase isoform IV (CA IV) to form CO_2 (Schwartz, 2002). This newly formed CO_2 then enters the tubule cell where it is converted to H^+ and HCO_3^-. The HCO_3^- is then transported at the basolateral membrane into the extracellular fluid by NBC1 (Na^+-HCO_3^- co-transporter isoform 1) and remaining H^+ that was formed is recycled via V-ATPase and NHE3 to further acidify the filtrate and promote HCO_3^- reabsorption (Soleimani, 2002). Most of the components in this model of HCO_3^- reabsorption have been identified in rainbow trout (Fig. 1-2). Trout NBC1 (Perry et al., 2003b), V-ATPase (Perry and Fryer, 1997; Perry et al., 2000), cytoplasmic CA isoform (tCAc; (Esbaugh et al., 2005) as well as CA IV (tCA IV; Georgalis et al., 2005) have been cloned and sequenced. The only major protein involved in mammalian renal HCO_3^- reabsorption that has not yet been identified in rainbow trout kidney is NHE3.

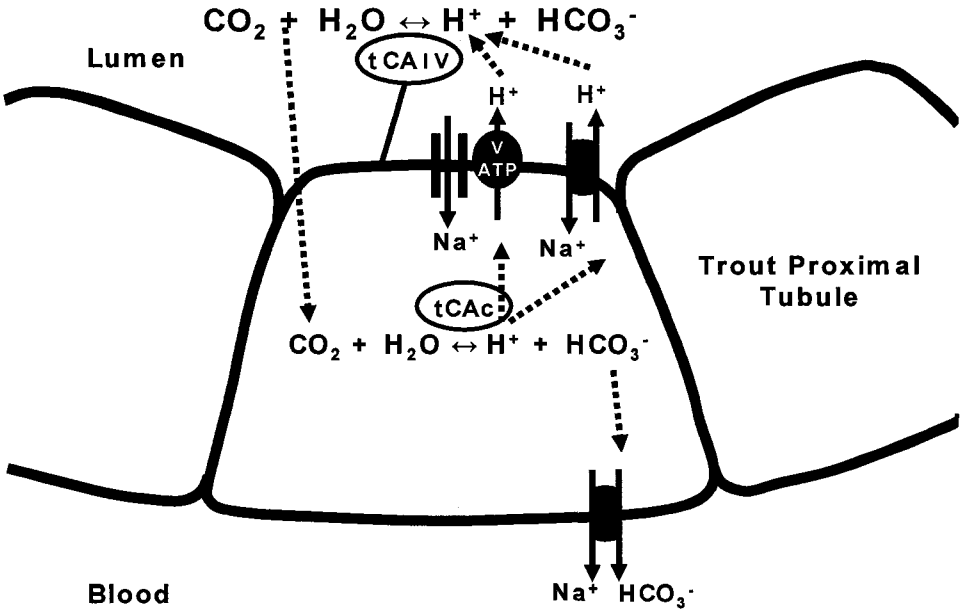


Figure 1-2. A model of a rainbow trout proximal tubular cell showing acid-base reactions occurring in the lumen and in cytoplasm. HCO_3^- reabsorption is accomplished by acidification of the filtrate by V-ATPase or Na^+/H^+ exchange. Filtered HCO_3^- is dehydrated catalytically in the presence of apically oriented tCAIV. The resultant CO_2 diffuses into the cytosol where it is hydrated to H^+ and HCO_3^- in the presence of tCAc. The HCO_3^- is absorbed into the blood by a basolateral Na^+/HCO_3^- co-transporter and additional H^+ enters the filtrate. In addition to V-ATPase, an apical NHE could be involved in acidification of filtrate; modified from Perry et al. (2003a).

Goals and hypothesis

There is a growing body of evidence supporting the involvement of NHE in acid base regulation in fish (Hirata et al., 2003). However, no studies have examined the role of NHE in rainbow trout, the one fish species for which acid-base regulating strategies

have been most fully described. Therefore the objective of this thesis is to increase our knowledge and understanding of the distribution and function of different NHE isoforms in rainbow trout by using molecular and physiological tools. Chapter 2 will examine the hypothesis that apical NHE2 and NHE3 are present in trout gills where they play a role in branchial net acid excretion. The approach will be to clone rainbow trout NHE2 and NHE3, and to localize them to the apical membrane of PNA^- (presumptive acid secreting) MRCs within the gills. In addition I will compare branchial NHE2 and NHE3 expression in trout maintained under control or hypercapnic conditions. Finally, Chapter 2 will examine the role of cortisol mobilisation in regulating NHE2 and NHE3 expression in the gills. In Chapter 3, I will test the hypothesis that apical NHE is present in kidney where it is involved in acidification of filtrate and systemic acid-base balance. Specifically, I will attempt to identify the predominant NHE isoforms in the kidney and to localize the mRNA and/or protein to proximal renal tubules. In addition it will be determined if mRNA and/or protein within the kidney increase with the exposure to hypercapnic conditions and if cortisol mobilisation might have a regulatory role in the renal expression of NHE.

CHAPTER 2

GILL ACID-BASE REGULATION

INTRODUCTION

Acid-base balance in teleost fish relies, in part, on the dynamic adjustment of acidic and basic equivalent fluxes across the gill (Goss et al., 1998; Claiborne, 1998; Claiborne et al., 2002; Perry et al., 2003a; Evans et al., 2005; Marshall and Grosell, 2006; Perry and Gilmour, 2006). Acid-base balance and ionic regulation are intricately related because of obligatory coupling between Na^+ uptake and acid excretion and Cl^- uptake and base excretion (reviewed by Evans et al., 2005; Marshall and Grosell, 2006; Perry and Gilmour, 2006). However, the specific mechanisms linking NaCl uptake to net branchial acid flux are not yet fully understood. Although Cl^- uptake and bicarbonate (HCO_3^-) efflux are tightly coupled, the specific gene product enabling Cl^-/HCO_3^- exchange and its cellular location have not been identified with certainty. Members of two gene families have been implicated as candidates for the Cl^-/HCO_3^- exchanger of the gill, namely paralogs within the SLC4 (Alper, 2006; Romero et al 2004) and SLC26 (Mount and Romero, 2004) HCO_3^- transporter gene families (Alper et al., 2002). In trout, the putative branchial Cl^-/HCO_3^- exchanger is thought to reside in a sub-type of mitochondria rich cell (MRC), termed the peanut lectin agglutinin positive MRC (PNA^+ MRC; Goss et al 2001; Galvez et al., 2002). It has been suggested (e.g. Tresguerres et al., 2006; Perry and Gilmour, 2006) that this cell type, containing an apical Cl^-/HCO_3^- exchanger, is functionally analogous to the base-secreting β-type intercalated cell of the mammalian collecting duct.

It is generally accepted that the linkage between branchial Na^+ uptake and acid excretion reflects either electroneutral Na^+/H^+ exchange (Maetz, 1973) and/or active H^+ extrusion via a V-type H^+-ATPase (V-ATPase) pump electrically coupled to apical

membrane Na^+ channels (Avella and Bornancin (1989). While there is empirical evidence in support of the V-ATPase – Na^+ channel model (Fenwick et al, 1999; Wilson et al; 2000; Reid et al 2003; Parks et al, 2007), the apparent absence of epithelial Na^+ channel (ENaC) genes in fish genomes suggests the participation of another conductive cation pathway that has yet to be identified. Based on thermodynamic constraints (Avella and Bornancin, 1989), it was thought that electroneutral Na^+/H^+ exchange, while likely in seawater (SW) fish would be unlikely to contribute to Na^+ uptake in freshwater (FW) species (Perry, 1997; Marshall, 2002). However, the results of more recent studies have clearly demonstrated that Na^+/H^+ exchangers (NHE) are expressed in the gills of both SW- and FW-acclimated fish thus indirectly suggesting their involvement in Na^+ uptake in both media (Claiborne et al., 1999; Edwards et al., 2001; 2002; Choe et al., 2002; 2005; 2007; Hirata et al., 2003; Scott et al., 2005; Edwards et al., 2005; Catches et al., 2006; Tresguerres et al., 2005; 2006). Specifically, two paralogs within the SLC9 (NHE) gene family (reviewed by Orlowski and Grinstein, 2004) have been implicated as likely candidates for apical Na^+/H^+ exchange, SLC9A2 (NHE2) and SLC9A3 (NHE3). The localization of NHEs to gill epithelial cells enriched with Na^+/K^+-ATPase (Edwards et al., 2002; Hirata et al., 2003; Tresguerres et al., 2006; Catches et al., 2006; Choe et al., 2005; 2007), suggests that the MRC is likely the principal site of Na^+/H^+ exchange. By analogy with the α-intercalated cells of the mammalian collecting duct and in consideration of the previous observations by Goss and co-workers (Galvez et al., 2002; Reid et al 2003; Parks et al 2007), it was recently proposed that in rainbow trout, the PNA^- MRC is the site of Na^+ uptake either by Na^+/H^+ exchange or conductance through Na^+ channels (Perry et al 2003b; Perry and Gilmour, 2006). Thus, the present study was

undertaken to test the hypothesis that NHEs are specifically expressed in the PNA^- MRC of the rainbow trout gill epithelium and that their expression is transcriptionally increased during respiratory acidosis, consistent with their potential involvement in Na^+ uptake and acid-base regulation. These ideas were tested by cloning rainbow trout NHE2 and NHE3 to provide tools to examine RNA and protein distribution and expression levels using in situ hybridization, immunocytochemistry, real time reverse transcriptase PCR and Western blotting.

MATERIALS AND METHODS

Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish were maintained on a 12h light and 12h dark photoperiod in circular fibreglass water tanks with flowing, aerated and dechloraminated City of Ottawa tapwater at 13° C. Animals were fed daily with a commercial trout diet and were acclimated for at least 2 weeks before any experiments were performed. Fish were not fed within 48 h of experimentation.

RNA and protein extraction

The fish were euthanized by a blow to the head and tissues were dissected, flash frozen in liquid N_2 and stored at -80° C until processing. Tissues were processed by grinding on dry ice using a mortar and pestle and then stored at -80° C until needed. Total RNA was extracted from 100 mg aliquots of processed frozen tissue samples using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA pellet was resuspended in 40 µl of nuclease-free H_2O and any remaining genomic DNA was removed using RNase-free DNase (8 units/RNA sample) for 20 min at room temperature. The RNA quality and concentration were assessed by gel electrophoresis and spectrophotometry (Eppendorf Biophotometer), respectively.

Gill and kidney proteins were extracted using 1X RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) with protease inhibitors (complete Mini protease inhibitor cocktail tablets; Roche). The tissues were

first ground under liquid N_2 with a pre-cooled mortar and pestle and then incubated on ice for 15 min. Samples were then sonicated two times for 1 sec at 50% full power and centrifuged at 10,000 rpm for 10 min at 4° C. The supernatant containing soluble protein was transferred to micro centrifuge tubes. Protein concentration was determined using a BioRad protein assay kit (Bio-Rad Laboratories) using BSA as a standard so that all of the protein samples could be diluted to a concentration of 20 $\mu g\ \mu l^{-1}$. The proteins were frozen and stored at -80° C until needed.

Molecular cloning of trout NHE2 and NHE3

Fish were euthanized by a blow to the head and gill and kidney tissues were dissected and immediately frozen in liquid N_2 before storage at -80° C. Total RNA was extracted from kidney or gill and assessed for quality and quantity as described above. Total RNA (gill RNA for NHE2 and kidney RNA for NHE3) was reverse transcribed using random hexamer primers and Stratascript reverse transcriptase (Stratagene). Degenerate primers for NHE2 and NHE3 (Table 2-1) were designed based on known fish and tetrapod sequences. PCR products for NHE2 and NHE3 were amplified using the following parameters: 4 min at 94° C followed by 40 cycles of 30 sec at 92° C, 30 sec at 58° C, and 1 min at 72° C. The final extension of the amplified products was 10 min at 72° C. The final composition of the PCR mix (25 µl) was: 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2 pmol each of forward and reverse primers, 2.5 units of Taq polymerase (Invitrogen) and 100 ng of cDNA. A PCR cloning kit (TA cloning kit; Invitrogen) was used to ligate the PCR products into a pCR II vector and subsequent transformation into competent DH5α *E. coli* cells. The desired clone was extracted using

a PureLink Quick Plasmid Miniprep Kit (Invitrogen). Purified plasmids were sequenced using M13 forward and reverse primers (Table 2-1). A search of GenBank protein databases using BLASTX revealed that the cloned NHE2 cDNA (~680 bp) exhibited highest amino acid identity with known fish NHE2 sequences. A BLASTX search also revealed that cloned NHE3 cDNA (1100 bp) sequence shared highest amino acid identity with known fish NHE3 sequences. Based on these sequences, primers were designed (Table 2-1) to be used for 3' and 5' RACE (rapid amplification of cDNA ends). For 5' and 3' RACE, total gill (for NHE2 RACE) and kidney (for NHE3 RACE) RNA was isolated using TRIzol reagent (Invitrogen). The cDNA was synthesized according to the protocol supplied in the FirstChoice RLM-RACE kit (Ambion). PCR was performed on the cDNA using gene specific primers (Table 2-1) and primers supplied in the RACE kit with the following parameters: 4 min at 94° C followed by 45 cycles of 30 sec at 92° C, 30 sec at 62° C, and 1 min 30 sec at 72° C. The final extension of the amplified products was at 72° C for 10 min. The PCR products were then cloned and sequenced as described above.

Sequence and phylogenetic analysis

Known NHE1, NHE2 and NHE3 amino acid sequences were used together with the complete sequence for NHE2 and the complete 752 AA sequence for NHE3 (NHE3a). Phylogenetic analysis was performed using the neighbour-joining method (TREE-PUZZLE v.5.2) with 1000 pseudo-replicates. An additional NHE3 gene (termed NHE3b) was identified by mining the Genomics Research on Atlantic Salmon Project (GRASP) rainbow trout data base. Consensus sequence data from three clusters

(2843046, 2850627 and 2869330) were assembled into a 862 AA sequence using contig assembly software (DNAMAN; Lynnon Biosoft v. 5.2.9). An examination of the NHE3b sequence revealed that a short segment between AAs 407 and 436 was missing from the GRASP database. Thus, to prevent bias in the phylogenetic analysis, the corresponding sequence from NHE3a (407 AA-WILNKYRLVPLEFIDQVVMSYGGLRGAVA-436 AA) was substituted. The NHE3b sequence was only used in the phylogenetic analysis; all the other experiments incorporated the NHE3a sequence, referred to as NHE3. GenBank accession numbers for the remainder of the sequences used in the phylogenetic analysis are as follows:

NHE1. *Oncorhynchus mykiss* (β NHE) Q01345, *Pseudopleuronectes americanus* AAO32340, *Amphiuma tridactylum* AAD33928, *Takifugu obscurus* BAE75800.1, *Rattus norvegicus* NP_036784.1, *Anguilla anguilla* CAB45085, *Cyprinus carpio* CAB45232, *Homo sapiens* NP_003038, *Drosophila melanogaster* AAF51559, *Gallus gallus* ABB82239.

NHE2. *Canis familiaris* XP_531775.2, *Gallus gallus* XP_416918.2, *Mus musculus* NP_001028461 XP_129721, *Oryctolagus cuniculus* P50482, 708 AA *Danio rerio* XP_001336127.1, 795AA *Danio rerio* XP_691364, *Homo sapiens* NP_003039, *Myoxocephalus octodecemspinosus* AAD46576, *Rattus norvegicus* P48763, partial 692 AA *Oncorhynchus mykiss* ABO32814, *Squals acanthias* ABC54565 *Tetraodon nigroviridis* CAG05798.

NHE3. 704 AA *Danio rerio* CAM47009.1, 829 AA *Danio rerio* XP_696670, 851 AA *Danio rerio* XP_696728, *Gallus gallus* XP_418895.2, *Mus musculus* NP_001074529.1, *Dasyatis sabina* AAT45738.2, *Homo sapiens* NP_004165.1, *Rattus norvegicus*

NP_036786.1, NHE3a; 752 AA *Oncorhynchus mykiss* ABO32815.1 and NHE3b; 862 AA *Oncorhynchus mykiss* assembled sequence, *Oreochromis mossambicus* BAF80347, *Tribolodon hakonensis* AB055466.

Northern blot analysis

10 μg of total RNA was extracted using Trizol (see above) and was given to Dr. Andrew Esbaugh who then completer the rest of the Northern blot protocol. The RNA was then fractionated by glyoxal/dimethyl sulphoxide (DMSO) denaturing electrophoresis on a 1% agarose gel and transferred to a Duralon nylon membrane (Stratagene, Missaussauga, ON, Canada) using 20X standard saline citrate (SSC). Membranes were ultraviolet-crosslinked (Fisher UV crosslinker) twice prior to hybridization.

Probe for NHE3 was generated from first strand cDNA from rainbow trout mRNA. A ~900 base pair probe was amplified and cloned using NHE3-F and NHE3-R primers (Table 2-1). The probe was then enzymaticaly cut from extracted plasmids with BamH I (Invitrogen) and Xho I (Invitrogen) for 2 h at 37° C. Probe was labelled using [-^{32}P]dCTP (specific activity 109 CPM μg^{-1} DNA) and the Ready-To-Go labelling system (Pharmacia, Piscataway, NJ, USA). Membranes were pre-hybridized at 60° C for 3 h in Church's buffer. Blots were then hybridized overnight in the same solution at 60° C, with approximately 109 CPM of denatured probe. The blots were then washed twice using 1X SSC/0.1% SDS solution (20 min, 60 °C) and once using 0.25X SSC/0.1% SDS (20 min, 60° C). Finally, blots were exposed to a phosphor screen (Kodak, Rochester, NY, USA)

and visualized and quantified using a phosphorimager (Molecular Devices, Sunnyvale, CA, USA) controlled by ImageQuant software.

Quantification of mRNA levels using RT-PCR

cDNA was synthesized from 1 μg of total RNA using random hexamer primers (Boehringer Mannheim) and RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas). Rainbow trout mRNA levels were measured by real-time PCR on samples of cDNA (1 μl) using a Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) and a Stratagene MX-4000 multiplex quantitative PCR system. ROX (Stratagene) was used as reference dye. The PCR conditions (final reaction volume= 12.5 μl) were as follows: cDNA template = 0.5 μl; forward and reverse primer = 300 nmol l^{-1}; 2X Master Mix = 12.5 μl; ROX = 1:30000 final dilution. The annealing and extension temperatures over 40 cycles were 56° C (30 sec) and 72° C (30 sec), respectively. All the primers used for real-time PCR (including the reference gene β-actin) were designed using web-based software (primer3; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Table 2-1). The specificity of the primers was verified by cloning and sequencing of the amplified products. To ensure that residual genomic DNA was not being amplified, control experiments were performed in which reverse transcriptase was omitted during cDNA synthesis. Relative expression of mRNA levels was determined (using β-actin as an endogenous standard) by a modification of the delta-delta Ct method (Pfaffll, 2001). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

Collection of tissues for immunocytochemistry and in situ hybridization

Adult rainbow trout was euthanized by a blow to the head and the gills were dissected and rinsed with 1X PBS to remove mucous. The gills were placed in 4% paraformaldehyde (PFA) overnight at 4° C. Samples were then placed in 15% sucrose for two h at room temperature followed by 30% sucrose at 4° C until sectioning. The gills were embedded in OCT cryosectioning medium (VWR), incubated for 20 min and sectioned horizontally (10 μm section) using a cryostat (Leica CM 1850). Tissue sections were placed on Superfrost Plus slides (VWR) and dried at room temperature for approximately 45 min prior to storage at -4 ° C until needed.

PNA labelling and Na^+/K^+-ATPase immunocytochemistry

Gill tissues were fixed in 4% PFA, cryoprotected in sucrose and sectioned as described previously. Sections were incubated for 2 h at room temperature with primary antibodies: α5 (1:100), a mouse monoclonal antibody against the α1 sub-unit of chicken Na^+/K^+-ATPase (University of Iowa Hybridoma Bank) and 25 mg ml^{-1} peanut lectin agglutinin (PNA) conjugated to biotin. The α5 antibody and biotinylated PNA have been used successfully for immunocytochemistry in rainbow trout (Wilson et al et al., 2000; Galvez et al. 2002). For negative controls, sections were incubated with 1X PBS buffer lacking primary antibodies or biotinylated PNA. Immunofluorescence was detected after incubating the sections with a 1:400 dilution of Alexa Fluor- 546 coupled to goat anti-mouse IgG and PNA was detected after incubating sections with Streptavidin conjugated to Alexa Fluor-488 (Fisher, Ottawa, ON, Canada). After washing (3 x 10 min in 0.1X

PBS), sections were mounted in Vectashield mounting medium (Vector Labs) and cover slipped.

PNA labelling and NHE3 immunocytochemistry

Gill tissues were fixed, cryoprotected in sucrose and sectioned as described previously. Custom affinity purified rabbit polyclonal antibodies (Abgent, San Diego, CA) raised against trout NHE2 and trout NHE3a were generated using synthetic multi-antigenic (8 chains) peptides. For trout NHE2, the synthetic peptide VPLHEEKKSSGKPKR corresponded to amino acids 440-455 of the rainbow trout NHE2 amino acid sequence (GenBank accession ABO32814). For trout NHE3a, the synthetic peptide ETKADVDFNKKFRAS corresponded to amino acids 578–593 of the rainbow trout NHE3a protein sequence (GenBank accession ABO32815.1). The corresponding region in NHE3b (E[redacted]KADVDFNKKF[redacted]A[redacted]) was 80% identical (12/15 AAs). Thus, it is likely that the antibodies recognize both NHE3a and NHE3b. Sections were incubated for 20 h at 4° C with NHE3 antibody (1:1000) and 25 mg ml^{-1} biotinylated PNA For negative controls, sections were incubated with 1X PBS buffer lacking primary antibodies and PNA. Immunofluorescence was detected after incubating the sections with a 1:400 dilution of Alexa Fluor 546-coupled to goat anti-mouse IgG and Streptavidin conjugated to Alexa Fluor 488 (Fisher, Ottawa, ON, Canada). Following the 3 x 10 min wash in 0.1X PBS sections were mounted in Vectashield mounting medium (Vector Labs) mounting medium (Vector Laboratories) and cover slipped.

Construction of NHE2 in situ hybridization probe

Primers were designed to produce a 942 bp DIG-labelled ribo-probe for rainbow trout NHE2 (Table 2-1). Gill total RNA (5 μg) was reverse transcribed using oligo-dT primer (Sigma Genosys) and Stratascript reverse transcriptase (Stratagene). PCR was performed on the resulting cDNA (0.5 μl in a 25 μl reaction) using appropriate primers. An aliquot of the PCR product was run on a 1.25% agarose gel and the rest was ligated into PCR II vector (Invitrogen). The desired clone was extracted using a PureLink Quick Plasmid Miniprep Kit (Invitrogen). Purified plasmids were sequenced using M13 forward and reverse primers (Table 2-1) to confirm identity and determine the orientation of the cloned sequence within the vector. Antisense DIG labelled RNA probes for NHE2 were synthesized by linearizing 1 μg of plasmid with BamH I (Invitrogen) followed by in vitro transcription with T7 RNA polymerase (New England Biolabs) for 1 h at 37° C. Sense DIG labelled RNA probes were created by linearizing 1 μg of plasmid with Xho I (Invitrogen) followed by in vitro transcription with SP6 RNA polymerase (Invitrogen) for 1 h at 37° C.

PNA labelling and NHE2 in situ hybridization

After desired PNA fluorescence images were taken, the same tissue sections were hydrated in 1X PBST (PBS with 0.1% Tween 20) twice for 15 min each and treated with proteinase K (Gibco BRL, Orand Island, NY) using 20 μg ml^{-1} in PBST for 15 min at room temperature. The slides were rinsed in 1X PBST, twice for 10 min each and then incubated 10 min at 60° C. 100 ng of digoxigenin-labelled RNA probe was denatured (boiled for 3 minutes and then cooled on ice) and added to the hybridization buffer (50% deionized formamide, 5X hybridization salts (0.75 M NaCl, 20 mM EDTA, 20 mM

PIPES, pH 6.8), 1X Denhardt's, 0.2% SDS, 5% dextran sulphate (Sigma)). Hybridization was performed for 20 h at 63° C in a humid chamber in a hybridization oven. The next day, the sections were washed twice in 2X SSC (15 min each at 60° C), twice in 0.2X SSC (15 min each at 60° C) and once in 0.1X SSC 50% PBS for 10 min at room temperature. For DIG detection, the sections were incubated first with 0.1 M PBS containing 1% goat serum, 2 mg ml^{-1} BSA and 0.3% triton-X at room temperature for 1 h. This was followed by incubation in anti-digoxigenin conjugated to alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:1000 in 0.1 M PBS containing 1% goat serum, 2 mg ml^{-1} BSA and 0.3% triton-X overnight at 4°C. The next day, the slides were washed twice in 0.1 M PB (15min each at room temperature). The slides were washed twice (5 min each) in coloration buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20). One nitroblue tetrazolium and 5-bromocresyl-3-indolyl phosphate (NBT/BCIP) tablet (Sigma) was dissolved in 10 ml water and this solution was layered over the sections. The chromogenic reaction was allowed to proceed in the dark until satisfactory coloration was achieved (at room temperature in a humid chamber). The slides were then washed twice with 0.1 M PBS (15 min each). The sections were covered with mounting media (60% glycerol) and cover slipped.

Microscopy and image acquisition

Both bright field and fluorescence images from tissue sections were visualized using a Zeiss Axiophot epifluorescence microscope. All images were taken using an Olympus DP70 digital microscope camera and image-Pro Plus v.6.0.0 (Media Cybernetics.Inc).

Western blots and antibody specificity

Proteins (50 to 100 μg per lane) were separated by SDS-PAGE on 10% tris-tricine polyacrylamide gels and then transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories) using a wet transfer unit. The membranes were blocked in 5% PBST-milk for 1 h at room temperature. After blocking, the membranes were probed with an NHE2 or NHE3 antibody (1:200) for 2 h at 37° C. To demonstrate specificity of the NHE2 and NHE3 antibodies, a second blot was incubated simultaneously with the appropriate primary antibody that was previously incubated over night at 4^0 C in the presence of 100X excess synthetic peptide. In addition, another blot was incubated with pre-immune serum only (1:100). All membranes were incubated in goat anti-rabbit IgG, horseradish peroxidase (1:5,000, Amersham Life Sciences) for 1 h at room temperature. After washing (3 times 5 min in TBST), the proteins were visualized using Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer). The protein size marker used was obtained from Fermentas Life Sciences. To estimate the changes in protein levels between control and hypercapnia tissue samples (see below), experimental and control protein samples were separated by SDS-PAGE and transferred onto the same 0.45-mm nitrocellulose membranes. The blots were blocked in 5% PBST-milk for 1 h at room temperature and the membrane was probed with NHE3 antibody (1:200) for 2 h at 37° C. To compensate for deviation in the amount of protein loaded, the same membrane was stripped using Re-Blot Plus mild stripping solution (CHEMICON). The membrane was then probed with a β-actin antibody (1:500, Sigma) for 1 h at 37° C, incubated in anti-mouse IgG, horseradish peroxidase (1:5,000) for 1 h at room temperature and

washed 3 times for 5 min in TBST. The proteins were visualized as described above. The size and the opacity of NHE3 bands, relative to the size and the opacity of β-actin bands were calculated using Image J analysis software.

Exposure of fish to hypercapnia

Adult fish were placed into black plastic boxes supplied with flowing and aerated water and were allowed to recover for 24 h. Fish were exposed to external hypercapnia for 24 h with an intended final water PCO_2 of 7.5 mm Hg. To achieve hypercapnia, a water equilibrium column was gassed with mixtures of CO_2 and air (Sierra C100L Smart-trak mass flow controllers; SRB Controls). Water PCO_2 was monitored by using a CO_2 electrode connected to a CO_2 meter (Cameron Instruments). Differences from the intended water PCO_2 were corrected by adjusting the gas and the water flows through the equilibration column. For investigating changes in protein levels, fish were killed and tissues were collected after 24 h of exposure to hypercapnia (N = 6) or normocapnia (controls; N = 6). To assess the changes in NHE2 and NHE3 mRNA levels using real-time PCR, tissues were collected after 3, 12, and 24 h (N = 6 at each time point) of exposure to hypercapnia. Control fish were also killed at 3, 12, and 24 h (N = 6 at each time point) of exposure to normocapnia.

Cortisol implants

Fish (N = 6) were lightly anesthetised with benzocaine (0.5g L^{-1} for ~30 sec or until they did not respond to touch), weighed and given an intra-peritoneal implant of cortisol (0.11 mg g^{-1} body weight; hydrocortisone 21-hemisuccinate sodium salt; Sigma-

Aldrich, Inc) dissolved in coco butter (22 mg of cortisol per 1 ml of coco butter). After 3 days, fish were anesthetised with benzocaine and blood was collected into heparinised syringes from the caudal vessels. Fish were then killed, gill tissues were dissected, frozen and the RNA was extracted for RT-PCR.

Statistical analyses

The effect of exposure to hypercapnia on gill NHE2 and NHE3 mRNA expression as determined by real-time PCR was analyzed using one-sample Student's *t*-tests. The effects of hypercapnia on NHE3 protein and the effects of cortisol implants on plasma cortisol levels were analysed by unpaired Student's *t*-tests and 1-way ANOVA.

Table 2-1. Oligonucleotide primers used for RT-PCR, cDNA cloning, real-time PCR and in situ probe construction.

Primer name	Primer Sequence	Uses
DNHE2-F	5'-AAY GAY GSI GTI ACI GTI GT-3'	Cloning of partial NHE2 cDNA
DNHE2-R	5'-GGI CKI ATI GTI ATI CCY TG-3'	Cloning of partial NHE2 cDNA
3NHE2F1	5'-TCA GGG AGA TAG AAC CCC TCT-3'	Outer 3'RACE
3NHE2F2	5'-TAT GGC CAT TGT GAC CTG TG -3'	Inner 3'RACE
5NHE2R1	5'-GAC CAC ACC CAG GAA GAA GA-3'	Outer 5'RACE
5NHE2R2	5'-AGA CCA GGG CAA AGG AGA TT-3'	Inner 5'RACE
QNHE2-F	5'-TGT GCC CTG ACC ATG AAG TA-3'	NHE2 RT-PCR
QNHE2-R	5'-CCC AGT TCC ACT CGT GTT CT-3'	NHE2 RT-PCR
NHE2-F	5'-CGGGCAGAGTCTAGCATTGT-3'	In situ Hybridization
NHE2-R	5'-TGT CGT CAC GGT CAC CAG-3'	In situ Hybridization
DNHE3-F	5'-CTT CAT GTT YCT KGG HAT CTC KGC-3'	Cloning of partial NHE3 cDNA
DNHE3-R	5'-CATGGTCCKSTGGAARATYTC-3'	Cloning of partial NHE3 cDNA
3NHE3F1	5'-ATC CTC CTC ACA CTC CTC TTC ATC TT-3'	Outer 3'RACE
3NHE3F2	5'-CAG GTG GTG ATG AGC TAC GGT -3'	Inner 3'RACE
5NHE3R1	5'-TTA TTC CCT GCA GCA TGA CA-3'	Outer 5'RACE
5NHE3R2	5'-GCC GAG GAA GAC AAA GAT GA-3'	Inner 5'RACE
NHE3-F	5' -GCT CCC TGG TTG GTA TTA TC- 3'	Northern blot
NHE3-R	5' -AAC CAG CAC AAC CAC CTC TC- 3'	Northern blot
QNHE3-F	5'-AGA GCA GCC GTG ACA GAA CT-3'	NHE3 RT-PCR
QNHE3-R	5'-AAC CAG CAC AAC CAC CTC TC-3'	NHE3 RT-PCR
Actin-F	5'- CCA ACA GAT GTG GAT CAG CAA-3'	RT-PCR Control
Actin-R	5'- GGT GGC AGA GCT GAA GTG GTA-3'	RT-PCR Control

RESULTS

Molecular cloning of trout NHE2 and NHE3

Using homology cloning techniques and data mining, three cDNA sequences were translated and assembled into open reading frames encoding for a deduced partial NHE2 protein of 692 amino acids, a deduced NHE3a protein of 752 amino acids and a deduced NHE3b protein of 862 amino acids (Fig. 2-1A). A BLAST search of the GenBank protein databases indicated that the rainbow trout NHE orthologs (NHE2, NHE3a and NHE3b) shared high sequence identity with other vertebrate NHE amino acid sequences (data not shown). Figure 2-1A illustrates the phylogenetic analysis of partial trout NHE2 and complete trout NHE3a and NHE3b sequences with other vertebrate NHEs. Included in the phylogenetic analysis is trout βNHE that is most closely related to NHE1. The tree clearly shows the grouping of rainbow trout NHE2, NHE3a and NHE3b paralogs with corresponding orthologous genes in the different species analysed. Confirming the presence of two NHE3 transcripts (NHE3a and NHE3b) the Northern blot (Fig. 2-1B) on kidney RNA showed two bands with putative NHE3a band being ~4200 bp and putative NHE3b ~4500 bp. Even though the estimated band sizes were larger than predicted based on the NHE3a and NHE3b coding sequences (probably due to the untranslated regions), the estimated difference between the two bands was ~300 bp and agrees with the difference between NHE3a and NHE3b sequences of 330 bp.

Tissue distribution of NHE2 and NHE3 mRNA

The relative levels of NHE2 and NHE3 mRNA across different tissues was examined using real-time PCR (Fig. 2-2) to reveal that the distribution of NHE mRNA was isoform specific. The highest amount of NHE2 mRNA (Fig. 2-2A) was detected in the gills followed by lower levels in posterior kidney and spleen. On the other hand, the level of NHE3 mRNA (Fig. 2-2B) was highest in the posterior kidney, followed by anterior kidney and the gill. On a relative scale, NHE2 was the predominant isoform in the gill and NHE3 was the predominant isoform in the kidney (see Chapter 3).

Localization of NHE2 and NHE3 to PNA$^+$ MRCs

Identification of PNA$^+$ and PNA$^-$ MRCs on gill tissue sections (Fig. 2-3) was used to localize NHE2 and NHE3 to specific MR cell sub types. Figure 2-3A clearly shows that a subset of the MR cells (red) co localized with apical PNA (arrows) and others did not (stars). It is evident that the majority of the PNA$^+$ MR cells are located near the bases of the lamellae whereas most of the PNA$^-$ MRCs are found on the lamellae but rarely near their bases (Fig. 2-3A). Figure 2-3B is a higher magnification of one PNA$^+$ MR cell supporting the basolateral localization of Na^+/K^+ ATPase (red) and apical localization of PNA (green). Immunofluorescence was eliminated after omission of primary antibody (Fig. 2-3C) or PNA (Fig. 2-3D) clearly indicating that non-specific binding of the secondary antibody and/or sreptavidin to gill sections was not a confounding issue.

The NHE3 antiserum yielded an immunoreactive band at 98.6 kDa that was not observed after the antibody was incubated with excess peptide against which it was raised (Fig. 2-4A). The size of the band was slightly larger than expected based on either the predicted NHE3a (84.4 kDa) or NHEb (95.8 kDa) sequences. Using the homologous

polyclonal antibodies against trout NHE3, it was possible to co- localize NHE3 to some, but not all MRC (Fig. 2-4B). Clearly, some of the MR cells (red) co localized with apical NHE3 (arrows) and others did not (stars). Moreover, as observed for the PNA^+ cells, (Fig. 2-3A), the $NHE3^+$ MRCs typically were localized to the basal portions of the lamellae. Indeed, after incubating the gill tissue with the NHE3 antibody and PNA (Fig. 2-4C) it is apparent that the apical NHE3 (red) co-localized with apical PNA (green), confirming the localization of NHE3 to PNA^+ MRCs. Immunofluorescence was eliminated after omission of the NHE3 primary antibody (Fig.2-4D).

On Western blots of gill protein, the homologous polyclonal antibodies against trout NHE2 yielded a single immunoreactive band of 61 kDa. The band was eliminated after the antibody was incubated in the presence of excess of peptide against which it was raised (data not shown). However, this same antiserum failed to produce any immunolabeling on trout gill sections even after multiple antigen retrieval techniques were performed (data not shown). Because the predicted size of trout NHE2 protein is 89.3 kDa and the smallest of the zebra fish NHE2s is 81.2 kDa, it is possible that the 61 kDa immunoreactive band observed on Western blots does not correspond with NHE2; without further verification, this antibody is unusable. Thus, it was necessary to develop other methods to identify the cell type(s) expressing NHE2. A technique was successfully developed to perform in situ hybridization using DIG-labeled antisense NHE2 probes on the same gill sections previously subjected to immunocytochemistry to identify PNA^+ and PNA^- MRCs. Thus it was possible to identify the MR cell type expressing NHE2 mRNA. Figure 2-5 clearly demonstrates that NHE2 mRNA is expressed in PNA^+ MRCs. In this particular instance it can be observed that four cells

showing distinct NHE2 mRNA expression (Fig. 2-5B; black arrows) are PNA^+ MRCs (Fig. 2-5A; white arrows) thus demonstrating that NHE2 expressing MRCs are indeed PNA^+ MRCs. Less clear, however, is whether all PNA^+ MR cells express NHE2 and whether any PNA^- MRCs also express NHE2. Negative control experiments incorporating either a DIG-labeled sense NHE2 probe (Fig. 2-5C) or omission of probe (Fig. 2-5D) on gill sections produced no obvious cellular staining.

Effects of hypercapnia on gill NHE2 and NHE3 levels

Changes in gill NHE2 and NHE3 mRNA levels during hypercapnia (PCO_2 = 7.5 mm Hg), as assessed by real-time PCR, appeared to be isoform specific (Fig. 2-6). Gill NHE2 mRNA levels were significantly increased after 3, 12 and 24 h exposure to hypercapnia (Fig. 2-6A), whereas NHE3 mRNA levels were unchanged (Fig. 2-6B).

Because the trout NHE2 antibody was unusable it was not possible to test if NHE2 protein levels were altered during hypercapnia. Consistent with the mRNA data, gill NHE3 proteins level were unchanged after 24 h of hypercapnia exposure (Fig. 2-7B).

Effects of elevated plasma cortisol levels on gill NHE2 mRNA expression

Fish subjected to intra-peritoneal cortisol implants exhibited significantly elevated plasma cortisol levels after 24, 48 and 72 h (Fig. 2-8A). The increase in plasma cortisol levels was associated with an increase in gill NHE2 mRNA level after 48 and 72 h.

DISCUSSION

In contrast to the extensive literature on NHE proteins in mammals (see reviews by Counillon and Pouysseégur, 2000; Hayashi et al., 2003; Orlowski and Grinstein, 2004), considerably less is known about the physiological roles of the various NHE paralogs in fish and indeed several members of the of the NHE gene family have yet to be identified or characterized. An obvious exception is the unique βNHE of rainbow trout red blood cells that was extensively characterized using pharmacological and molecular techniques (Nikinmaa and Boutilier 1995; Malapert et al., 1997). In the present study, we report the identification of two additional rainbow trout NHE isoforms, NHE2 and NHE3 (Fig. 2-1). The physiological role of the two NHE isoforms may be tissue specific; relatively high levels of NHE2 mRNA were detected in the gills, kidney and intestine (Fig. 2-2A), all of which express acid/base excreting epithelia (Marshall and Grosell, 2006). The kidney and gill exhibited abundant expression of NHE3 mRNA when compared to other tissues (Fig. 2-2B). The predominant NHE isoform in the gill, however, was NHE2 (Fig. 2-2C).

Sequence analysis and northern blot results indicated that there are at least two different NHE3 transcripts present in the rainbow trout (Figure 2-1). Similar results were obtained by Yan et al., (2007) who reported two NHE3 genes, NHE3a and NHE3b, in zebrafish (*Danio rerio*) of which only NHE3b was detectable in the gill. A search of GenBank revealed three NHE3 genes ranging between 2112 and 2553 base pairs. Because the antibody we developed for NHE3 is likely to recognize both of the trout

isoforms, we cannot determine if there is preferential expression of one form as in the zebrafish.

The presence of NHE in the gills of rainbow trout is not particularly surprising because the occurrence of branchial NHE2 or NHE3 was previously documented in several species within the agnathans (Edwards et al., 2001; Choe et al., 2002; Tresguerres et al., 2005), elasmobranchs (Edwards et al., 2002; Choe et al., 2002, 2005; 2007; Tresguerres et al., 2005) and teleosts (Claiborne et al., 1999; Edwards et al., 1999, 2005; Wilson et al., 2000a, b; Hirata et al., 2003; Scott et al., 2005; Catches et al., 2006). These studies not only included examples from FW and SW acclimated fish but also demonstrated that transferring euryhaline species to lowered salinity could induce an increase in mRNA levels of NHE2 (*Fundulus heteroclitus*, Scott et al., 2005) or NHE3 (*Dasyatis sabina*, Choe et al., 2005). The finding of NHEs in FW fish is particularly significant because it provides indirect evidence that electroneutral Na^+/H^+ exchange may function even when the concentration gradient for Na^+ across the apical membrane apparently is unfavourable. Perhaps even more surprising is the localization of an NHE3 isoform to the apical membrane of MRCs in Osorezan dace (*Tribolodon hakonensis*) inhabiting highly acidic (pH 3.5) water (Hirata et al., 2003). In this case, an apical NHE would be required to function against both Na^+ and H^+ concentration gradients (see below).

A subset of MRCs expresses NHEs in rainbow trout

Aside from the study of Wilson et al. (2000a) which provided evidence for NHE localization to a sub-population of MRCs and pavement cells (PCs) in tilapia

(*Oreochromis mossambicus*), all previous studies demonstrated that NHE2 and NHE3 are co-localized with Na^+/K^+-ATPase enriched MRCs. The novel finding of the present study was the demonstration that in trout, both NHE2 and NHE3 are confined to a specific sub-type of MRC, the PNA^+ MRC (Galvez et al., 2002). Unlike the PNA^- MRCs which tended to be situated along the more distal segments of the lamellae, the PNA^+ cells were typically found on the lamellar surfaces more proximal to the filament as well as within the interlamellar regions. The localization of NHEs to PNA^+ MRCs obviously does not support our initial hypothesis that was based on the model of Perry and Gilmour (2006) that positioned NHEs on the apical membrane of PNA^- MRCs. Because the NHE2 antibody was ineffective it was not possible to determine the sub-cellular distribution of NHE2. However, it is generally accepted that NHE2 is an apical membrane isoform (Brant et al., 1995; Malakooti et al., 1999). The result of immunocytochemistry utilising the NHE3 antibody clearly showed that NHE3 was confined to the MRC apical membrane. Although not attempted in this study, previous experiments have demonstrated that the cells expressing apical NHEs generally do not contain high levels of V-ATPase (Choe et al 2005; 2007; Catches et al 2006). Two exceptions appears to be Pacific hagfish (*Eptatretus stoutii*) in which NHE2, V-ATPase and Na^+/K^+-ATPase are localised to the same gill epithelial cells (Tresguerres et al 2006) and zebrafish (Yan et al 2007) in which NHE3b is confined to cells enriched with apical membrane V-ATPase and not Na^+/K^+-ATPase.

Are the PNA^+ MRCs acid- or base-secreting cells?

The PNA^+ MRC of trout is characterized by a tubular network arising from extensive infolding of the basolateral plasma membrane. The PNA^- MRC has no such elaboration of the basolateral membrane and levels of Na^+/K^+-ATPase, while high, are lower than in the PNA^+ cells (Galvez et al 2002). Thus, the PNA^+ MRC is presumed to be analogous to the so-called "chloride cell" of FW fish (Perry, 1997). These PNA^+ MRCs or chloride cells are believed to be the site of apical membrane Cl^-/HCO_3^- exchange and hence are thought to function as net base-secreting cells (Perry et al 2003; Perry and Gilmour 2006; Tresguerres et al 2006). Indeed, there is extensive indirect evidence to suggest that net base excretion in trout (Goss and Perry, 1993; Goss et al 1994a; Perry and Goss, 1994) and other species (Goss et al., 1992a; 1994b) is regulated by the number of PNA^+ MRCs (chloride cells) exposed to the water. Thus, the increase in net acid excretion during acidosis required for pH regulation is thought to arise, in part, from reduced rates of Cl^-/HCO_3^- exchange reflecting the physical covering of chloride cells (now presumed to be PNA^+ MRCs) by adjacent PCs (see reviews by Goss et al., 1992b; 1995; 1998; Laurent and Perry, 1995; Perry and Gilmour, 2006). The unexpected finding of this study that NHEs are also expressed on the apical membrane (assumed for NHE2) of PNA^+ MRCs clearly complicates the current model of a net base-secreting PNA^+ MRC because if operating at equal rates, apical membrane Cl^-/HCO_3^- and Na^+/H^+ exchanges would be acid-base neutral. At rest, the net branchial fluxes of Na^+ and Cl^- are roughly equivalent and so predictably, the rates of net acid- or base-excretion at the gill are low (Wood et al., 1984). Thus, under resting conditions, the placement of Na^+/H^+ and Cl^-/HCO_3^- exchangers on the apical membrane of PNA^+ MRCs, if operating at similar

rates, would promote acid-base neutrality. Moreover, the excretion of H^+ via NHE might actually aid Cl^-/HCO_3^- exchange as proposed in the model of intestinal HCO_3^- excretion in SW acclimated rainbow trout (Grosell et al., 2007). In that model, H^+ originating either from NHE or V-ATPase facilitates HCO_3^- efflux via Cl^-/HCO_3^- exchange by promoting the recycling of extracellular CO_2. Regardless of whether NHE aids Cl^-/HCO_3^- exchange, a critical remaining question is "how can conditions of net acid or base excretion be achieved if NHEs and Cl^-/HCO_3^- exchangers are both positioned on the apical membrane of PNA^+ MRCs"? We propose that two additional requirements must be satisfied; first, there must be differential regulation of NHE and Cl^-/HCO_3^- exchangers during acid-base disturbances and second, there must be a second site of H^+ excretion, the PNA^- MRC. During acidosis, the physical covering of PNA^+ MRCs by adjacent PCs, while causing both the Cl^-/HCO_3^- and Na^+/H^+ exchangers to be uncoupled from the water, could lead to increased acid excretion and retention of HCO_3^- if there was a corresponding transcriptional increase in NHE expression combined with an increase in acid excretion via PNA^- MRCs. In the present study there were significant increases in mRNA levels during hypercapnia for the predominant NHE isoform in the gill, NHE2 (NHE3 levels were unchanged) supporting the idea that net acid excretion by the PNA^+ MRC could be increased by preferential loss of apical membrane Cl^-/HCO_3^- exchange sites. Previous studies examining the effects of acidosis on NHEs in other species have produced conflicting results. For example, while expression of NHE3 in Atlantic hagfish (*Myxine glutinosa*; Edwards et al., 2001) and NHE2 in *Fundulus heterolclitus* (Edwards et al., 2005) and *Squalus acanthias* (Tresguerres et al., 2005) increased during acidotic conditions, there were no detectable changes caused by acidosis in NHE2 expression in

the sculpin (*Myoxocephalus octodecimspinosus*; Catches et al., 2006) or NHE3 expression in the Atlantic stingray (*Dasyatis sabina*; Choe et al., 2005). The other postulated requirement, increased H^+ excretion by the PNA^- MRC, is supported by numerous observations. Perhaps most significant are the observations that the PNA^- cells express more V-ATPase than PNA^+ cells and that hypercapnia elicits an increase in expression of V-ATPase exclusively the in PNA^- cells (Galvez et al., 2002). Further, only the PNA^- cells exhibit acid-stimulated Na^+ flux that is prevented by application of the Na^+ channel blocker phenamil (Reid et al., 2003). Because it is likely that the mitochondria rich PCs described in earlier literature (Goss et al., 1994) are the same cells as the PNA^- MRCs described in more current literature (Galvez et al., 2002), earlier observations of apically oriented V-ATPase whose expression is increased during hypercapnia (Laurent et al 1994; Sullivan et al 1996) are consistent with a role for the PNA^- MRC in Na^+ uptake and net acid excretion.

How is a favourable gradient for electroneutral Na^+/H^+ exchange established?

Clearly, if Na^+/H^+ exchange is to operate in FW, mechanisms must exist to establish a favourable chemical gradient within the microenvironment that the NHE functions. As discussed by Hirata et al (2003), it is possible that activity of the basolateral Na^+/K^+-ATPase in close proximity to the apical membrane, creates pockets of low cytoplasmic Na^+ to establish an inwardly directed gradient for Na^+ diffusion. Although our previous model (e.g. Perry and Gilmour, 2006) placed V-ATPase on the basolateral membrane of PNA^+ cells, its presence there would likely reduce or abolish an otherwise favourable gradient for H^+. Moreover, its continuing activity during acidosis

would be counter productive with respect to pH regulation. Given that with two exceptions (Tresguerres et al., 2006; Yan et al., 2007), all previous studies have failed to demonstrate V-ATPase expression in cells co-expressing NHEs, its presence in the NHE-containing PNA^+ cells of trout must be reconsidered.

Regulation of NHE by cortisol

Concomitant with the increased expression of NHE2, plasma cortisol levels increased significantly during hypercapnia (see Chapter 3). Because previous research on mammals has shown that NHEs in kidney are transcriptionally regulated by glucocorticoids (Hayashi et al 2002), we sought to determine if cortisol might be playing a role in regulating NHE2 levels in trout gill. The data showing a significant increase in branchial NHE2 mRNA are consistent with the notion that cortisol mobilization during hypercapnic acidosis contributes to pH regulation by activation of NHE2.

Figure 2-1. Phylogenetic relationship of selected NHE1-3 proteins from different species and orthologous rainbow trout NHE2, NHE3a and NHE3b amino acid sequences. The grouping of each rainbow trout ortholog with its corresponding form is an indication that the rainbow trout clone is most closely related to that NHE isoform (A). The existence of the two rainbow trout NHE3 transcripts is indicated in a representative northern blot (B). The tree was constructed using the neighbour-joining method, and numbers indicate bootstrap values for 1,000 replicates. GenBank accessions are as follows:

NHE1. *Oncorhynchus mykiss* (βNHE) Q01345, *Pseudopleuronectes americanus* AAO32340, *Amphiuma tridactylum* AAD33928, *Takifugu obscurus* BAE75800.1, *Rattus norvegicus* NP_036784.1, *Anguilla anguilla* CAB45085, *Cyprinus carpio* CAB45232, *Homo sapiens* NP_003038, *Drosophila melanogaster* AAF51559, *Gallus gallus* ABB82239.

NHE2. *Canis familiaris* XP_531775.2, *Gallus gallus* XP_416918.2, *Mus musculus* NP_001028461 XP_129721, *Oryctolagus cuniculus* P50482, 708 AA *Danio rerio* XP_001336127.1, 795AA *Danio rerio* XP_691364, *Homo sapiens* NP_003039, *Myoxocephalus octodecemspinosus* AAD46576, *Rattus norvegicus* P48763, partial 692 AA *Oncorhynchus mykiss* ABO32814, *Squalus acanthias* ABC54565 *Tetraodon nigroviridis* CAG05798.

NHE3. 704 AA *Danio rerio* CAM47009.1, 829 AA *Danio rerio* XP_696670, 851 AA *Danio rerio* XP_696728, *Gallus gallus* XP_418895.2, *Mus musculus* NP_001074529.1, *Dasyatis sabina* AAT45738.2, *Homo sapiens* NP_004165.1, *Rattus norvegicus* NP_036786.1, NHE3a; 752 AA *Oncorhynchus mykiss* ABO32815.1 and NHE3b; 862 AA *Oncorhynchus mykiss* assembled sequence, *Oreochromis mossambicus* BAF80347, *Tribolodon hakonensis* AB055466.

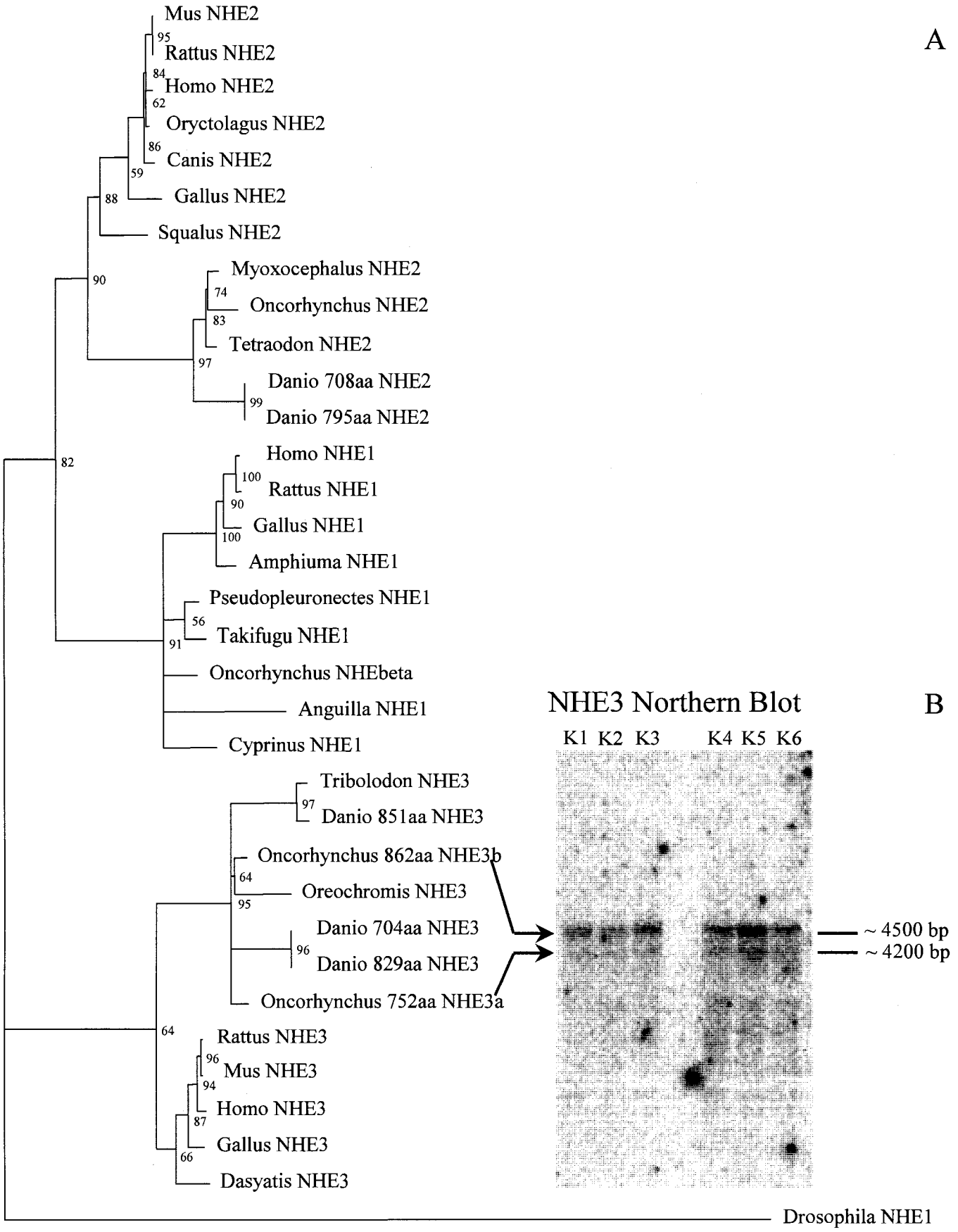

A
Mus NHE2
Rattus NHE2
Homo NHE2
Oryctolagus NHE2
Canis NHE2
Gallus NHE2
Squalus NHE2
Myoxocephalus NHE2
Oncorhynchus NHE2
Tetraodon NHE2
Danio 708aa NHE2
Danio 795aa NHE2
Homo NHE1
Rattus NHE1
Gallus NHE1
Amphiuma NHE1
Pseudopleuronectes NHE1
Takifugu NHE1
Oncorhynchus NHEbeta
Anguilla NHE1
Cyprinus NHE1
Tribolodon NHE3
Danio 851aa NHE3
Oncorhynchus 862aa NHE3b
Oreochromis NHE3
Danio 704aa NHE3
Danio 829aa NHE3
Oncorhynchus 752aa NHE3a
Rattus NHE3
Mus NHE3
Homo NHE3
Gallus NHE3
Dasyatis NHE3
Drosophila NHE1
0.1
NHE3 Northern Blot
B
K1 K2 K3 K4 K5 K6
~ 4500 bp
~ 4200 bp

Figure 2-2. Tissue distribution analysis of rainbow trout NHE2 (A) and NHE3 (B) mRNA as determined by real-time PCR and expressed relative to a reference tissue (brain) set to a relative value of 1. The inset (C) represents the relative mRNA levels of NHE2 and NHE3 in the gill. Values shown are means + 1 SEM; asterisks indicate significant differences ($P<0.05$) from the reference tissue. P values for NHE2 mRNA levels in gill, anterior kidney, posterior kidney, spleen and white muscle were 0.004, 0.058, 0.039, 0.017 and 0.087, respectively. P values for NHE3 mRNA levels in gill, anterior kidney, posterior kidney, heart and intestine were 0.016, 0.008, 0.009, 0.148 and 0.142, respectively. The P value for gill NHE2 mRNA levels relative to NHE3 gill mRNA levels (inset in A) was 0.045.

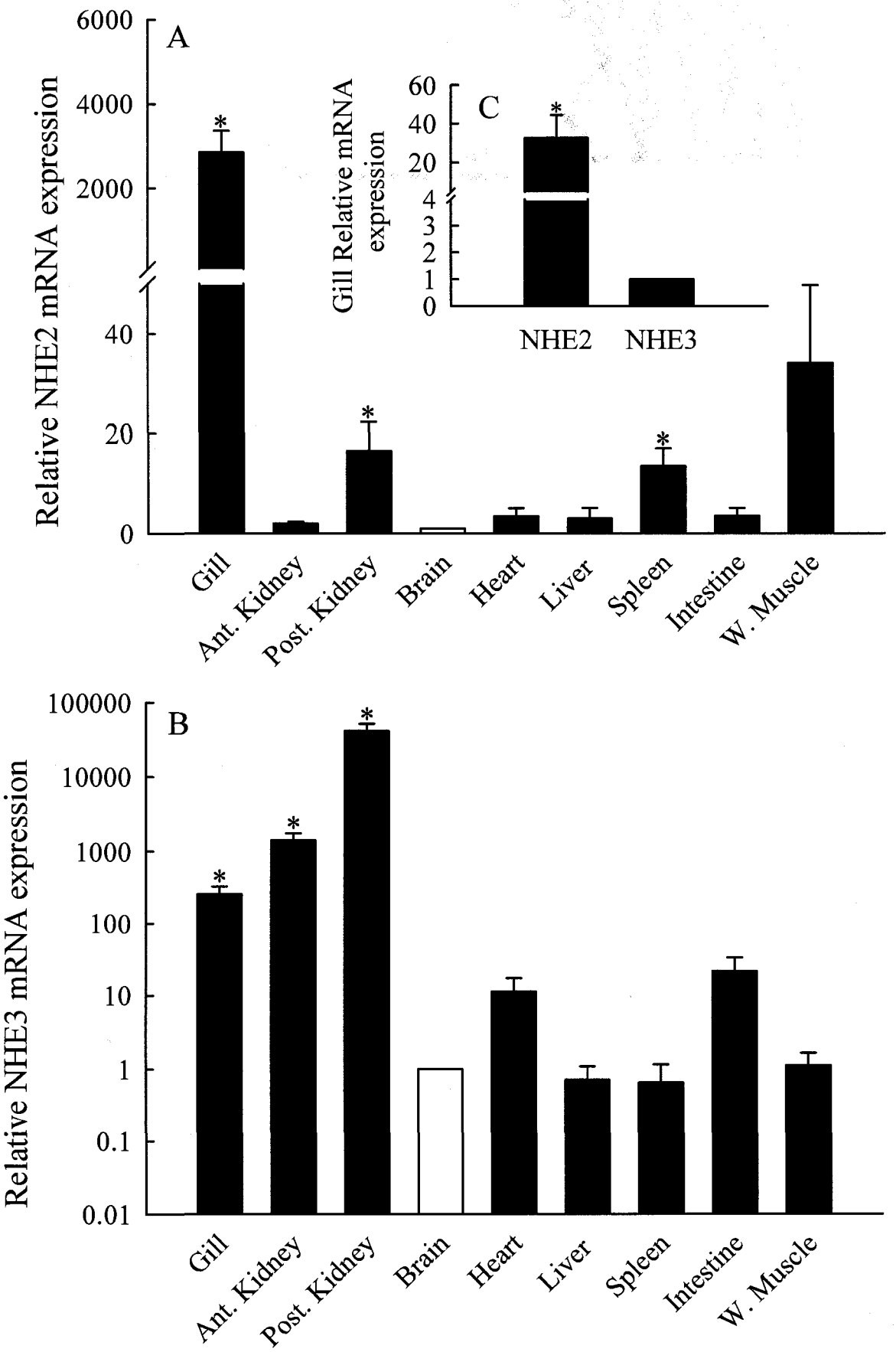

A
Relative NHE2 mRNA expression
6000
4000
2000
40
20
0
C
Gill Relative mRNA expression
60
40
20
4
3
2
1
0
NHE2
NHE3
Gill
Ant. Kidney
Post. Kidney
Brain
Heart
Liver
Spleen
Intestine
W. Muscle
B
Relative NHE3 mRNA expression
100000
10000
1000
100
10
1
0.1
0.01
Gill
Ant. Kidney
Post. Kidney
Brain
Heart
Liver
Spleen
Intestine
W. Muscle

Figure 2-3. (A) Identification of PNA^+ and PNA^- negative MRCs in gill sections from adult rainbow trout. PNA^+ MRCs are labelled with arrows; stars indicate PNA^- MRCs. MRCs were identified on the basis of immunoreactivity against Na^+/K^+-ATPase (red); green indicates apical PNA and blue indicates nuclei (DAPI). (B) Higher magnification image of a PNA (green) positive MRC. Negative controls included (C) omission of primary antibody against Na^+/K^+-ATPase and (D) omission of biotinylated PNA. The images in (C) and (D) were captured using the same exposure and microscope settings as A). Bar in (A) 20 μm and in (B) 5 μm.

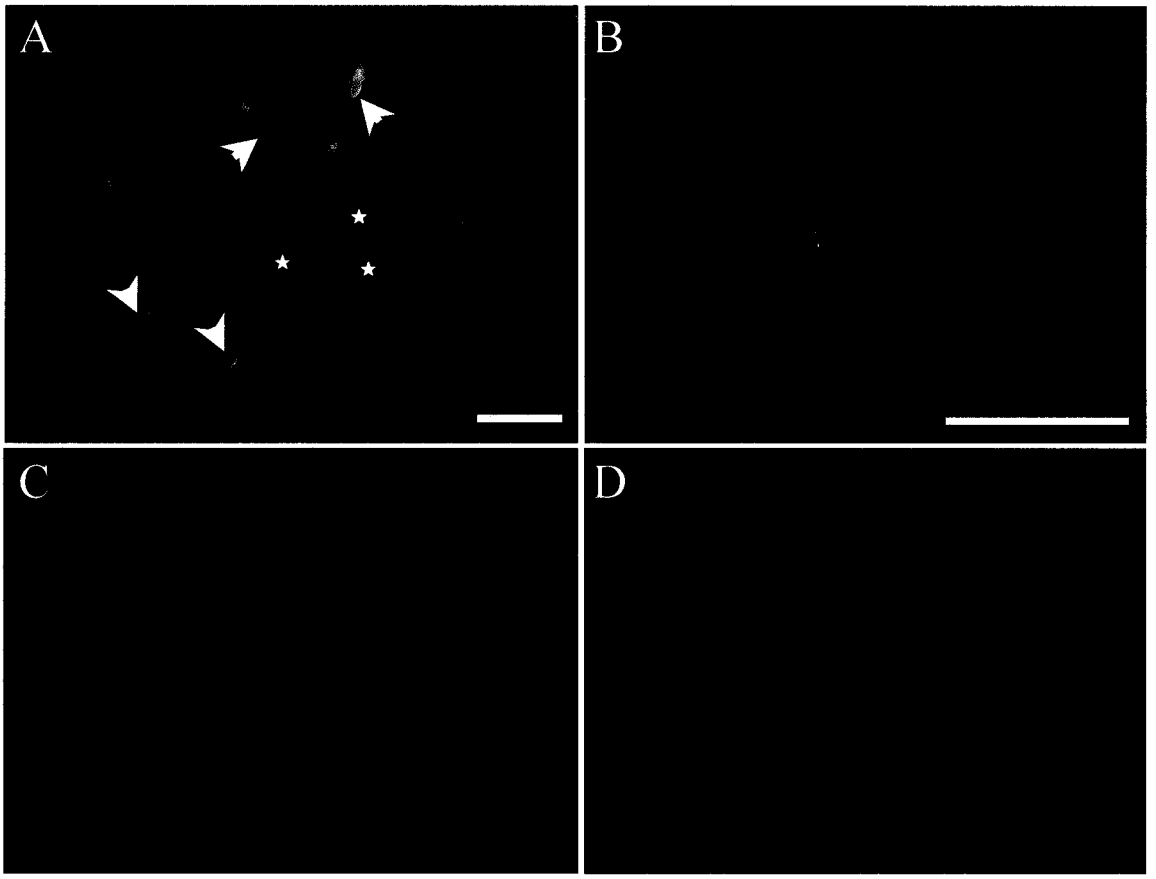

A
B
C
D

Figure 2-4. Identification of NHE3 expressing cells as PNA^+ MRCs in gill sections from adult rainbow trout. (A) NHE3 protein was detected in gill (G) and kidney (K) protein extracts using Western blots (N = 6) to reveal a 98 kDa immunoreactive band (lanes 1 and 2) that was absent after the NHE3 antibody was incubated with excess peptide against which the antibody was raised (lanes 3 and 4). (B) Co-localization of NHE3 (green) and Na^+/K^+-ATPase (red) in MRCs (nuclei are stained blue); NHE3 expressing MRCs are labelled with arrows and NHE3 negative MR cells are indicated by stars. (C) Localization of NHE3 to the apical membrane of PNA^+ MRCs; red indicates NHE3, green indicates PNA and nuclei are stained blue. (D) The NHE3 immunofluorescence disappeared with omission of primary NHE3 antibody. Bar in (A) 40 μm and bar in (C) 10 μm.

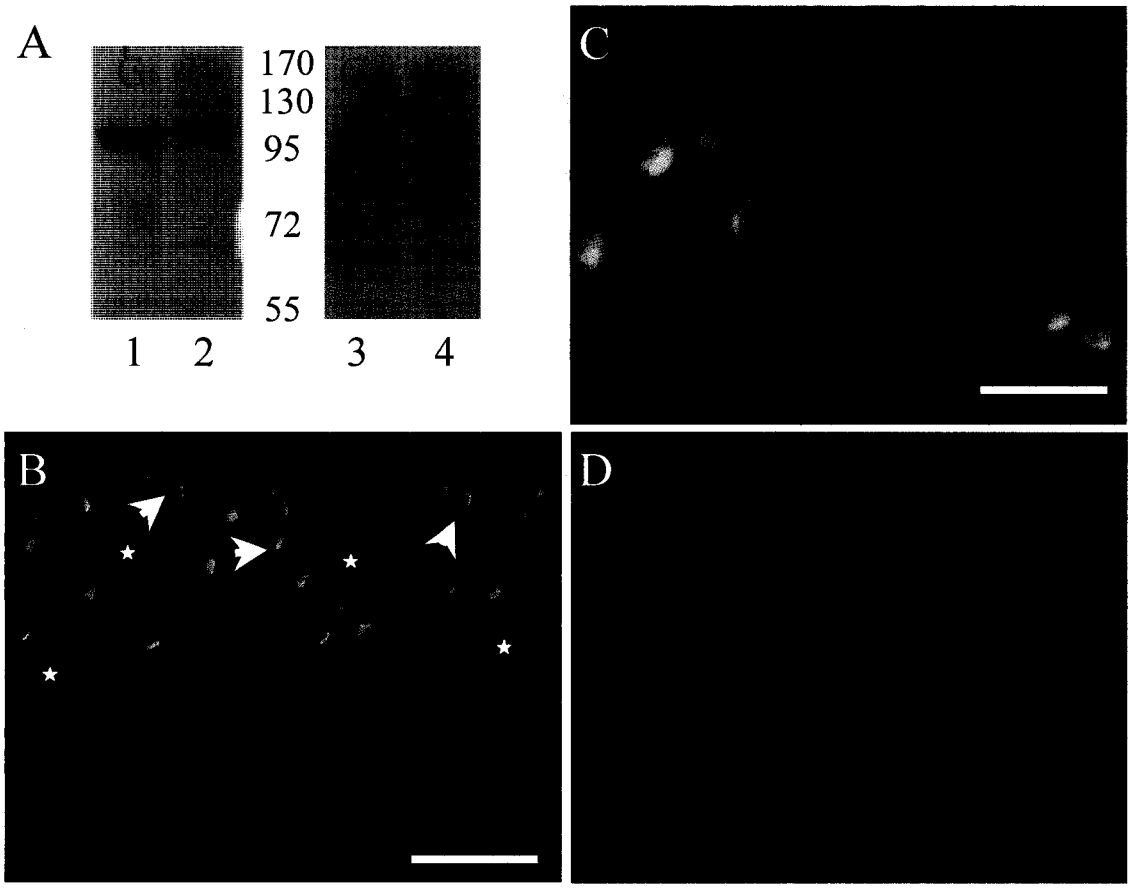

A
170
130
95
72
55
1
2
3
4
B
C
D

Figure 2-5. Detection of NHE2 expressing cells in rainbow trout gill by combined immunofluorescence and in situ hybridization. (A) PNA^+ MRCs (labelled with arrows) were detected using a primary antibody against Na^+/K^+-ATPase (red) and biotinylated PNA (green). (B) In situ hybridization on the same section demonstrated that the PNA^+ MRCs were enriched with NHE2 mRNA (black arrowheads). (C) No staining was observed when a sense probe was used or (D) when probe was omitted. Bar 20 μm.

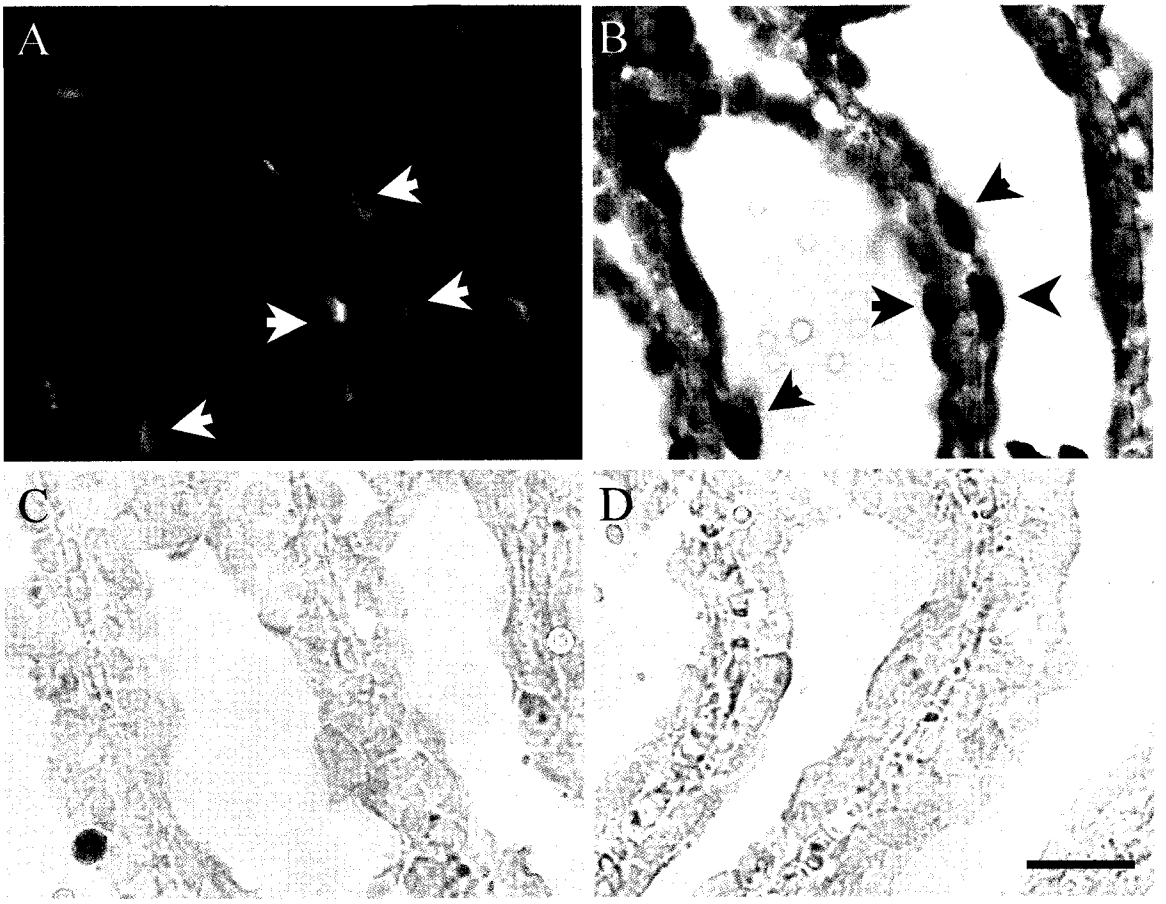

A
B
C
D

Figure 2-6. The effects of hypercapnia (water PCO_2 = 7.5 mm Hg) on trout gill (A) NHE2 and (B) NHE3 relative mRNA levels. Gill NHE2 mRNA levels were significantly increased at 3, 12 and 24 h of hypercapnia (P values of 0.014, 0.031 and 0.040, respectively). Gill NHE3 mRNA levels were unchanged throughout the period of hypercapnia exposure.

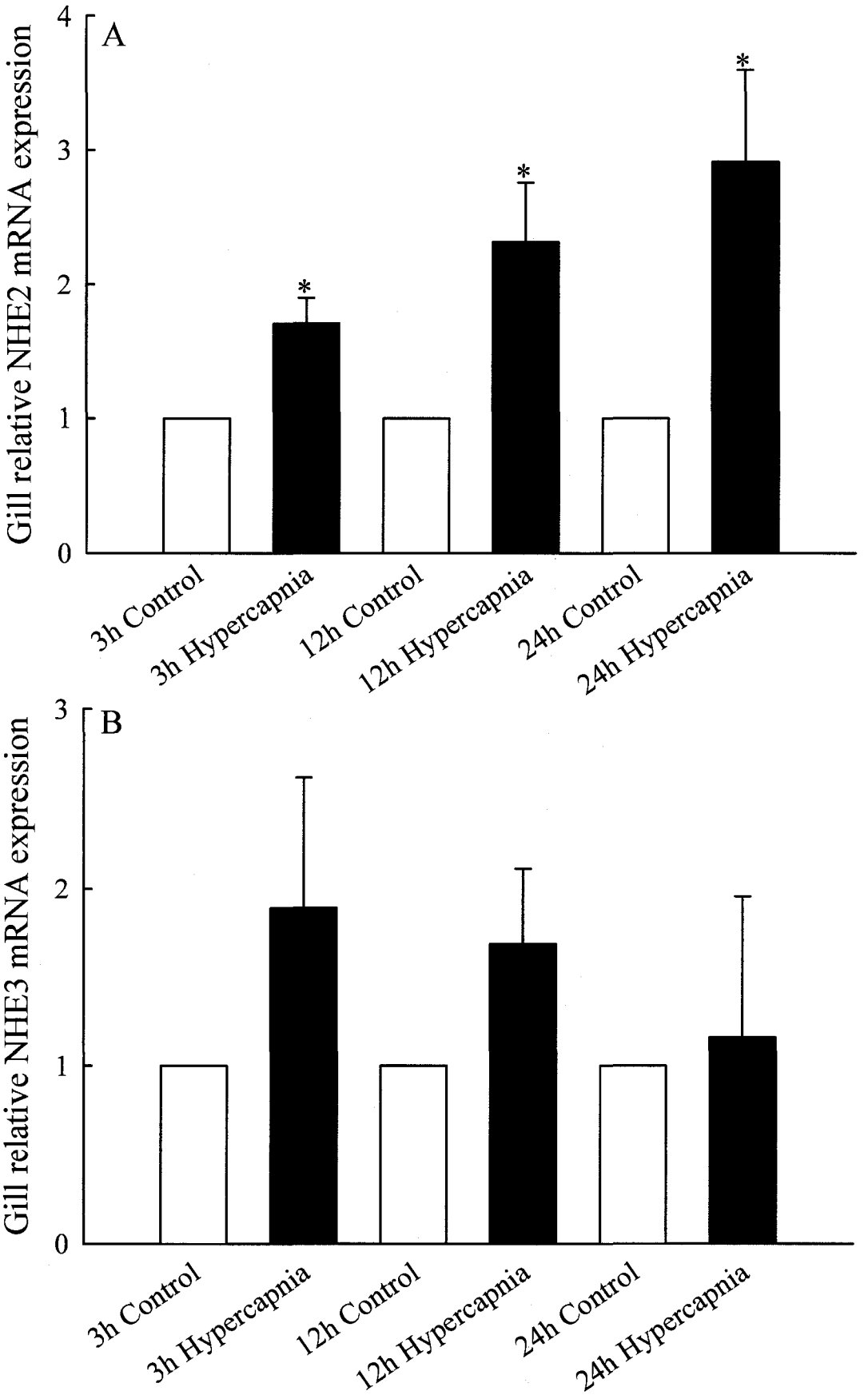

A
Gill relative NHE2 mRNA expression
4
3
2
1
0
*
*
*
3h Control
3h Hypercapnia
12h Control
12h Hypercapnia
24h Control
24h Hypercapnia
B
Gill relative NHE3 mRNA expression
3
2
1
0
3h Control
3h Hypercapnia
12h Control
12h Hypercapnia
24h Control
24h Hypercapnia

Figure 2-7. The effects of exposure to hypercapnia for 24 h (water PCO_2 = 7.5 mm Hg) on trout gill NHE3 protein levels as revealed by Western blot analysis (N = 6; each lane represents a different fish). Densitometric analysis of Western blots (B) normalized to β-actin intensities, revealed unaltered levels of NHE3 protein after 24 h of hypercapnia.

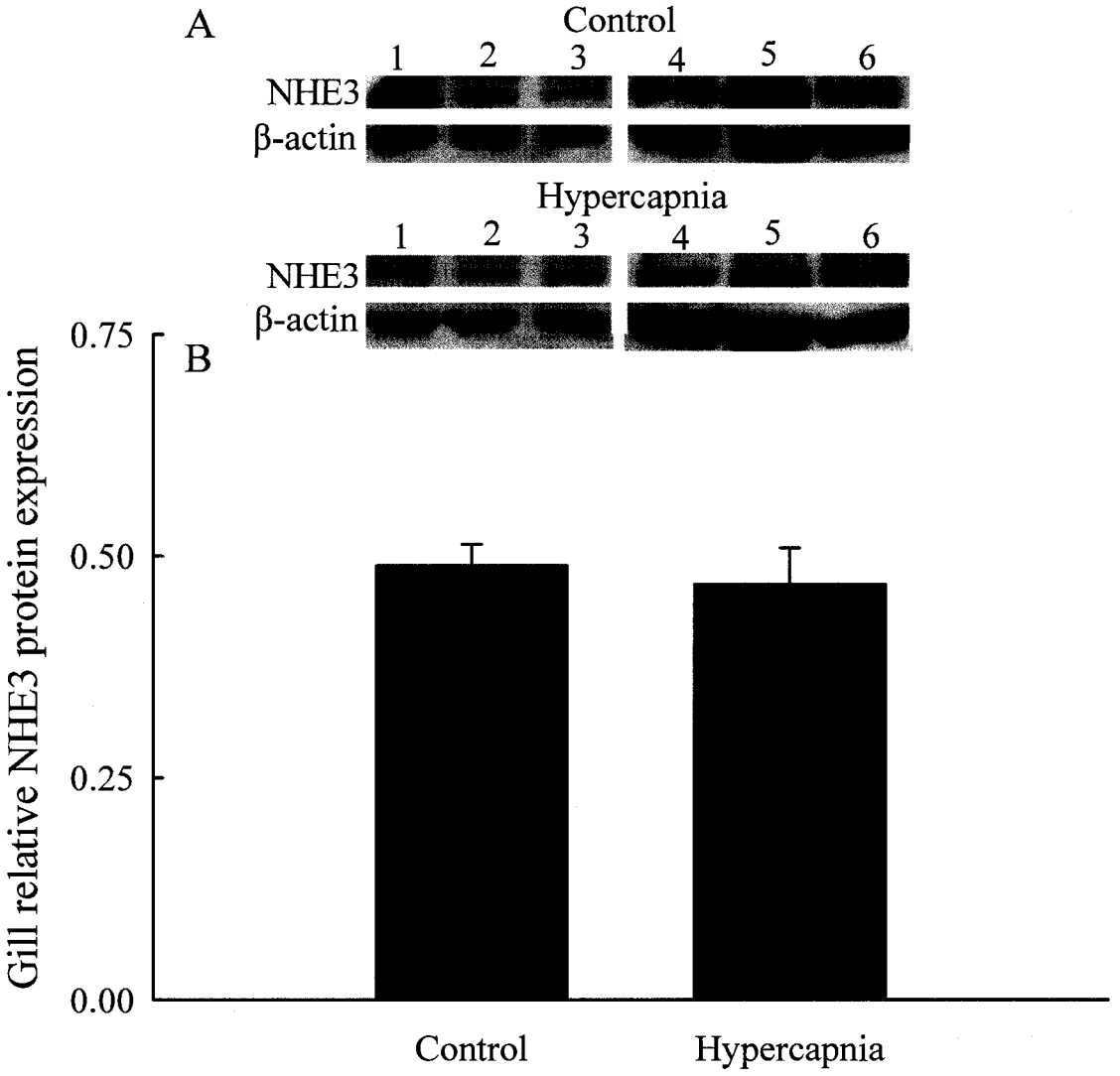
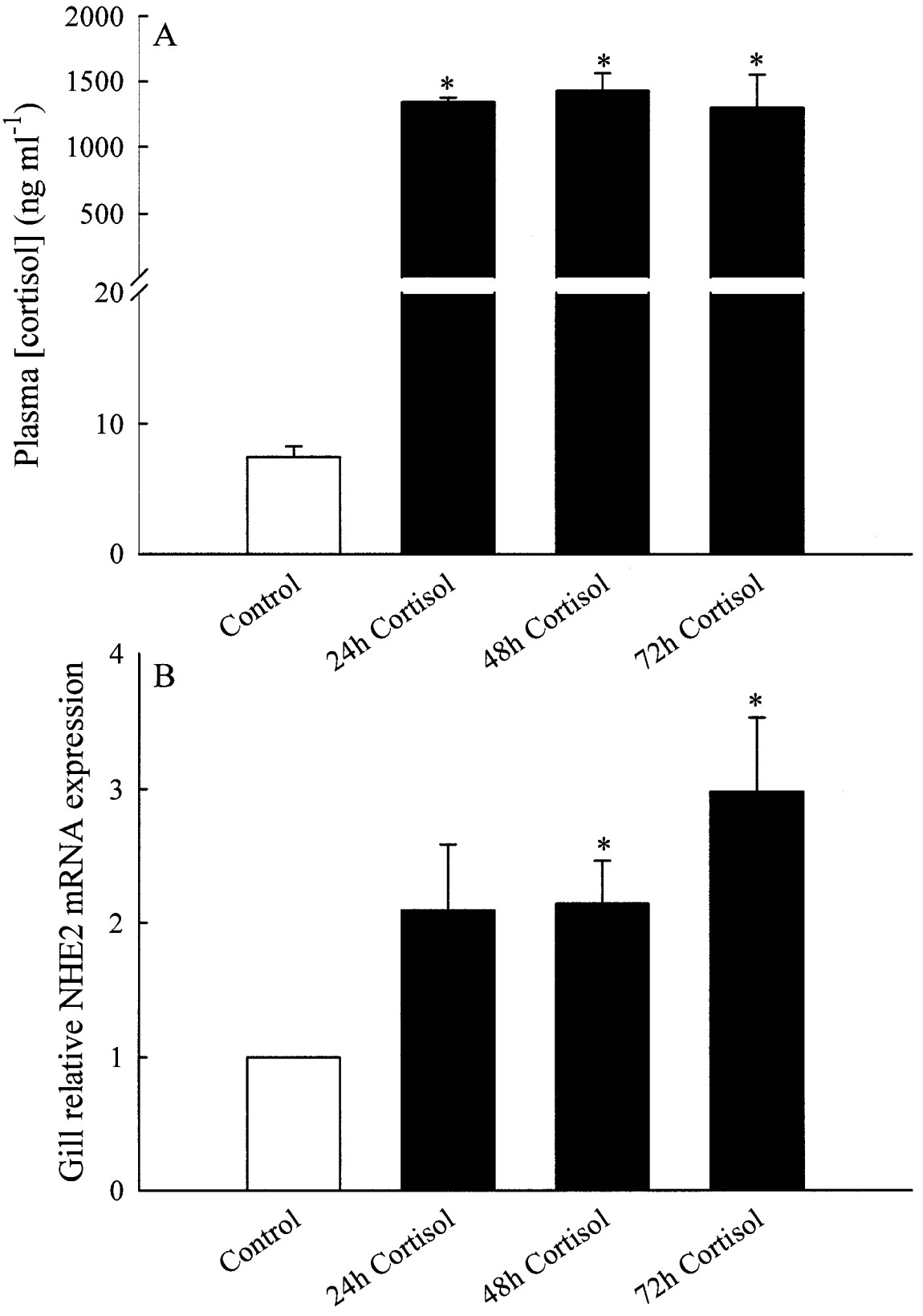

A
Control
1 2 3 4 5 6
NHE3
β-actin
Hypercapnia
1 2 3 4 5 6
NHE3
β-actin
B
Gill relative NHE3 protein expression
0.75
0.50
0.25
0.00
Control
Hypercapnia

Figure 2-8. The effects of elevated plasma cortisol levels on gill relative NHE2 mRNA expression after 24 (N = 5), 48 (N = 6) and 72 h (N = 6) of administration of exogenous cortisol. (A) Plasma cortisol levels were markedly elevated after fish were given cortisol implants. (B) NHE2 mRNA levels were significantly elevated at 48 (P = 0.017) and 72 h (P = 0.014) post injection.


A
Plasma [cortisol] (ng ml-1)
2000
1500
1000
500
20
10
0
*
*
*
Control
24h Cortisol
48h Cortisol
72h Cortisol
B
Gill relative NHE2 mRNA expression
4
3
2
1
0
*
*
Control
24h Cortisol
48h Cortisol
72h Cortisol

CHAPTER 3

RENAL ACID-BASE REGULATION

INTRODUCTION

The metabolic regulation of acid-base disturbances in fish, like in other vertebrates, relies on adjustments of plasma HCO_3^- levels at constant PCO_2. For example, respiratory acidosis associated with external hypercapnia is compensated by gradual accumulation of HCO_3^- within the plasma (Cameron, 1978). The rise in plasma HCO_3^- concentration reflects a marked increase in net acid excretion at the gill which, in turn, is caused by differential modulation of the rates of branchial Cl^- and Na^+ net fluxes (Evans et al., 2005). Cl^- uptake is linked to base efflux via Cl^-/HCO_3^- exchange and Na^+ uptake is coupled to H^+ excretion either by Na^+/H^+ exchange (Krogh, 1938) or by diffusion of Na^+ through channels, a process energised by V-type H^+-ATPase (V-ATPase) mediated H^+ secretion (Avella and Bornancin, 1989). Thus, during hypercapnic acidosis, the increase in branchial acid excretion typically reflects stimulation of Na^+ uptake and inhibition of Cl^- uptake (Cameron, 1976). In rainbow trout, a reduction in the rate of branchial Cl^-/HCO_3^- exchange is the principal adjustment underlying the increase in plasma HCO_3^- levels; increased Na^+ uptake linked to H^+ secretion is less important in this species (Perry et al., 1987).

Although the gill is the major site of acid-base equivalent fluxes in fish, the kidney also plays an essential role by controlling the extent of HCO_3^- reabsorption (Wood et al., 1999). For example, as discussed by Perry and Gilmour (2006), the accumulation of HCO_3^- within the plasma as a strategy to regulate hypercapnic acidosis can only succeed if the accumulating HCO_3^- filtered at the kidney is reabsorbed. In mammals, the renal reabsorption of HCO_3^- involves acidification of the filtrate predominantly at the

proximal tubule (Romero and Boron, 1999). Acidification of the filtrate is accomplished by H^+ pumping via the V-ATPase (reviewed by Nakhoul and Hamm, 2002) and electroneutral Na^+/H^+ exchange. In the mammalian proximal tubule, the specific Na^+/H^+ exchanger thought to mediate the bulk of filtrate acidification is SLC9A3 or NHE3 (Knepper and Brooks, 2001; Fenton and Knepper, 2007). Many of the components required for HCO_3^- reabsorption in the mammalian proximal tubule have been identified in rainbow trout. NBC1 (Perry et al., 2003b), V-ATPase (Perry and Fryer, 1997; Perry et al., 2000), cytoplasmic carbonic anhydrase (tCAc, the functional equivalent of CA II; (Esbaugh et al., 2005) as well as CA IV (tCAIV; (Georgalis et al., 2005)) have been identified in rainbow trout kidney. Moreover, increased expression of NBC1, V-ATPase, tCAc and tCA IV occur in trout exposed to hypercapnia, presumably to increase HCO_3^- reabsorption (Perry et al., 2003b; Georgalis et al., 2005).

One key component required for HCO_3^- reabsorption (at least in the mammalian model) yet to be identified in trout kidney is luminal (apical) NHE. In Chapter 2, two of the apical isoforms, NHE2 and NHE3, were cloned and demonstrated to be present in gill and kidney. The expression of branchial NHE2 was markedly enhanced during hypercapnia possibly owing to a concomitant elevation of plasma cortisol levels; NHE3 expression was unaffected. To complete the model of HCO_3^- reabsorption in trout kidney, the primary goal of the present study was to establish whether, by analogy to the mammalian proximal nephron, the NHE3 isoform is localized to the apical membrane of proximal tubule cells and whether its expression increases during hypercapnia concurrently with increased renal HCO_3^- reabsorption. Because glucocorticoids have been implicated in the regulation of mammalian NHEs (Baum et al., 1993; Baum and

Quigley, 1993) and renal acidification (reviewed by Hayashi et al., 2002), a final objective was to determine whether renal NHE3 is transcriptionally regulated by cortisol.

MATERIALS AND METHODS

Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish were maintained on a 12h light : 12h dark photoperiod in circular fibreglass water tanks supplied with flowing, aerated and dechloraminated City of Ottawa tap water at 13° C. Animals were fed daily with a commercial trout diet and were acclimated for at least 2 weeks before any experiments were performed. Food was withheld for 48 h prior to experimentation.

RNA and protein extractions

Fish were killed by a blow to the head and dissected tissues were ground on dry ice with a mortar and pestle and stored at -80° C until needed. Total RNA was extracted from 100 mg aliquots of frozen tissue samples using Trizol reagent (Invitrogen). The RNA pellet was resuspended in 40 µl of nuclease-free H_2O and treated with RNase-free DNase (8 units per RNA sample) for 20 min at room temperature to remove any remaining genomic DNA. The RNA concentration and quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf Biophotometer). Kidney proteins were extracted using 1X RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) with protease inhibitors (complete Mini protease inhibitor cocktail tablets; Roche). The tissues were first ground under liquid N_2 with a pre-cooled mortar and pestle and then incubated on ice for 15 min. Samples were sonicated (2 X 1 sec pulsed at medium power; micro ultrasonic cell disruptor by Kontes),

centrifuged at 10,000 rpm for 10 min at 4° C and the supernatant containing soluble proteins was collected. Protein concentration was determined using a BioRad protein assay kit (Bio-Rad Laboratories) using BSA as a standard. Ultimately, all of the protein samples were diluted to a concentration of ~20 μg ml^{-1} and frozen and stored at -80° C until needed.

Quantification of mRNA levels using real-time PCR

cDNA was synthesized from 1 μg of RNA using random hexamer primers (Boehringer Mannheim) and RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas). Rainbow trout mRNA levels were measured by real-time PCR on samples of cDNA (1 μl) using a Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) and a Stratagene MX-4000 multiplex quantitative PCR system. ROX (Stratagene) was used as reference dye. The PCR conditions (final reaction volume = 12.5 μl) were as follows: cDNA template = 0.5 μl; forward and reverse primer = 300 nmol l^{-1}; 2X Master Mix = 12.5 μl; ROX = 1:30000 final dilution. The annealing and extension temperatures over 40 cycles were 56° C (30 sec) and 72° C (30 sec), respectively. The primers used for real-time PCR (including the reference gene β-actin) were designed using online software (Primer 3; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 3-1). The specificity of the primers was verified by cloning and sequencing of amplified products. To ensure that residual genomic DNA was not being amplified, control experiments were performed in which reverse transcriptase was omitted during cDNA synthesis. Relative expression of mRNA levels was determined (using β-actin as an endogenous standard) by

a modification of the delta-delta Ct method (Pfaffl, 2001). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

Collection of tissues for immunocytochemistry and in situ hybridization

Rainbow trout were killed by a blow to the head and the kidney tissue was dissected, placed in 4% paraformaldehyde (PFA) and kept overnight at 4° C. Samples were then placed in 15% sucrose for 2 h at room temperature followed by 30% sucrose at 4° C until sectioning. The tissue was embedded in OCT cryosectioning medium (VWR) and incubated for 20 min. Horizontal sections (10 μm) were obtained using a cryostat (Leica CM 1850) and placed onto glass microscope slides (VWR superfrost plus). Slides were dried at room temperature for approximately 45 min and then stored at -4° C until required.

NHE and Na^+/K^+-ATPase immunocytochemistry

Sections were incubated for 2 h at room temperature with the following primary antibodies:

1. α5 (1:100), a mouse monoclonal antibody (University of Iowa Hybridoma Bank) raised against the α1 sub-unit of chicken Na^+-K^+-ATPase that has been used extensively to localize Na^+/K^+-ATPase in fish tissues.

2. A custom affinity-purified rabbit polyclonal antibody (Abgent, San Diego, CA) raised against trout NHE3 that was generated using 8 chain multi-antigenic peptides. The synthetic peptide ETKADVDFNKKFRAS corresponded to amino acids 578-593 of the rainbow trout NHE3a protein sequence (GenBank accession ABO32815.1) that is also

likely to recognize AA's 578-593 of the NHE3b protein sequence (E[redacted]KADVDFNKKF[redacted]A[redacted]; see Chapter 2).

3. To identify V-ATPase, a rabbit polyclonal antibody raised against the A subunit of the killifish (*Fundulus heteroclitus*) was generously provided by Dr. Toyoji Kaneko (Katoh et al., 2003.

Sections were incubated for 20 h at 4° C with the NHE3 antibody (1:1000). For negative controls, sections were incubated with 1X PBS buffer lacking primary antibodies. Immunofluorescence was detected after incubating the sections with a 1:400 dilution of Alexa Fluor 546-coupled to goat anti-mouse IgG and goat anti-rabit IgG conjugated to Alexa Fluor 488 (Fisher, Ottawa, ON, Canada). Following the 3 x 10 min washes in 0.1X PBS, sections were mounted with Vectashield mounting medium (Vector Laboratories) containing DAPI to stain nuclei, and cover slipped. For negative controls, sections were incubated with 1XPBS buffer lacking primary antibodies and processed as above.

NHE3 in situ hybridization probes

Primers were designed to produce a 772 bp DIG labelled ribonucleotide probe for detection of rainbow trout NHE2 by in situ hybridization (Table 3.1). Gill total RNA (5 μg) was reverse transcribed using oligo-dT primer (Sigma Genosys) and Stratascript reverse transcriptase (Stratagene). PCR was performed on the resulting cDNA (0.5 μl in a 25 μl reaction). The final constitution of the PCR mix was: 1XPCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2 pmol each of forward and reverse primers, 2.5 units of Taq polymerase (Invitrogen), and 100 ng of trout cDNA. The following cycling parameters

were used: 4 min at 94° C followed by 40 cycles of 30 sec at 92° C, 30 sec at 58° C and 1 min at 72° C. The final extension of the amplified products was at 72° C for 10 min. A PCR cloning kit was used to clone the PCR products into a pCR II vector (Invitrogen) and was transformed into chemically competent DH5 alpha *e. coli* cells. The desired clone was extracted using a PureLink Quick Plasmid Miniprep Kit (Invitrogen). Purified plasmids were sequenced using M13 forward and reverse primers to confirm identity and determine the orientation of the cloned sequence within the vector. An antisense DIG labelled RNA probe was synthesized by linearizing 1 μg of plasmid with BamH I (Invitrogen) followed by in vitro transcription with T7 RNA polymerase (New England Biolabs) for 1 h at 37° C. Sense DIG labelled (Roche) RNA probes were created by linearizing 1 μg of plasmid with Xho I (Invitrogen) followed by in vitro transcription with SP6 RNA polymerase (Invitrogen) for 1 h at 37° C.

NHE3 in situ hybridization

The slides were incubated for 10 min at 60° C. The DIG-labelled RNA probe (100 ng) was denatured (boiled for 3 min and then rapidly chilled on ice) and added to the hybridization buffer [50% deionized formamide, 5X hybridization salts (0.75 M NaCl, 20 mM EDTA, 20 mM PIPES, pH 6.8), 1X Denhardt's, 0.2% SDS, 5% dextran sulphate (Sigma)]. Hybridization was performed for 20 h at 63° C in a humid chamber in a hybridization oven. The next day, the sections were washed twice in 2X SSC (15 min each at 60° C), twice in 0.2X SSC (15 min each at 60° C), and once in 0.1X SSC for 10 min at room temperature. For DIG detection, the sections were incubated first with 0.1 M PBS containing 1% goat serum, 2 mg ml^{-1} BSA and 0.3% triton-X at room temperature

for 1 h. This was followed by incubation in anti-digoxigenin conjugated with alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:1000 in 0.1 PBS (containing 1% goat serum, 2 mg ml^{-1} BSA and 0.3% triton-X) overnight at 4° C. The following day the slides were washed twice in 0.1 M PBS or (15 min each at room temperature). The slides were washed twice (5 min each) in coloration buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20). One nitroblue tetrazolium and 5-bromocresyl-3-indolyl phosphate (NBT/BCIP) tablet (Sigma) was dissolved in 10 ml water and this solution was layered over the sections. The chromogenic reaction was allowed to proceed in the dark until satisfactory coloration was achieved (at room temperature in a humid chamber). The slides were then washed twice with 0.1 M PBS (15 min each). The sections were covered with mounting media (60% glycerol) and cover slipped.

PAS staining of proximal tubules following immunocytochemistry

After the appropriate imunocytochemistry was completed in kidney sections and the images were taken, a periodic acid Schiff (PAS) staining protocol was performed according to manufacturers directions (Sigma-Aldrich). Briefly, the sections were hydrated (2 X 10 min) in 1X PBS and then periodic acid solution was applied for 5 min. Sections were then rinsed with water (2 X 10 min) and then Schiff's reagent was applied for 15 min. After washing the sections for 5 min in running tap water, Heamotoxylin solution was applied as counter stain for 90 sec. The images were taken in the same area that they were taken previously for immunocytochemistry.

Microscopy and image acquisition

Bright filed and fluorescence images were acquired using a Zeiss Axiophot epifluorescence microscope equipped with an Olympus DP70 digital camera. Images were processed using image-Pro Plus v.6.0.0 (Media Cybernetics.Inc).

Western blots and antibody specificity

Proteins (50 to 100 μg per lane) were separated by SDS-PAGE on 10% tris-tricine polyacrylamide gels and then transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories) using a wet transfer unit. The membranes were blocked in 5% PBST-milk for 1 h at room temperature. After blocking, the membranes were probed with an NHE3 antibody (1:200) for 2 h at 37° C. The specificity of the NHE3 antibody was established in Chapter 2. All membranes were incubated in goat anti-rabbit IgG, horseradish peroxidase (1:5,000, Amersham Life Sciences) for 1 h at room temperature. After washing (3 times 5 min in TBST), the proteins were visualized using Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer). The protein size marker used was obtained from Fermentas Life Sciences. To estimate the changes in protein levels between control and hypercapnia tissue samples (see below), experimental and control protein samples were separated by SDS-PAGE and transferred onto the same 0.45-mm nitrocellulose membranes. The blots were blocked in 5% PBST-milk for 1 h at room temperature and the membrane was probed with NHE3 antibody (1:200) for 2 h at 37° C. To compensate for deviation in the amount of protein loaded, the same membrane was stripped using Re-Blot Plus mild stripping solution (CHEMICON). The membrane was then probed with a β-actin antibody (1:500, Sigma) for 1 h at 37° C, incubated in anti-mouse Igg, horseradish peroxidase (1:5,000) for 1 h at room temperature and washed

3 times for 5 min in TBST. The proteins were visualized as described above. The size and the opacity of NHE3 bands, relative to the size and the intensity of ß-actin bands were calculated using Image J analysis software.

Exposure of fish to hypercapnia

Adult fish were placed into black plastic boxes supplied with flowing and aerated water and were allowed to recover for 24 h. After acclimation fish were exposed to external hypercapnia for 24 h with intended final water PCO_2 of 7.5 mm Hg. To achieve hypercapnia, a water equilibrium column was gassed with mixtures of CO_2 and air (Sierra C100L Smart-trak mass flow controllers; SRB Controls). Water PCO_2 was monitored by using a CO_2 electrode connected to a CO_2 meter (Cameron Instruments). Differences from the intended water PCO_2 were corrected by adjusting the gas and the water flows through the equilibration column. For investigating changes in protein levels, fish were killed and kidney tissues were collected after 24 h of exposure to hypercapnia (N = 6) or normocapnia (controls; N = 6). To assess the changes in NHE3 mRNA levels using real-time PCR, tissues were collected after 3, 12, and 24 h (N = 6 at each time point) of exposure to hypercapnia. Control fish were also killed at 3, 12, and 24 h (N= 6 at each time point) of exposure to normocapnia.

Cortisol implants

Fish (N = 6) were lightly anesthetised with benzocaine (0.5g L^{-1} for ~30 sec or until they did not respond to touch), weighed and given an intra-peritoneal implant of cortisol (0.11 mg g^{-1} body weight; hydrocortisone 21-hemisuccinate sodium salt; Sigma-

Aldrich, Inc) dissolved in coco butter (22 mg of cortisol per 1 ml of coco butter). After 3 days, fish were anesthetised with benzocaine and blood was collected into heparinised syringes from the caudal vessels. Fish were then killed, kidney tissues were dissected, frozen and then process for real-time PCR or Western blotting.

Statistical analyses

The effect of exposure to hypercapnia on kidney NHE3 mRNA expression as determined by real-time PCR was analyzed using one-sample Student's *t*-tests. Differences in band size and intensities on Western blots were assessed using unpaired Student's *t*-tests.

Table 3-1 Oligonucleotide primers used for real-time PCR, and in situ probe construction.

Primer name	Sequence	Use
NHE3-F	5'-GCT CCC TGG TTG GTA TTA TC- 3'	In situ hybridization
NHE3-R	5'-AAC CAG CAC AAC CAC CTC TC- 3'	In situ hybridization
QNHE3-F	5'-AGA GCA GCC GTG ACA GAA CT-3'	NHE3 RT-PCR
QNHE3-R	5'-AAC CAG CAC AAC CAC CTC TC-3'	NHE3 RT-PCR
Actin-F	5'- CCA ACA GAT GTG GAT CAG CAA-3'	RT-PCR reference
Actin-R	5'- GGT GGC AGA GCT GAA GTG GTA-3'	RT-PCR reference

RESULTS

Relative abundance of NHE2 and NHE3 mRNA in the kidney

The distribution of NHE2 and NHE3 mRNA levels within the kidney tissues was examined using real-time PCR (Fig. 3-1). Relative NHE3 to NHE2 kidney mRNA distribution revealed that NHE3 was the predominant isoform in the kidney and exceeded NHE2 expression by approximately 15,000X.

Localization of NHE3 and V-ATPase to the proximal nephron

In situ hybridization clearly demonstrated that a subset of renal tubules contained NHE3 mRNA (Fig. 3-2). Tissues processed as negative controls, either using a DIG-labelled sense probe (Fig. 3-2C) or by omission of probe (Fig. 3-2D), exhibited background staining only. Using a homologous NHE3 polyclonal antibody (Chapter 2), NHE3 protein was also localized to the apical membrane of a sub population of renal tubules (Fig. 3A) expressing basolateral Na^+/K^+-ATPase. Some tubules that were particularly enriched with Na^+/K^+-ATPase exhibited no NHE3 immunoreactivity (Fig. 3-3A). Subsequent treatment with PAS (to identify brush borders) of the same tissue sections processed for immunofluorescence, identified the proximal tubule as the site of apical NHE3 (Fig. 3-3B). Immunoflorescence was eliminated by omission of primary antibodies Inset in panel A) indicating that non specific binding of the secondary antibody to kidney tissue sections was negligible. A similar staining pattern was observed using a rabbit antibody raised against killifish (*Fundulus heteroclitus*) A-subunit of V-ATPase (Katoh et al., 2003) in which V-ATPase protein was localized to

apical membrane of proximal tubules (Fig. 3-4A, B). Immunofluorescence was eliminated after omission of primary antibodies (inset in panel A).

Effects of hypercapnia and cortisol on kidney NHE3 levels

Kidney NHE3 mRNA levels were significantly increased after 3, 12 and 24 h of exposure to hypercapnia (Fig. 3-5). Consistent with the increase in mRNA expression, kidney NHE3 protein levels increased significantly after hypercapnia exposure (Fig. 3-6). Plasma cortisol concentrations were significantly elevated after 24 h of hypercapnia (Fig. 3-7A) suggesting that cortisol could be regulating renal NHE3 at the transcriptional level. In support of this idea, elevating plasma cortisol levels using intraperitoneal cortisol implants (see Chapter 2) was associated with increased NHE3 mRNA expression after 48 and 72 h (Fig. 3-7B).

DISCUSSION

Although the fish gill is the major site of acidic equivalent exchanges between the internal and external environments, the kidney also plays an essential role in systemic acid-base balance by regulating the extent of HCO_3^- reabsorption (Georgalis et al., 2006; Perry et al., 1987; Wheatly et al, 1984; Wood and Jackson, 1980; Wood et al., 1999). For example, during metabolic compensation of respiratory acidosis, plasma HCO_3^- levels can become markedly elevated (e.g. > 50 mmol l^{-1} in European eel (*Anguilla anguilla*) (Mckenzie et al., 2002) owing to increased net branchial acid excretion (Cameron, 1976). To sustain elevated levels of plasma HCO_3^-, the additional HCO_3^- being filtered at the kidney must be reabsorbed. The reabsorption of HCO_3^- requires equimolar secretion of H^+ into the filtrate and thus while the net output of acid by the kidney may be minor compared to the gill, H^+ secretion is increased markedly. For example, to sustain plasma levels of HCO_3^- at 20 mmol l^{-1} (a typical value for rainbow trout experiencing metabolic compensation of hypercapnic acidosis (Goss and Perry, 1993), would require additional renal H^+ secretion of approximately 150 μmol kg^{-1} h^{-1} [this estimate assumes a glomerular filtration rate of 7 ml kg^{-1} h^{-1} or 1.77 X urine flow rate (Perry et al 1987; Wheatly et al, 1984)]. This estimate of renal acid secretion is similar to measured rates of branchial net acid excretion during compensation of hypercapnia (Goss and Perry, 1993). Moreover, unlike the transient increase in branchial acid excretion that is required to elevate plasma [HCO_3^-], the increased rates of renal acid secretion must be maintained even after metabolic compensation is complete. The mechanisms underlying renal acid secretion and HCO_3^- reabsorption in fish have not been fully elucidated. Indeed, current

models for tubular acid secretion in fish (Perry et al., 2003b; Perry and Gilmour, 2006; Perry et al., 2003d;) are largely based on extrapolation from mammalian models. In these models, intracellular H^+ is generated via the hydration of CO_2 by a cytosolic isoform of carbonic anhydrase (tCAc; (Esbaugh et al., 2005) within the proximal convoluted tubule, the predominant site of HCO_3^- reabsorption. Secretion of H^+ across the apical membrane is achieved via V-ATPase (Wagner et al, 2004) and NHE3 (Aronson, 2002; Wu et al., 1996). It was recently demonstrated that in trout kidney, the secretion of H^+ and associated HCO_3^- reabsorption is facilitated by externally oriented apical (luminal) membrane bound CA IV (Georgalis et al., 2006). As is mammalian models (Schwartz, 2002; Winkler et al., 1997), the facilitation of HCO_3^- reabsorption by CA IV stems from its role in catalyzing the dehydration of filtered HCO_3^- within the lumen of the proximal tubule. The CO_2 thus formed diffuses into the tubule cells where it is hydrated to HCO_3^- and H^+, a reaction catalysed by tCAc; the HCO_3^- is moved across the basolateral membrane via a Na^+/HCO_3^- co-transporter (Romero and Boron, 1999). Overall, HCO_3^- reabsorption is favored because H^+ is recycled via NHE3 and V-ATPase. An important contribution of the current study was the first demonstration of NHE3 in proximal tubule of any fish species and its co-localization with V-ATPase thereby substantiating two putative components of the models for piscine renal acid secretion and HCO_3^- reabsorption (Perry et al., 2003b). Although Perry and Fryer (1997) previously demonstrated the presence of apical membrane V-ATPase expression in trout proximal tubules, they did not comment on the co-expression of NHE3. Another significant outcome of the present study was the finding that renal NHE3 protein expression is increased by respiratory acidosis owing to transcriptional regulation. The increased levels

of NHE3 protein during hypercapnia presumably reflect the need to secrete greater quantities of H^+ into the filtrate to reabsorb the greater quantities of HCO_3^- being filtered at the glomerulus. Evidence for increased activity of renal Na^+/H^+ exchange during hypercapnia was provided by Georgalis et al. (2005) by demonstrating that inhibition of renal CA (a key component of the $NaHCO_3$ reabsorption process) using acetazolamide caused a much larger increase in urinary Na^+ levels during hypercapnia compared to normocapnia. Although not assessed in this study, previous research has shown VATPase, tCAc and tCA IV mRNA in the trout kidney also increase during hypercapnic acidosis (Georgalis et al., 2006; Perry et al., 2003b;). Thus, the net consequence of systemic respiratory acidosis in rainbow trout is the induction of the cellular machinery required to enhance renal HCO_3^- reabsorption. We believe that elevated levels of cortisol (from 35.3 ± 9.4 to 100.1 ± 30.9 ng ml^{-1}; Figure 3-7A) during hypercapnia are responsible, at least in part, for the transcriptional activation of renal NHE3. Only a few studies have examined the impact of hypercapnia on plasma cortisol levels in fish and these have yielded variable results. Although elevated levels of cortisol have been reported in hypercapnic white sturgeon (*Acipenser transmontanus*) (Crocker et al., 2000) and Atlantic salmon smolts (*Salmo salar*) (Fivelstad et al., 1999), the levels in the European eel (*Anguilla anguilla*) (Mckenzie et al., 2002) remained unaltered during exposure to high ambient PCO_2. It is likely that species differences account for the inconsistent data. While indirect, there is evidence for the involvement of cortisol in the increased expression of NHE3. First, the elevation of plasma cortisol levels in hypercapnic trout occurred concurrently with increasing NHE3 mRNA and protein expression. Second, the administration of exogenous cortisol resulted in an increase in

NHE3 mRNA. This conclusion is also supported by previous studies on mammals demonstrating that glucocorticoids transcriptionally activate renal NHE3 expression (Hayashi et al., 2002; Kandasamy and Orlowski, 1996) and promote renal tubular acidification (Baum and Quigley, 1993). Further studies should be directed at teasing apart the relative participation of glucocorticoids, other hormones including endothelin (Laghmani et al, 2001) and angiotensin II (Li and Zhuo, 2007), and the direct effects of acidosis (Hayashi et al., 2002).

Figure 3-1. Relative NHE2 and NHE3 mRNA level in rainbow trout kidney as determined by real-time PCR (N = 6). Statistical analysis of the data demonstrated that on a relative basis, NHE3 mRNA (means ± 1 SEM) was approximately 15,000X more abundant in kidney than NHE2 (P = 0.002). The reference gene used was ß-actin and data were compared relative to NHE2 mRNA which was given a relative value of 1.

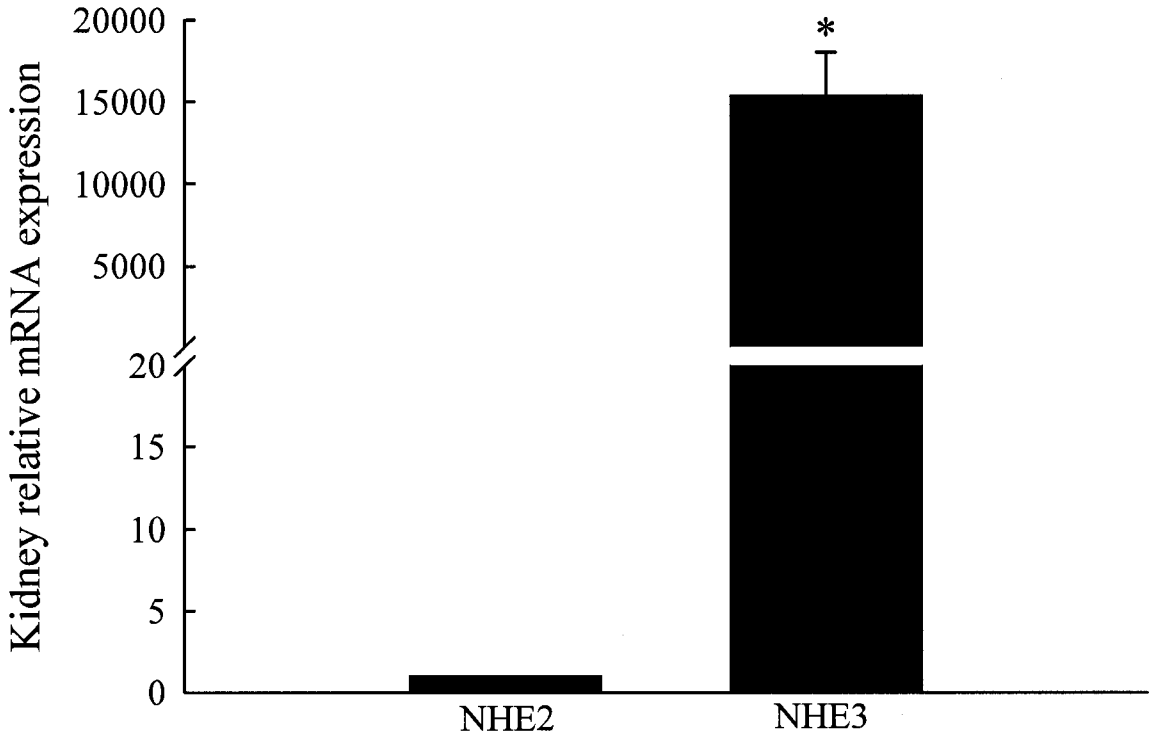

Kidney relative mRNA expression
20000
15000
10000
5000
20
15
10
5
0
*
NHE2
NHE3

Figure 3-2. Identification of NHE3 mRNA enriched renal tubules on 10 μm sections of rainbow trout kidney. As indicated by the black arrowheads (A) and (B), DIG-labelled antisense NHE3 probe was detected in some renal tubules and not in others. Using labelled sense strand probe for NHE3 (C) and no-probe control (D) did not result in any localization pattern. Bar 40 μm.

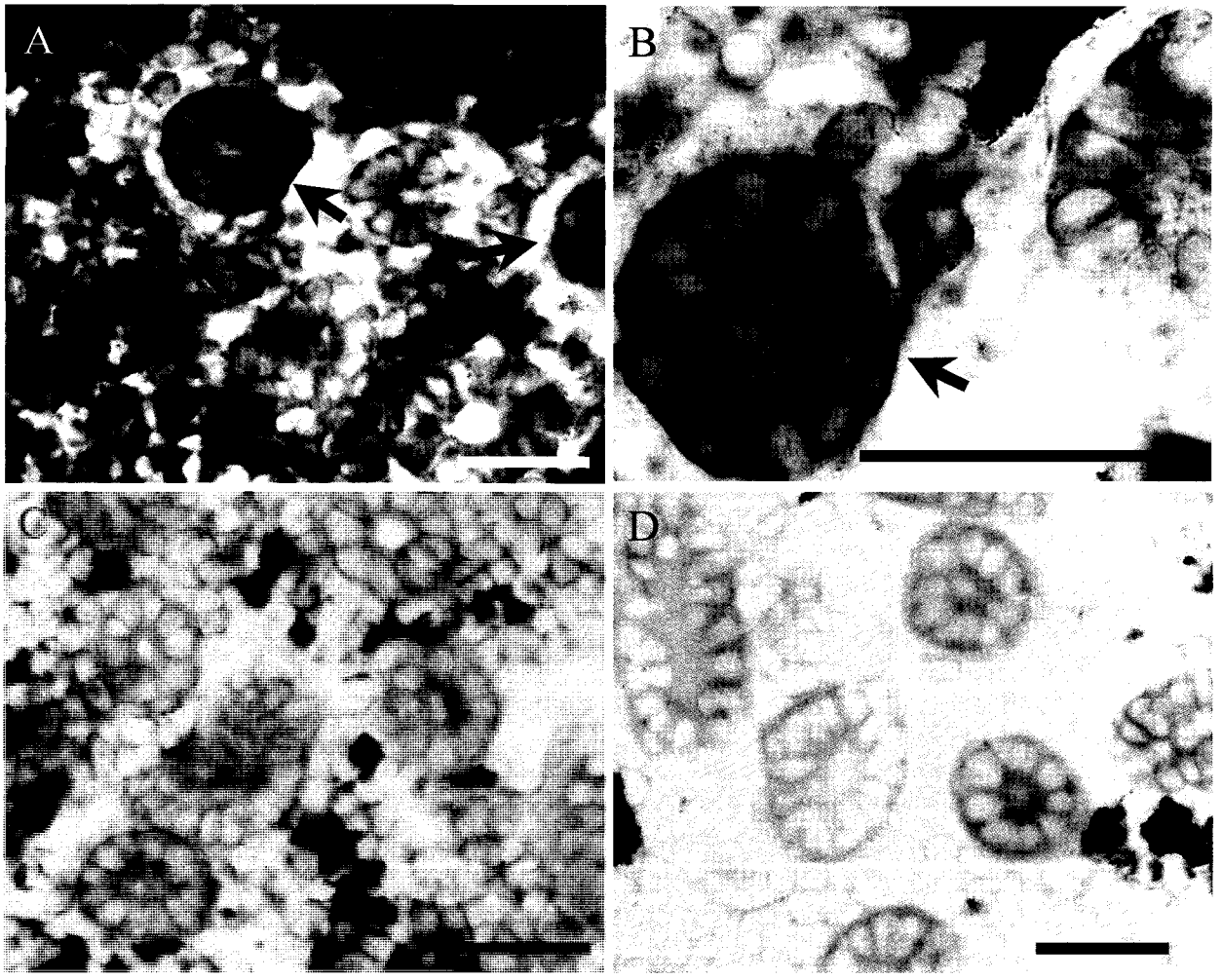

A
B
C
D

Figure 3-3. Localization of apical NHE3 to proximal renal tubules on 10 μm sections of rainbow trout kidney. (A) Apical NHE3 (green) was co-localized with Na^+/K^+ ATPase (red); nuclei are stained blue (DAPI). PAS staining of the same section (B) demonstrated that NHE3 is specifically localized to the brush border of proximal tubule cells. Apical NHE3 expressing renal tubules are labelled with white arrows. The NHE3 and Na^+/K^+ ATPase immunofluorescence disappeared with omission of primary antibodies (inset in A) after sections were incubated with appropriate concentration of secondary antibody and image was taken under the same exposure as in (A). Bar 50 μm.

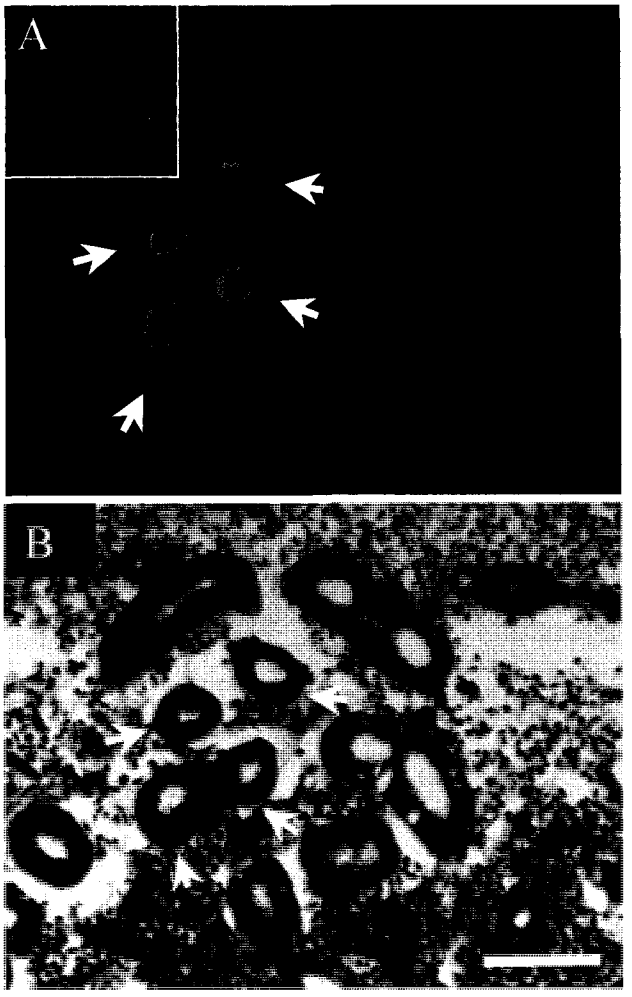

A
B

Figure 3-4. Localization of apical V-ATPase to proximal renal tubules on 10 μm serial sections of rainbow trout kidney. (A)Apical V-ATPase (green) expressing renal tubules identified with rabbit polyclonal antibody against *Fundulus* H-ATPase co-localize with Na^+/K^+ ATPase (red) and also co-localize with proximal tubule brush border stain, PAS, that was done on an adjacent section (B). Apical V-ATPase expressing renal tubules are labelled with white arrows. The V-ATPase and Na^+/K^+ ATPase immunofluorescence disappeared with omission of primary antibodies (inset in A) after sections were incubated with appropriate concentration of secondary antibody and image was taken under the same exposure as in (A) respectively. Bar 40 μm.

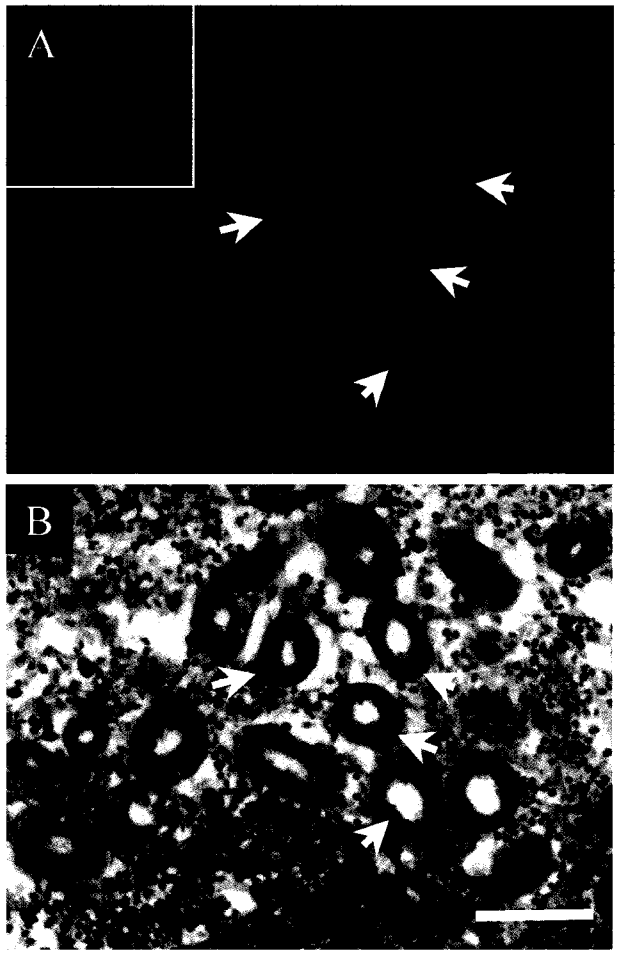

A
B

Figure 3-5. Effects of 3, 12 and 24 h of hypercapnia exposure (water PCO_2 = 7.5 mm Hg) on trout kidney NHE3 mRNA levels as determined by real-time PCR. Kidney NHE3 mRNA levels were significantly increased at all periods of hypercapnia exposure (P values = 0.0001, 0.0051, 0.0009, respectively; Student's t-test).

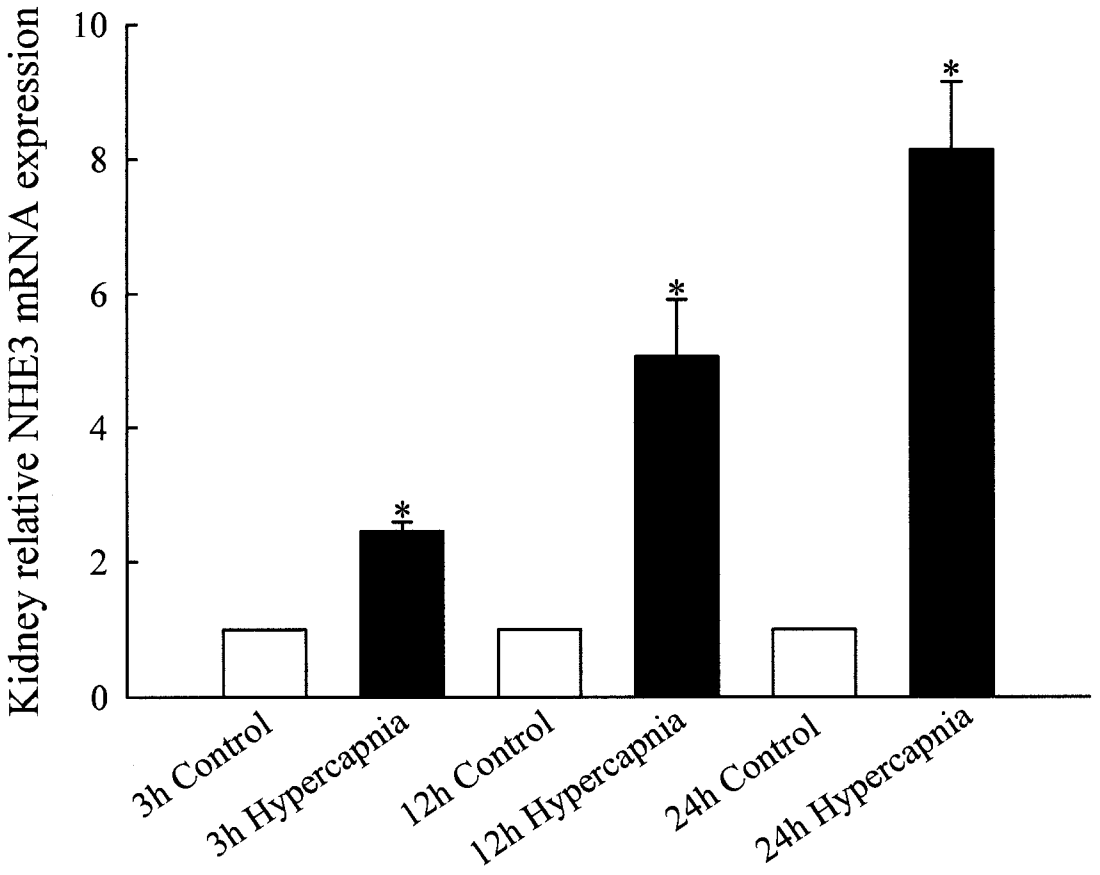

Kidney relative NHE3 mRNA expression
10
8
6
4
2
0
*
*
*
3h Control
3h Hypercapnia
12h Control
12h Hypercapnia
24h Control
24h Hypercapnia

Figure 3-6. Effects of 24 h of hypercapnia exposure (water PCO_2 = 7.5 mm Hg) on trout kidney NHE3 protein level as determined by analysis of Western blots. Western blots using trout NHE3 antibody (A) show increase in kidney NHE3 protein levels after exposure to 24 h hypercapnia (water PCO_2 = 7.5 mm Hg). Densitometric analysis of blots from normocapnic and hypercapnic tissues (B) confirmed these data and revealed significant increase (P = 0.033; Student's *t*-test) of NHE3 protein levels after 24 h hypercapnia exposure.

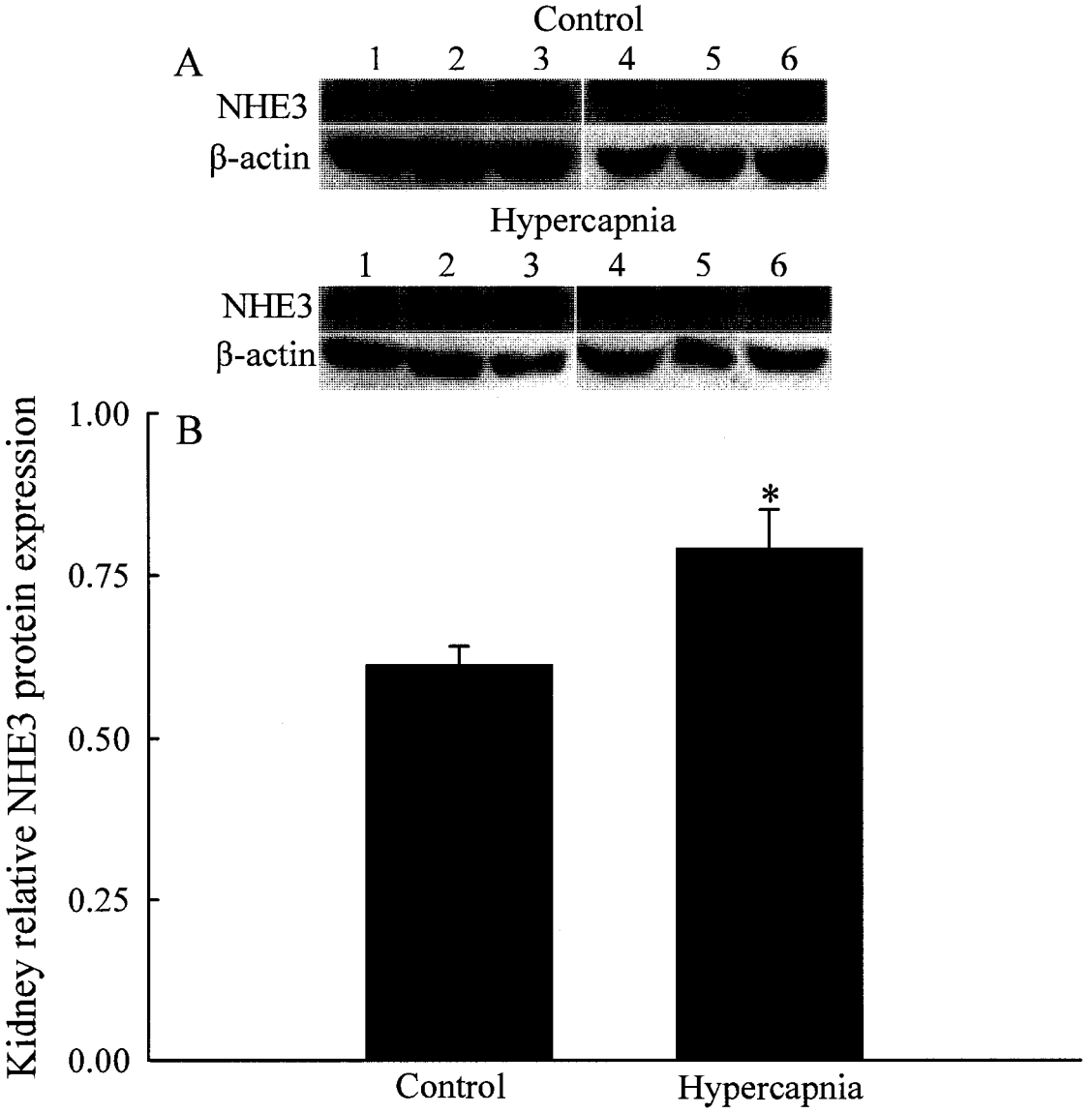
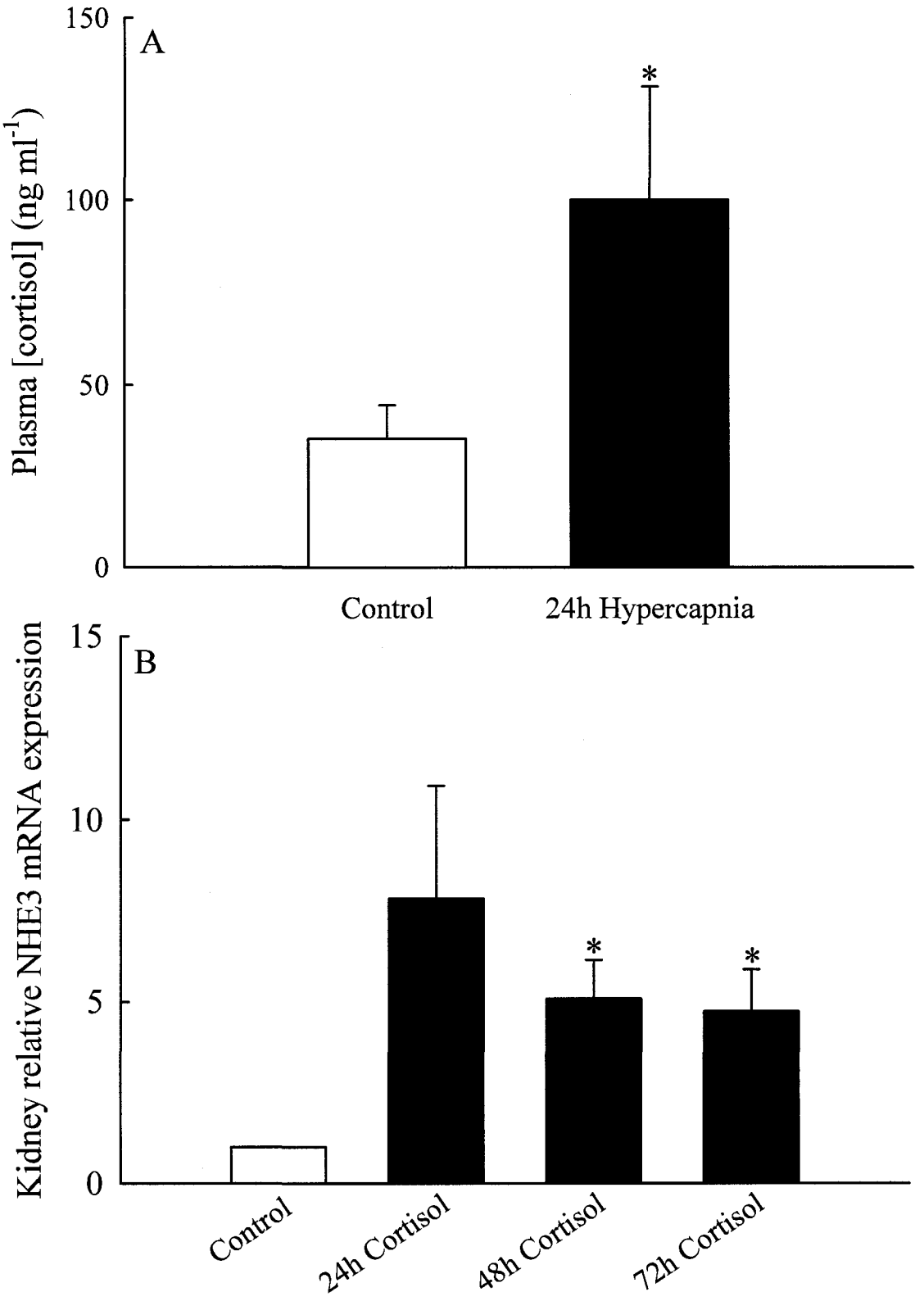

A
Control
1 2 3 4 5 6
NHE3
β-actin
Hypercapnia
1 2 3 4 5 6
NHE3
β-actin
B
Kidney relative NHE3 protein expression
1.00
0.75
0.50
0.25
0.00
*
Control
Hypercapnia

Figure 3-7. Effects of elevated plasma cortisol on kidney NHE3 mRNA levels 24, 48 and 72 h after cortisol implants were injected into the body cavity of fish (N = 5 for 24 h; N = 6 for 48 and 72 h) as measured by real-time PCR. Plasma cortisol levels (A) after fish were implanted with 0.11 mg of cortisol per 1g of body weight (0.5 ml coco butter implant containing 22 mg cortisol/ml coco butter for 100g fish) showing highly elevated plasma cortisol levels 24 h post injection with P values 0.0001, (Student's *t*-test). Kidney NHE3 mRNA levels (B) were significantly elevated 24 h post injection of cortisol, but was statistically increased only after 48 and 72 h post injection [(P values = 0.0901, 0.0119, 0.0230, respectively (Student's *t*-test)]. The reference gene used was ß-actin and control fish (N = 6) were used to compare the data at 24, 48 and 72 h.


A
150
100
50
0
Plasma [cortisol] (ng ml-1)
*
Control
24h Hypercapnia
B
15
10
5
0
Kidney relative NHE3 mRNA expression
*
*
Control
24h Cortisol
48h Cortisol
72h Cortisol

CHAPTER 4

GENERAL DISCUSSION

Freshwater fish regulate their internal acid-base status by a combination of branchial, renal, and metabolic processes (Goss et al., 1995; Claiborne et al., 2002a; Perry et al., 2003d; Evans et al., 2005b) with the gills and the kidney functioning as the major sites of acid-base regulation (Cameron et al., 1982; Wood et al., 1999; Perry et al., 2003). This thesis provides the first direct demonstration of SLC9 Na^+/H^+ exchange genes (NHE) in rainbow trout gill and kidney. Using a combination of techniques including immunocytochemistry, in situ hybridization, immunoblotting and real-time PCR, the results of this thesis provide evidence for the involvement of two NHE isoforms, SLC9A2 (NHE2) and SLC9A3 (NHE3) in branchial and renal acid-base and ionic regulation.

The involvement of NHEs in the gill

The results of this study demonstrate that in trout, both NHE2 and NHE3 are confined to the PNA^+ MRC and not to the PNA^- MRC as expected (Perry and Gilmour, 2006). This implies that the model of gill acid base balance must be refined, with NHE2 and NHE3 located in PNA^+ MRCs. This clearly complicates the current model of a net acid/base-secreting PNA^+ MRC because if operating at equal rates, apical membrane Cl^-/HCO_3^- exchange and Na^+/H^+ exchange would be acid-base neutral. Therefore, I propose a new model in which the apical Na^+/H^+ and Cl^-/HCO_3^- exchangers are differentially regulated within the PNA^+ MRC to allow net excretion of acid or base (Fig. 4-1).

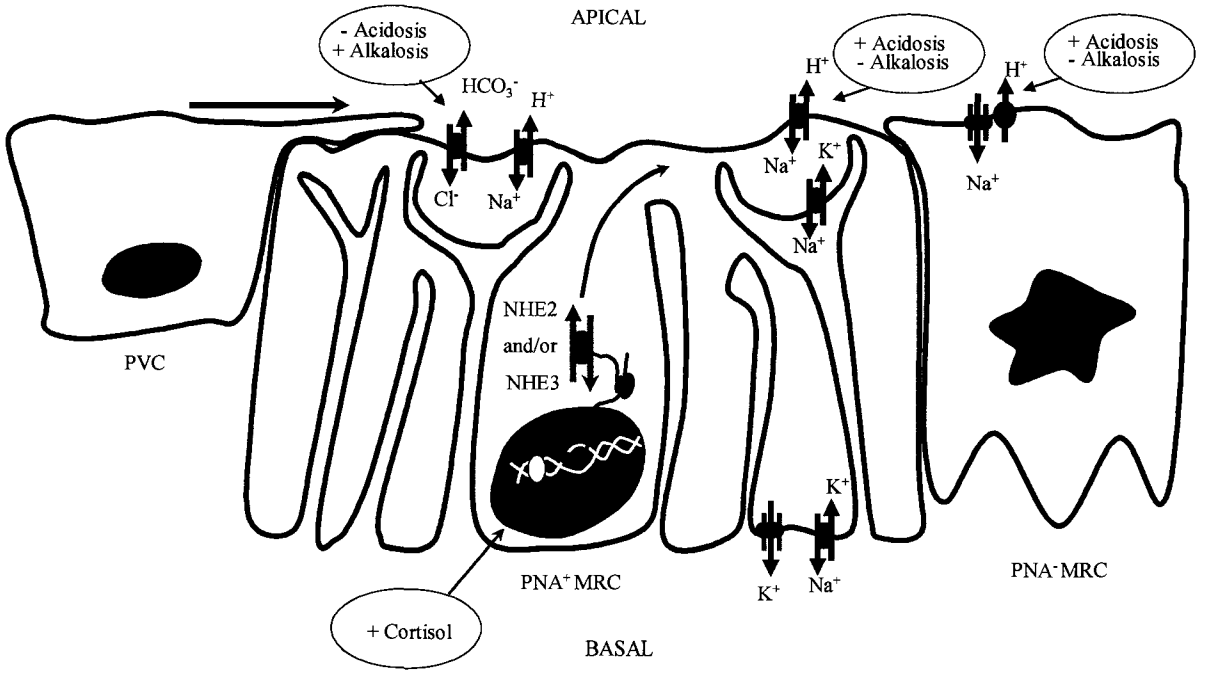


Figure 4-1: A novel model for acid-base balance and ion uptake in FW rainbow trout showing proposed roles of NHE2 and NHE3 in PNA^+ MRCs and V-ATPase/Na^+ channel in PNA^- MRCs. Cytoplasmic carbonic anhydrase (CA) catalyses the hydration of intracellular CO_2 to generate HCO_3^- for apical Cl^-/HCO_3^- exchange and H^+ for pumping (PNA^- cell) or electroneutral exchange with Na^+ (PNA^+ cell).

According to this model, physical covering of PNA^+ MRCs by adjacent pavement cells (PCs) during acidosis would decrease equally the rates Cl^-/HCO_3^- and Na^+/H^+ exchange. However, based on the findings of this thesis with indicate that NHE2 mRNA levels increase during acidosis, it is possible that a transcriptional increase in the numbers of Na^+/H^+ exchangers could transform the previously acid/base neutral PNA^+ MRC into an acid secreting cell during acidosis. Additional help in dealing with acidosis could be provided by an increase in acid excretion via PNA^- MRCs, presumably through V-ATPase coupled to Na^+ channel (Reid et al 2003). The findings of this thesis further suggest that the transformation of the PNA^+ MRC into a net acid excreting cell during hypercapnia may be linked to elevated circulating levels of the stress hormone .

The involvement of NHEs in the kidney

The results of this study demonstrate that in the trout proximal nephron, NHE3 is confined to the apical membrane as proposed in previous models (e.g. Perry and Gilmour, 2006). Figure 4-2 is a revised version of the previous models that incorporates the findings of this thesis.

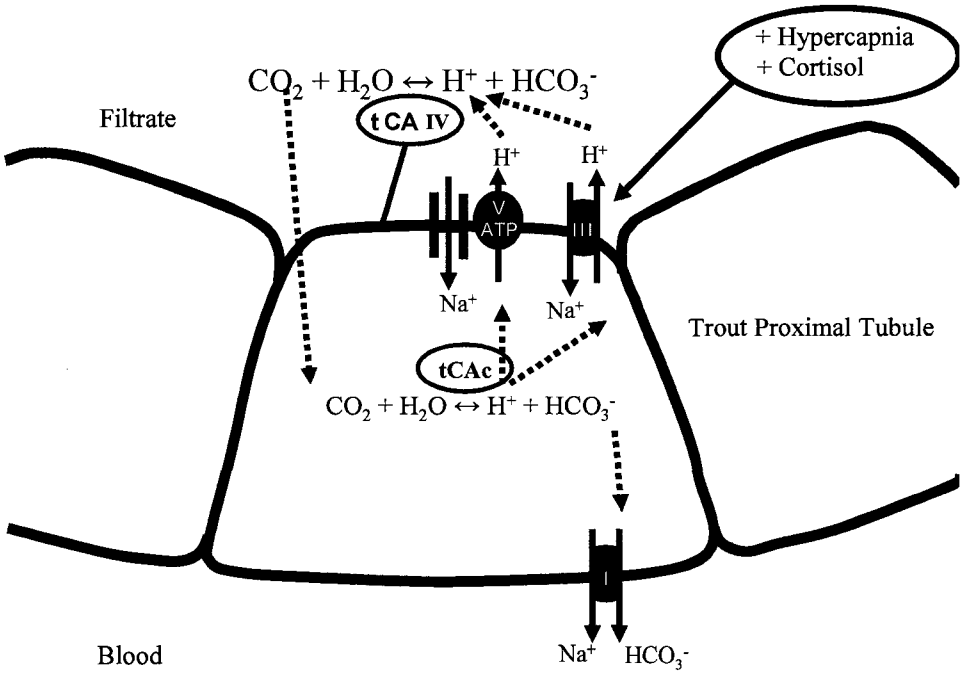


Figure 4-2: Renal acid-base balance and HCO_3^- reabsorption model showing the proposed role of NHE3 and V-type H-ATPase in the proximal nephron. Trout cytoplasmic carbonic anhydrase (tCAc) catalyses the hydration of intracellular CO_2 generating HCO_3^- for uptake by Na^+-HCO_3^- co-transporter I (NBC I) and H^+ for excretion across the apical membrane via NHE3 and V-ATPase.

The results of this thesis indicate that NHE3 and V-ATPase are co-localized to the apical membrane of proximal tubule cells where they are presumed to play key roles in acidification of the filtrate and hence HCO_3^- reabsorption. Secretion of H^+ results in the rapid formation of filtrate CO_2 in the presence of membrane bound tCA IV (Georgalis et al., 2005). The CO_2 diffuses back into the tubule cells where it is catalytically hydrated to H^+ and HCO_3^- by tCAc. The resultant H^+ enters the lumen via NHE3 or V-ATPase while the HCO_3^- exits the cell at the basolateral membrane via NBC1 (Swenson 2000; Perry et al., 2003a; Perry and Gilmour, 2006). Overall, HCO_3^- reabsorption is favored because H^+ is recycled via NHE3 and V-ATPase. Based on quantitative analysis of the mRNA and protein levels (Chapter 3), this thesis provides evidence for transcriptionally mediated increased numbers of NHE3 proteins during hypercapnia. I propose that the hormonal trigger for the regulation of renal NHE activity during hypercapnia is an elevation of plasma cortisol levels given that cortisol treatment caused a significant increase in renal NHE3 mRNA expression. Cortisol-induced transcriptional regulation of Na^+/H^+ exchange activity was documented previously in the mammalian proximal nephron (Baum and Quigley, 1993).

Although the results of this thesis have advanced the model of renal acid base balance in rainbow trout, further experiments are needed to localize NBC1 to the basolateral membrane of the proximal tubule. Additional investigations should also focus on whether other membrane-associated isoforms, such as NHE2 are also present, as is the case in mammals (Schwartz 2002; Ledoussal et al., 2001).

Conclusions

The results of this study provide the first direct evidence for the existence of apical membrane NHE isoforms in the gills and the kidney of rainbow trout. The predominant branchial isoform, NHE2, is likely to play a key role in acid excretion during hypercapnia. Both NHE2 and NHE3 are confined to PNA^+ MR cells while renal NHE3 is localized specifically to the proximal segment of the nephron where it is presumed to play an important role in renal HCO_3^- reabsorption, thereby contributing to systemic acid-base regulation. The fact that both gill and kidney isoforms are up-regulated during the hypercapnia indicates that trout rely on integrated compensatory mechanisms involving both the gill and kidney during acid-base disturbances and that these responses may be linked to the stress hormone cortisol.

REFERENCES

Alper S. L. 2006. Molecular physiology of SLC4 anion exchangers. *Exp. Physiol.* 91 (1):153-161.

Aronson PS. 2002. Ion exchangers mediating NaCl transport in the renal proximal tubule. *Cell Biochem. Biophys.* 36: 147-153.

Avella M. and M. Bornancin. 1989.A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* 142:155-175.

Baum M.and R. Quigley. 1993. Glucocorticoids stimulate rabbit proximal convoluted tubule acidification. *J. Clin. Invest.* 91:110-114.

Baum, M., Cano, A., Alpern, R.J., 1993. Glucocorticoids stimulate Na^+/H^+ antiporter in OKP cells. Am. J. Physiol. 264, F1027-F1031.

Biemesderfer D, Pizzonia J, Abu-Alfa A, Exner M, Reilly RF, Igarashi P, Aronson PS. 1993. NHE3: a Na^+/H^+ exchanger isoform of renal brush border. *Am. J. Physiol.* 265:F736–F742.

Biemesderfer D, Rutherford PA, Nagy T, Pizzonia JH, Abu-Alfa AK, Aronson PS. 1997. Monoclonal antibodies for high resolution localization of NHE3 in adult and neonatal rat kidney. *Am. J. Physiol.* 273: F289–F299.

Bioche Heisler, N., 1984. Acid-base regulation in fishes. In: W.S. Hoar, D.J. Randall (Eds.), *Fish Physiology Vol XA. Academic Press, New York*, pp. 315-401.

Brant S. R., Yun C. H. C., Donowitz M., and C. M. Tse. 1995. Cloning, tissue distribution, and functional analysis of the human Na+/H+ exchanger isoform, NHE3. *Amer. J. Physiol-Cell. Physiol.* 38 (1):C198-C206.

Brett C.L., Donowitz M., Rao R. 2005. Evolutionary origins of eukaryotic Na^+/H^+ exchangers. *Amer. J. Physiol.* 288: (C)223-239.

Cameron J. N. and G. A. Kormanik. 1982. The acid-base responses of gills and kidneys to infused acid and base loads in the channel catfish, *Ictalurus punctatus*. *J. Exp. Biol.* 99:143-160.

Cameron, J.N., 1976. Branchial ion uptake in Arctic grayling: resting values and the effects of acid-base disturbance. *J. Exp. Biol.* 64, 711-725.

Cameron, J.N., 1978. Regulation of blood pH in teleost fish. *Respir. Physiol.* 33, 129-144.

Catches J. S., Burns J. M., Edwards S. L., and J. B. Claiborne. 2006. Na^+/H^+ antiporter, V-H+-ATPase and Na^+/K^- -ATPase immunolocalization in a marine teleost (Myoxocephalus octodecemspinosus). *J. Exp. Biol.* 209 (Pt 17):3440-3447.

Chambrey R., Warnock D.G., Podevin R.A., Bruneval P., Mandet, C., Belair M.F., Bariety J., Paillard, M., 2001. Immulocalization of Na+/H+ exchanger isoform NHE2 in rat kidney. *Am. J. Physiol.* 275, F379 –F386.

Choe K. P., A. I. Morrison-Shetlar, B. P. Wall, and J. B. Claiborne. 2002. Immunological detection of Na(+)/H(+) exchangers in the gills of a hagfish, Myxine glutinosa, an elasmobranch, Raja erinacea, and a teleost, Fundulus heteroclitus. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 131 (2):375-385.

Choe K. P., Edwards S. L., Claiborne J. B., and D. H. Evans. 2007. The putative mechanism of Na(+) absorption in euryhaline elasmobranchs exists in the gills of a stenohaline marine elasmobranch, *Squalus acanthias*. *Comp. Biochem. Physiol A Mol. Integr. Physiol* 146 (2):155-162.

Choe, K. P. and Evans, D. H. 2003. Compensation for hypercapnia by a euryhaline elasmobranch: effect of salinity and roles of gills and kidneys in fresh water. *J. Exp. Zool. 297A: 52-63.*

Choe, K. P., O'Brien, S., Evans, D. H., Toop, T., and Edwards, S. L. 2004. Immunolocalization of Na^+/K^+-ATPase, carbonic anhydrase II, and vacuolar H^+-ATPase in the gills of freshwater adult lampreys, *Geotria australis. J. Exp. Zool.* 301A: 654-665.

Claiborne J. B., Blackston C. R., Choe K. P., D. C. Dawson, S. P. Harris, L. A. Mackenzie, and A. I. Morrison-Shetlar. 1999. A mechanism for branchial acid excretion in marine fish: identification of multiple Na^+/H^+ antiporter (NHE) isoforms in gills of two seawater teleosts. *J. Exp. Biol.* 202:315-324.

Claiborne J. B.. Acid-base regulation. 1998.The Physiology of Fishes. Anonymous. Evans DH. Boca Raton:CRC Press. 2:171-198.

Claiborne J.B., Edwards S.L., Alison I. Shetlar A.I.M. 2002a. Acid–Base Regulation in Fishes: Cellular and Molecular Mechanisms. *J. Exp. Zool.* 293: 302-319.

Claiborne, J.B., Edwards, S.L., Morrison-Shetlar, A.I., 2002b. Acid-base regulation in fishes: cellular and molecular mechanisms. *J. Exp. Zool.* 293, 302-319.

Counillon L. and J. Pouyssegur 2000. The expanding family of eucaryotic Na^+/H^+ exchangers *J. Biol. Chem.* 275(1):1-4.

Crocker CE, Farrell AP, Gamperl AK and Cech JJ, Jr. 2000.Cardiorespiratory responses of white sturgeon to environmental hypercapnia. *Am. J. Physiol.* 279: R617-R628.

Edwards S. L., Claiborne J. B., A. I. Morrison-Shetlar, and T. Toop. 2001.Expression of Na^+/H^+ exchanger mRNA in the gills of the Atlantic hagfish (*Myxine glutinosa*) in response to metabolic acidosis. *Comp. Biochem. Physiol. A* 130:81-91.

Edwards S. L., Donald J. A., T. Toop, M. Donowitz, and C. M. Tse. 2002. Immunolocalisation of sodium/proton exchanger-like proteins in the gills of elasmobranchs. *Comp. Biochem. Physiol A Mol. Integr. Physiol* 131 (2):257-265.

Edwards S. L., Wall B. P., A. Morrison-Shetlar, S. Sligh, J. C. Weakley, and J. B. Claiborne. 2005. The effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (Fundulus heteroclitus). *J. Exp. Zoolog. A Comp. Exp. Biol.* 303 (6):464-475.

Esbaugh A., Perry S.F., Bayaa M., Georgalis T., Nickerson J.G., Tufts B.L., Gilmour K.M. 2005. Cytoplasmic carbonic anhydrase isozymes in rainbow trout Oncorhynchus mykiss: comparative physiology and molecular evolution. *J. Exp. Biology*. 208: 1951-1961.

Evans, D.H., 2002. Cell signaling and ion transport across the fish gill epithelium. *J. Exp. Zool.* 293, 336-347.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005a. The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste. *Physiol. Rev.* 85, 97-177.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005b. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 85, 97-177.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005c. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 85, 97-177.

Evans, D.H., Piermarini, P.M., Potts, W.T.W., 1999. Ionic transport in the fish gill epithelium. J. Exp. Zool. 283, 641-652.

Fenton, R.A., Knepper, M.A., 2007. Mouse Models and the Urinary Concentrating Mechanism in the New Millennium. *Physiol. Rev.* 87, 1083-1112.

Fenwick J. C., Wendelaar Bonga S. E., and G. Flik.1999. In vivo bafilomycin-sensitive Na(+) uptake in young freshwater fish. *J. Exp. Biol.* 202 (Pt 24):3659-3666.

Fivelstad S, Olsen AB, Kloften H, Ski H and Stefansson S. 1999. Effects of carbon dioxide on Atlantic salmon (*Salmo salar* L.) smolts at constant pH in bicarbonate rich freshwater. *Aquaculture.* 178: 171-187.

Galvez F., Adamia S., and. G. G. Goss. 2001. Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 281 (5):R1718-R1725.

Galvez F., Reid S. D., Hawkings G., and G. G. Goss. 2002. Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 282 (3):R658-R668.

Georgalis T., Gilmour K.M., Yorston J., Perry S.F. 2006. The roles of cytosolic and membrane bound carbonic anhydrase in the renal control of acid-base balance in rainbow trout Oncorhynchus mykiss. *American journal of physiology. Renal physiology American Journal of Physiology. Submitted.*

Georgalis T., Perry S. F., and K. M. Gilmour. 2006. The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 209:518-530.

Goss G. G., Adamia S., and F. Galvez. 2001.Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 281 (5):R1718-R1725.

Goss G. G., Perry S. F., Fryer J. N., and P. Laurent. 1998.Gill morphology and acid-base regulation in freshwater fishes. *Comp. Biochem. Physiol. A* 119:107-115.

Goss G. G., Wood C. M., Laurent P., and S. F. Perry. 1994b. Morphological responses of the rainbow trout (*Oncorhynchus mykiss*) gill to hyperoxia, base ($NaHCO_3$) and acid (HCl) infusions. *Fish. Physiol. Biochem.* 12:465-477.

Goss GG, Laurent P, Perry SF 1992 Evidence for a morphological component in acid-base regulation during environmental hypercapnia in the brown bullhead (Ictalurus nebulosus). *Cell Tissue Res* 268(3):539-552

Goss GG, Laurent P, Perry SF 1994a Gill morphology during hypercapnia in brown bullhead (*I. Nebulosus*): role of chloride cells and pavement cells in acid-base regulation. *J. Fish. Biol.* 45:705-718

Goss, G.G., Perry, S.F., Laurent, P., 1995. Ultrastructural and morphometric studies on ion and acid-base transport processes in freshwater fish. In: C.M. Wood, T.J. Shuttleworth (Eds.), Cellular and Molecular Approaches to Fish Ionic Regulation. *Academic Press, Inc.*, San Diego, pp. 257-284.

Goyal S., Vanden Heuvel G., and Aronson P.S. 2003. Renal expression of novel Na/H exchanger isoform NHE8. *Amer. J. Physiol. Renal. Physiology.* 284: (F)467–473.

Hayashi H., Szaszi K., and S. Grinstein. 2002. Multiple Modes of Regulation of Na+/H+ Exchangers. *Ann NY Acad Sci* 976 (1):248-258,

Hirata T., Kaneko T., Ono T., Nakazato T., Furukawa N., Hasegawa S., Wakabayashi S., Shigekawa M., Chang M.-H., Romero M.F., Hirose S., 2003. Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Amer. J. Physiol.*. 284:(R)1199-1212.

Hirose, S., Kaneko, T., Naito, N., Takei, Y., 2003. Molecular biology of major components of chloride cells. Comparative Biochemistry and Physiology Part B:

Jensen F.B. 2004. Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O2 and CO2 transport. *Acta Physiolo. Scandin.* 182: 215–227.

Kandasamy RA and Orlowski J. 1996. Genomic organization and glucocorticoid transcriptional activation of the rat Na^+/H^+ exchanger *Nhe3* gene. *J. Biol. Chem.* 271: 10551-10559.

Keith P. Choe, Akira Kato, Shigehisa Hirose, Consuelo Plata, Aleksandra Sindic, Michael F. Romero, J. B. Claiborne, and David H. Evans. 2005. NHE3 in an ancestral vertebrate: primary sequence, distribution, localization, and function in gills. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289 (5):R1520-R1534.

Knepper, M.A., Brooks, H.L., 2001. Regulation of the sodium transporters NHE3, NKCC2 and NCC in the kidney. *Curr. Opin. Nephrol. Hypertens.* 10, 655-659.

Krogh A., 1938. The active absorption of ions in some freshwater animals. *Z. Vergl. Physiol.* 25, 335-350.

Laghmani K, Preisig PA, Moe OW, Yanagisawa M and Alpern RJ. 2001. Endothelin-1/endothelin-B receptor-mediated increases in NHE3 activity in chronic metabolic acidosis. *J. Clin. Invest.* 107: 1563-1569.

Laurent P., Goss G. G., and S. F. Perry. 1994. Proton pumps in fish gill pavement cells? *Arch. Int. Physiol. Biochim. Biop.* 102:77-79.

Ledoussal C., Lorenz, J.N., Nieman, M.L., Soleimani, M., Schultheis, P.J., Shull, G.E., 2001. Renal salt wasting in mice lacking NHE3 Na+/H+ exchanger but not mice lacking NHE2. Am. J. Physiol. 281, F718 –F727.

Li XC and Zhuo JL. 2007.Selective knockdown of AT1 receptors by RNA interference inhibits Val5-ANG II endocytosis and NHE-3 expression in immortalized rabbit proximal tubule cells. *Am. J. Physiol.* 293: C367-C378.

Maetz J. 1973. Na^+/H^+ exchanges and NH_4 movement across the gill of *Carassius auratus*. *J. Exp. Biol.* 58:255-275.

Malakooti J., Dahdal R.Y., Schmidt L., Layden T.J., Dudeja P.K., Ramaswamy K. 1999. Molecular cloning, tissue distribution, and functional expression of the human Na^+/H^+ exchanger NHE2. *Am. J. Physiol.* 277:(G)383–390.

Malapert M., Guizouarn H., Fievet B., Jahns R., Garciaromeu F., Motais R., and F. Borgese. 1997. Regulation of Na^+/H^+ antiporter in trout red blood cells. Anonymous. Anonymous. *J. Exp. Biol.* 200(2):353-360.

Marshall W. S.. 2002. Na+, Cl-, Ca2+ and Zn2+ transport by fish gills: Retrospective review and prospective synthesis. *J. Exp. Zool.* 293 (3):264-283,

Marshall, W. S. and Grosell, M. 2006. Ion transport and osmoregulation in fish. In *The Physiology of Fishes* (ed. D. Evans), pp. 177-230.

Mckenzie DJ, Taylor EW, Dalla Valle AZ and Steffensen JF. 2002.Tolerance of acute hypercapnic acidosis by the European eel (*Anguilla anguilla*). *J. Comp. Physiol. [B]* 172: 339-346.

Mello-Aires M. and Malnic G. 2002. Distal tubule bicarbonate transport. *J. Nephro.* 5:97-111.

Nakhoul N. L. and L. L. Hamm. Vacuolar H(+)-ATPase in the kidney. *J Nephrol.* 15 Suppl 5:S22-S31, 2002.

Nakhoul N.L. and Hamm L.L. 2002. Vacuolar H^+-ATPase in the kidney.*J. Nephro.* 5:22-31.

Nikinmaa M. and R. G. Boutilier. 1995. Adrenergic control of red cell pH, organic phosphate concentrations and haemoglobin function in teleost fish. edited by N. Heisler, *Berlin:Spinger-Verlag*, p. 107-133.

Numata M., Orlowski J. 2001. Molecular cloning and characterization of a novel $(Na^+/K)/H^+$ exchanger localized to the trans-Golgi network. *J. Bio. Chem.* 276:17387–17394.

Numata M., Petrecca K., Lake N., Orlowski J. 1998. Identification of a mitochondrial Na/H exchanger. *J. Bio. Chem.* 273:6951–6959.

Orlowski J. and S. Grinstein. 2004. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflugers Arch.* 447 (5):549-565.

Parks S. K., Tresguerres M., and G. G. Goss. 2007. Interactions between Na+ channels and Na^+-$HCO3^-$ cotransporters in the freshwater fish gill MR cell: a model for transepithelial Na+ uptake. *Am. J. Physiol. Cell Physiol.* 292 (2):C935-C944.

Pelis, R. M., Zydlewski, J. and McCormick, S. D. 2001. Gill Na^+,K^+,2Cl cotransporter abundance and location in Atlantic salmon: effects of seawater and smolting. *Am. J. Physiol.* 80, R1844-R1852.

Perry S. F. and K. M. Gilmour. 2006.Acid-base balance and CO_2 excretion in fish: Unanswered questions and emerging models. *Respir.Physiol. Neurobiol.* 154:199-215.

Perry S.F., Beyers M.L., Johnson D.A. 2000. Cloning and molecular characterisation of the trout (*Oncorhynchus mykiss*) vacuolar H^+-ATPase B subunit. *J. Ex. Bio.* 203: 459-470.

Perry S.F., Fryer J.N. 1997. Proton pumps in the fish gill and kidney. Fish *Physiol. Bio.* 17: 363-369.

Perry, S.F., Furimsky, M., Bayaa, M., Georgalis, T., Nickerson, J.G., Moon, T.W., 2003a. Integrated involvement of Na+/HCO3- cotransporters and V-type H+-ATPases in branchial and renal acid-base regulation in freshwater fishes. Biochem. Biophys. Acta 1618, 175-184.

Perry, S.F., Furimsky, M., Bayaa, M., Georgalis, T., Shahsavarani, A., Nickerson, J.G., Moon, T.W., 2003b. Integrated responses of Na^+,HCO_3^- cotransporters and V-type H^+-ATPases in the fish gill and kidney during respiratory acidosis. *Biochim. biophys. Acta* 1618, 175-184.

Perry, S.F., Malone, S., Ewing, D., 1987. Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ionic fluxes and blood acid-base status. *Can. J. Zool.* 65,

Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S., 2003d. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: Their role in ionic and acid-base regulation. *J. Exp. Zool.* 300A, 53-62.888-895.

Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L.Y., 2003c. Channels, pumps and exchangers in the gill and kidney of freshwater fishes: Their role in ionic and acid-base regulation. *J. Exp. Zool.* 300, 53-62.

Piermarini PM, Evans DH (2001) Immunochemical analysis of the vacuolar proton-ATPase B-subunit in the gills of a euryhaline stingray (*Dasyatis sabina*): effects of salinity and relation to Na(+)/K(+)-ATPase. *J. Exp. Biol.* 204(Pt 19):3251-3259

Reid S. D., Hawkings G. S., Galvez F. and G.G. Goss. 2003. Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells. *J. Exp. Zool.* 206, 551-559.

Ritter M. Fuerst J., Wöll E., Chwatal S., Gschwentner M., Lang F., Deetjen P., Paulmichl M. 2001. Na^+/H^+Exchangers: Linking Osmotic Dysequilibrium to Modified Cell Function *Cell. Physiol. Bio.* 11:1-18.

Romero M. F., Fulton C. M., and W. F. Boron. 2004a. The SLC4 family of HCO_3^- transporters. *Pflugers Arch.* 447 (5):495-509.

Romero M.F. and. D.B. Mount. 2004b.The SLC26 gene family of multifunctional anion exchangers. *Pflugers Arch.* 447 (5):710-721.

Romero, M.F., Boron, W.F., 1999. Electrogenic Na+/HCO3- cotransporters: Cloning and physiology. *Annu. Rev. Physiol* 61, 699-723.

Schwartz G.J. 2002. Physiology and molecular biology of renal carbonic anhydrase. *J. Neph.* 15(S): 61-74.

Scott G. R., Claiborne J. B., Edwards S. L., Schulte P. M, and C. M. Wood. 2005.Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. *J. Exp. Biol.* 208 (14):2719-2729.

Soleimani M. 2002. $Na^+HCO_3^-$ cotransporters (NBC): expression and regulation in the kidney. *J. Nephrol.* 5: 32-40.

Sullivan G. V., Fryer J. N., and S. F. Perry. 1996.Localization of mRNA for proton pump (H^+-ATPase) and Cl^-/HCO_3^- exchanger in rainbow trout gill. *Can. J. Zool.* 74 (11):2095-2103.

Swenson E. R. 2000.Respiratory and renal roles of carbonic anhydrase in gas exchange and acid-base regulation. *EXS* (90):281-341.

Tresguerres M., Katoh F., Fenton H., Jasinska E., and G. G. Goss. 2005.Regulation of branchial V-$H^{(+)}$-ATPase, $Na^{(+)}/K^{(+)}$-ATPase and NHE2 in response to acid and base infusions in the Pacific spiny dogfish (Squalus acanthias). *J. Exp. Biol.* 208 (Pt 2):345-354.

Tresguerres M., Parks S. K., and G. G. Goss. 2006.V-$H^{(+)}$-ATPase, $Na^{(+)}/K^{(+)}$-ATPase and NHE2 immunoreactivity in the gill epithelium of the Pacific hagfish (Epatretus stoutii). *Comp. Biochem. Physiol A Mol. Integr. Physiol.* 145 (3):312-321.

Tsuganezawa H., Kobayashi K., Lyori M., Araki T., Koizumi A., Watanabe S., Kaneko A., Fukao T., Monkawa T., Yoshida T., Kim D., Kanai Y., Endou H., Hayashi M.,

Saruta T. 2001. A new member of the HCO_3^- transporter superfamily is an apical anion exchanger of b-intercalated cells in the kidney. *J. Bio. Chem.* 276:8180–8189.

Wagner CA, Finberg KE, Breton S, Marshansky V, Brown D and Geibel JP. 2004. Renal vacuolar H+-ATPase. *Physiol. Rev.* 84: 1263-1314.

Wheatly MG, Hobe H and Wood CM. 1984.The mechanisms of acid-base and ionoregulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia II The role of the kidney. *Respir. Physiol.* 55: 155- 173.

Wilson J.M., Laurent P., Tufts B.L., Benos D.J., Donowitz M., Vogl A.W., Randall D.J. 2000a. NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J. Exp. Bio.* 203: 2279-2296.

Wilson R. J., M Harris. B., Remmers J. E., and S. F. Perry. 2000b.Evolution of air-breathing and central CO_2/H^+ respiratory chemosensitivity: new insights from an old fish? *J. Exp. Biol.* 203 (Pt 22):3505-3512.

Winkler CA, Kittelberger AM and Schwartz GJ.1997.Expression of carbonic anhydrase IV mRNA in rabbit kidney: stimulation by metabolic acidosis. *Am. J. Physiol.* 272: F551-F560.

Woo A.L., Noonan, W.T., Schultheis, P.J., .Neumann, J.C., Manning, P.A., Lorenz, J.N., Shull, G.E., 2003. Renal function in NHE3-deficient mice with transgenic rescue of small intestine absorptive defect. *Am. J. Physiol.* 284, F1190–F1198.

Wood C. M. and Jackson E. B.1980.Blood acid-base regulation during environmental hyperoxia in the rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* 42: 351-372.

Wood C. M., Wheatly M. G., and Hobe H..1984. The mechanisms of acid-base and ionoregulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. III. Branchial exchanges. *Respir. Physiol.* 55:175-192.

Wood C.M., Milligan C.L., Walsh P.J. 1999. Renal responses of trout to chronic respiratory and metabolic acidoses and metabolic alkalosis. *Amer. J. Physio.* 277: (R)482-492.

Wu M. S, Biemesderfer D, Giebisch G, and Aronson P. S.1996. Role of NHE3 in mediating renal brush border Na^+-H^+ exchange - Adaptation to metabolic acidosis. *J. Biol. Chem.* 271: 32749-32752.

Yan J.J., Chou M. Kaneko Y., T. and P. P. Hwang. 2007. Gene expression of Na+/H+ exchanger in zebrafish H+-ATPase-rich cells during acclimation to low-Na+ and acidic environments Hwang Articles in PresS. *Am. J. Physiol. Cell. Physiol.* 152-358.

Yun C., Tse C., Nath S., Levine S., Brant S., Donowitz M. 1995. Mammalian Naþ/Hþ exchanger gene family: structure and function studies. *Amer. J. Physio.* 269:(G)1–11.